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Animal models to study cancer-initiating cells from Glioblastoma

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1. ABSTRACT

Three main subtypes of gliomas with distinct molecular pathologies have been modeled in animals to better understand their biology. Genetically engineered mouse models that take advantage of genetic abnormalities observed in human gliomas have been instrumental in this process. These models better recapitulate signaling transduction pathways and the microenvironment that play crucial roles in glioma formation than *in vitro* systems or transplantation models. An increasing amount of data supports the existence of cells functionally defined by their self-renewal ability and tumor-initiating potential upon serial transplantation. As the issue of these cells with stem cell character in gliomagenesis becomes more illusive, animal models that provide an accurate experimental system where the stem cell character can be manipulated and studied are urgently needed. This review provides an overview of the current state of the literature with respect to animal models used in the study of gliomas and cells with stem cell character in their native environment.

2. INTRODUCTION

Glioblastoma Multiforme (GBM) is the most lethal form of primary brain tumors with treatment regimen that is rather palliative. The median survival rate of GBM patients is approximately 15 months, prognosis that has not improved significantly over the past decades (1). Its tendency to invade the neighboring parenchyma and heterogeneous nature at both molecular and cellular levels have thwarted the development of an effective treatment for the disease. Adding another layer of complexity, the genetic heterogeneity of GBM is found not only among the tumors of the same subtype but also between individual tumors within the group. The mainstays of current therapeutic regimen include surgical resection followed by chemotherapy and radiation (2). While the majority of the tumor bulk can be removed and targeted with conventional therapies, there seems to exist a population of resistant tumor stem-like cells that survive and eventually repopulate the tumor.

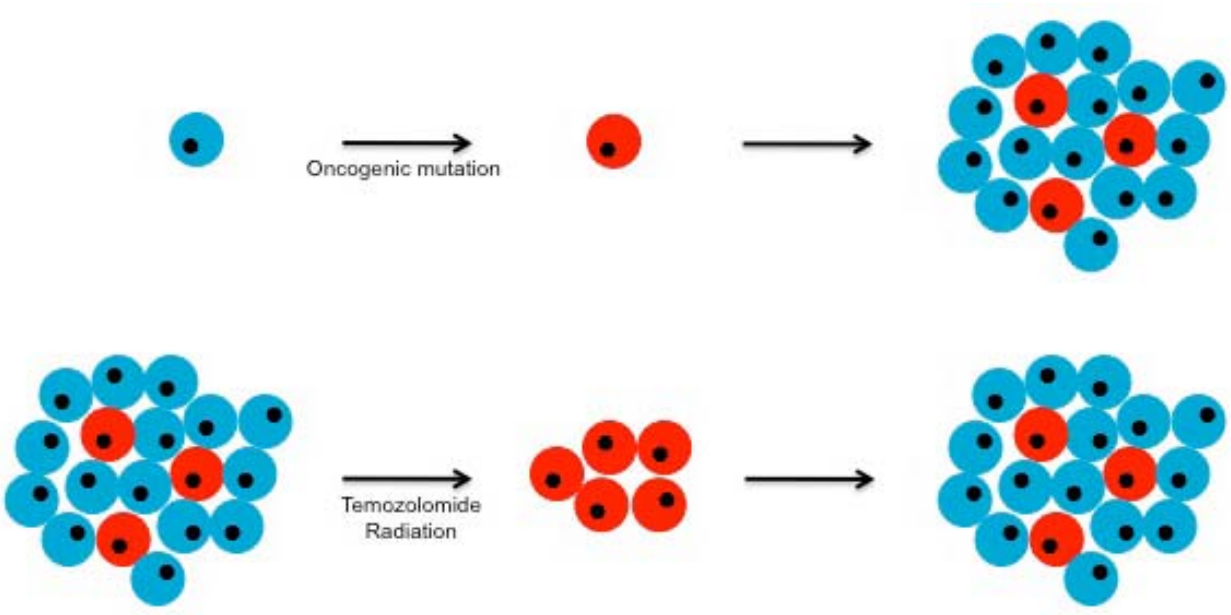

Figure 1. Cancer stem-like cells (CSCs) may give rise to primary tumors after the accumulation of oncogenic mutations or survive therapies and give rise to drug-resistant recurrent tumors.

In recent years, studies supporting the existence of a subpopulation of cells with the ability to initiate the tumor and another subpopulation of cells without this property have received increasing amount of attention. Cancer stem-like cells (CSCs) or tumor-initiating cells are functionally defined as a subset of cells that can self-renew and possess the capacity to initiate and propagate tumors upon serial transplantation (3). These cells have been identified in leukemia as well as in solid tumors such as prostate, head and neck, pancreatic, colon, and brain cancers (4-10). In addition to their self-renewing property, these cells can differentiate into different lineages and have the ability to recapitulate the tumor (11). Although the origin of CSCs has not been specifically determined, data suggests that CSCs may originate from normal stem cells, progenitor cells, or de-differentiated mature cells following the accumulation of oncogenic mutations. These cells may then become the origin of primary tumors as a result of genetic instability or drug-resistant recurrent tumors (Figure 1).

CSCs are commonly studied by utilizing their functional properties. The existence of brain tumor-initiating cells (BTICs) has been demonstrated by several groups using CSC markers such as CD133 and functional assays such as side population analysis (12-14). Isolated BTICs have been shown to possess the ability to self-renew, express neural stem cell markers, form neurospheres, and differentiate into multiple lineages (15). Although *in vitro* assays provide a robust, controlled system to study these cells, it is difficult to mimic the cellular heterogeneity of tumors or the complexity of tumor-stroma interactions *in vitro*. Therefore, the animal modeling is necessary to better understand the tumor pathology in a more clinically relevant setting, which may be further used as a platform to test potential therapeutics. This review will cover some of the murine models that have been useful in better understanding gliomagenesis as well as BTICs.

3. MOLECULAR PATHOLOGY

3.1. Cell of origin in glioma formation

The specific cell of origin for gliomas is unknown and in fact the tumors may arise from multiple cell types. There are three major underlying theories postulated to explain the cell type responsible for the initiation of gliomas. The first suggests that terminally differentiated mature glia acquire mutations that endow them with a capacity for transformation. The second suggests that restricted neural progenitor cells, which normally terminally differentiate following successive divisions, could serve as the origin for gliomas with the retention of progenitor characteristics that contribute to tumorigenesis. The third suggests that adult neural stem cells acquire mutations that allow them to bypass the regulatory mechanisms that control their activity and thus facilitate the progression of tumorigenesis. A significant amount of data exists to support the retrograde differentiation of committed glial cells as the source of tumor-initiating cells in gliomas. Based on histological and immunohistological similarities between the primary glioma subtypes (astrocytomas and oligodendrogliomas) and their normal counterparts (astrocytes and oligodendrocytes), it has been postulated that astrocytes and/or their precursors and oligodendrocytes and/or their precursors are the cells of origin for astrocytomas and oligodendrogliomas respectively (16, 17). The ability for these cells to act as the cell of origin is supported by the fact that several signaling pathways affecting glial differentiation and proliferation are dysregulated in gliomas, and the forced expression of these signaling pathways in glial cells *in vitro* and *in vivo* results in the

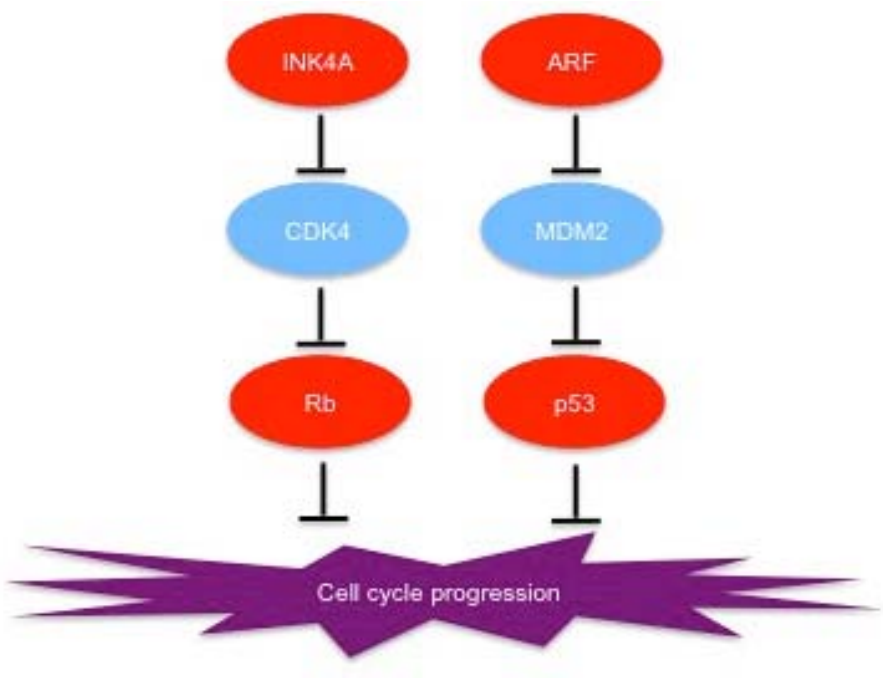

Figure 2. Cell cycle genes widely used in modeling primary astrocytic and oligodendroglial brain tumors *in vivo*

formation of gliomas in mice (18-24). For example, the genetic loss of *Ink4a/Arf*, genes involved in the regulation of the cell cycle, in mature glial cells in mice with the concurrent activation of EGFR results in the formation of gliomas (20). In addition, cell type-specific gene transfer of the transcription factor *c-myc* into mature astrocytes downregulated the expression of an astrocyte-specific marker, glial fibrilary acidic protein (GFAP), and enhanced the expression of Nestin. Nestin is an intermediate filament protein used as a marker for a progenitor phenotype and used to identify neural stem cells. Nestin-expressing cells possess the ability to differentiate into different cell types such as glial cells and neurons (9). *C-myc* in combination with *Ras* and *Akt* induced gliomas, while *Ras* and *Akt* alone were insufficient to induce tumorigenesis (25). These studies suggest that terminally differentiated glial cells can acquire the ability to initiate tumor formation.

3.2. Signaling pathways disrupted in GBMs

In modeling primary astrocytic and oligodendroglial brain tumors *in vivo*, several molecular abnormalities commonly found to be disrupted in patients with glioma have been widely utilized: 1) cell cycle genes such as Retinoblastoma (*RB*) and *p53*, 2) receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (*EGFR*) and platelet-derived growth factor receptor (*PDGFR*), and 3) components of RTK downstream pathways including PI3K/AKT and RAS/MAPK signaling pathways.

Recently, the Cancer Genome Atlas Research Network has demonstrated that *RB* and *p53* tumor suppressor genes are mutated or deleted while negative regulators of these suppressors such as cyclin-dependent kinase 4 (*CDK4*) and *MDM2* are amplified in human gliomas (Figure 2). The *p53* gene mutations or deletions have been found in secondary tumors arising from low-grade astrocytic tumors as well as sporadic low-grade astrocytomas. Mutations at the *RB* gene and its functional silencing by amplified *CDK4* were also found in a significant number of astrocytomas. The pathways that these two tumor suppressors are involved in were dysregulated in the majority of GBMs analyzed (~80-90%). Furthermore, the *CDKN2A* locus that encodes positive regulators of *RB* and *p53*, *INK4A* and *ARF* respectively, was deleted in ~50% of high-grade astrocytomas as well as in anaplastic oligodendrogliomas (26, 27). These findings confirm and emphasize the importance of cell cycle regulation in gliomagenesis, and its direct involvement in glioma formation has been explored using various mouse models that will be further discussed below.

In the same study, a significant number of amplification and activating mutations of RTKs was striking. Genomic amplification as well as activating mutations of *EGFR* were observed in ~40% of primary GBMs. A constitutively active deletion mutant *EGFRvIII* was the most common (20-30%). In addition, *PDGFR* was upregulated in low-grade astrocytic and oligodendroglial tumors as well as in ~13% of GBMs, and its ligand *PDGF* was upregulated. Similarly, hepatocyte growth factor receptor (*HGFR* or *MET*) and its ligand *HGF* were increased, suggesting enhanced signaling by autocrine and paracrine loops between ligands and receptors (26, 27).

The effects of amplification and activating mutations of RTKs are further mediated by downstream signaling pathways, notably PI3K/AKT and RAS/MAPK

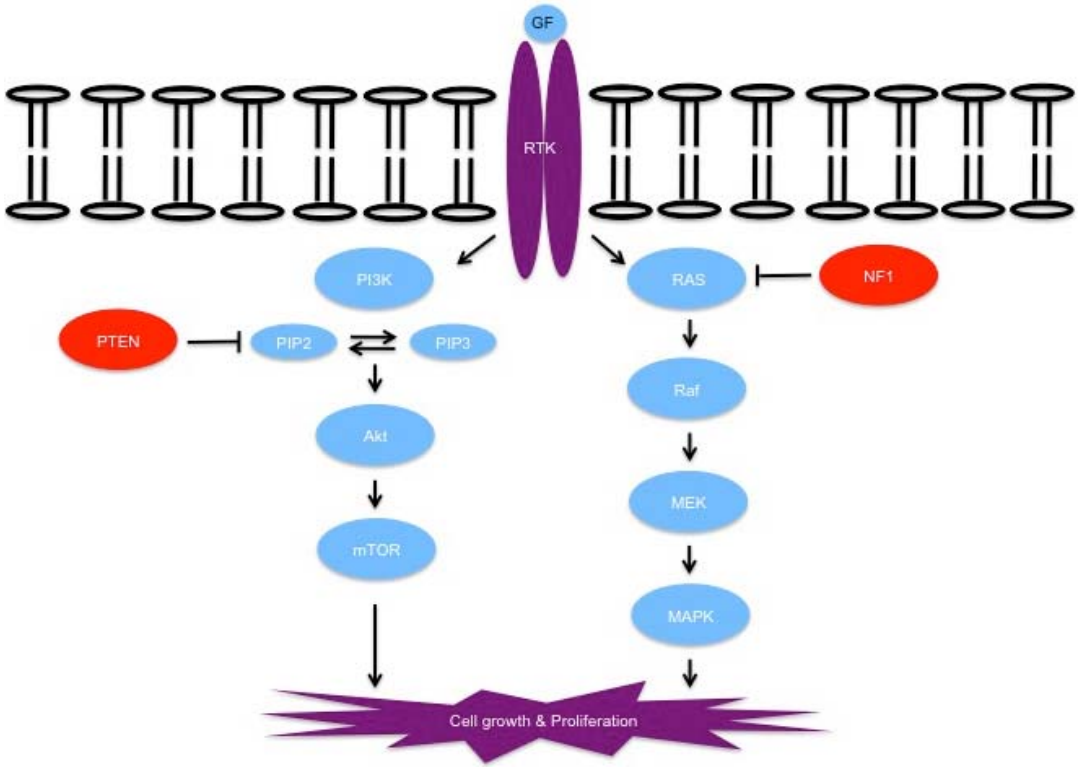

Figure 3. Receptor tyrosine kinases (RTKs) and downstream signaling pathways commonly used in modeling primary astrocytic and oligodendroglial brain tumors *in vivo*

pathways (Figure 3). These pathways are crucial in cell growth and proliferation and have been shown to exhibit enhanced signaling in ~85% of GBM. One of the most typical loss-of-function mutations was found in phosphatase and tensin homolog (*PTEN*) that negatively regulates PI3K/AKT pathway (~36%). Most commonly, the mutation or deletion of *PTEN* gene is achieved by means of the complete loss of its locus on chromosome 10q. This leads to enhanced signaling by *AKT* to facilitate gliomagenesis. In addition, RAS/MAPK pathway exhibited enhanced signaling in astrocytic gliomas via activating mutation of *RAS* and targeted deletions in neurofibromatosis type-1 (*NF1*) tumor suppressor gene in 15-18% glioma samples analyzed (26, 27).

4. CANCER STEM CELLS

As discussed earlier, terminally differentiated mature glial cells with multiple mutations that confer transformative capability, restricted neural progenitor cells with intact progenitor characteristics, and adult neural stem cells with mutations resulting in dysfunctional regulatory mechanisms have been postulated to serve as the cell of origin for gliomas. Based on more recent findings, an alternative hypothesis that involves restricted neural progenitor cells with extended capacity to self-renew through the accumulation of oncogenic mutations has been discussed. The rest of the review will focus on murine models that have been instrumental in studying gliomagenesis with particular emphasis on those used to study tumor-initiating cells.

4.1. Cancer stem cell hypothesis

By definition, stem cells are a rare population of cells that possesses the ability to self-renew and generate differentiated mature cells of a particular tissue (11). CSCs are functionally defined as a population of cells that can self-renew and possess the capacity to initiate and propagate tumors upon serial transplantation *in vivo* (3). These cells have the ability to differentiate into multiple lineages and have the potential to recapitulate the tumor. CSCs were first identified and isolated in hematopoietic stem cells (HSCs) using their functional properties. HSCs were purified from the mouse bone marrow by taking advantage of various surface markers and fluorescence-activated cell sorting (FACS) technique (28). Subsequently, cells exhibiting similar properties as stem cells were found in the studies of acute myelogenous leukaemia (AML). A subpopulation of leukemic stem cells with normal HSC surface markers such as CD34+/CD38- were shown to promote AML in non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice (4, 29). The engrafted leukemia cells were subjected to serial transplantation to test for the self-renewal ability of these so-called tumor-initiating cells. Since then, serial transplantation has been one of the main criteria in functionally defining CSCs in various tumors. The existence of CSCs has recently been strengthened by the identification of such cells in solid tumors such as breast, prostate, head and neck, pancreatic,

and colon cancers as well as in brain tumors (5-10). Although the origin of CSCs has not been specifically determined, data suggests that CSCs may originate from normal stem cells, progenitor cells, or de-differentiated mature cells following the accumulation of oncogenic mutations (3).

4.2. Brain tumor-initiating cells (BTICs)

The existence of BTICs has been demonstrated by several groups using cell surface markers and functional assays (12-14, 30, 31). These cells were shown to self-renew, express neural stem cell markers, form neurospheres, and differentiate into multiple lineages (15). Moreover, neurospheres derived from primary tumors were shown to maintain genetic profiles similar to the original tumor when grown in serum-free media supplemented with appropriate growth factors (32). Characterizing and honing of the tools to study BTICs are critical in successfully identifying and subsequently isolating these cells from tumors that arise in animal models.

Several groups have independently demonstrated BTICs from GBMs using marker-dependent (i.e. CD133) and marker-independent (i.e. FACS) methods (12, 13, 33, 34). One group observed that cells capable of forming neurospheres in serum-free condition expressed a cell surface marker, CD133 (also known as prominin 1), which has been previously found on normal human neural stem cells. After enriching this population using antibody-coated magnetic beads, the group utilized xenograft models to investigate the tumor-initiating potential of these cells when injected into the brains of NOD/SCID mice. As few as 10^2 CD133+ gave rise to tumors that show similar tumor histology and cellular heterogeneity as the original patient's tumor, whereas 10^5 CD133- cells were required for a similar phenotype. In addition, CD133+ cells self-renewed as determined by serial transplantation assay and gave rise to both CD133+ and CD133- populations (12). Although CD133 expression has been correlated with poor clinical prognosis, there is still considerable debate as to whether tumor-initiating ability is a unique feature of CD133+ population (35). CD133- cells have been shown to give rise to both CD133+ and CD133- cells and able to initiate tumors as effectively as CD133+ cells in nude rats (36, 37). Furthermore, the stability of stem cell properties of CD133+ cells is questionable since it shows dramatic changes depending on how primary human tumor samples were handled and cultured (38). This suggests that the CD133 marker alone may not be sufficient to isolate tumor-initiating cells.

Another group demonstrated that stage-specific embryonic antigen 1 (SSEA-1/CD15/Lewis X [LeX]) can also be used to isolate BTICs (30). SSEA-1 is a neural stem cell marker that is also expressed by pluripotent embryonic stem cells (39). From freshly dissociated primary human GBM cultures, SSEA-1+ cells were isolated and cultured in serum-free media. SSEA-1+ cells were highly tumorigenic when injected into xenografts, tumors arising as early as ~10 weeks, whereas SSEA-1- cells rarely formed tumors. As few as 10^3 SSEA-1+ cells were required for tumor formation. SSEA-1+ cells formed more neurospheres than SSEA-1- cells and were able to differentiate into multiple lineages as marked by Nestin, GFAP, and Tuj-1. In a lineage tracking experiment, SSEA-1+ cells gave rise to both SSEA-1+ and SSEA-1- cells, while SSEA-1- cells were unsuccessful in producing either population (30). Most SSEA-1+ cells were also CD133+, confirming the notion that multiple markers are necessary in enriching and identifying BTICs.

In addition, the drug-resistance or drug-transporting property of ATP-binding cassette (ABC) transporters can be utilized to isolate and study stem-like cells in a subset of gliomas (40). The CSC model hypothesizes that drug resistance is mediated by a population of stem-like cells that express drug transporters such as ABC transporters and survive after therapy to mediate tumor recurrence. Recently, the ABCG2 expression levels in BTICs have been correlated with the grade of gliomas and mitoxantrone resistance; higher-grade gliomas were shown to have increased levels of ABCG2 expression and were more resistant to mitoxantrone treatment (41). ABC transporters are membrane pumps that play a key role in pumping out various organic molecules as well as chemotherapeutic drugs. ABC transporters hydrolyze ATP molecules and use the energy derived from the hydrolysis reaction to efflux endogenous bile acids, cholesterol, ions, and peptides as well as cytotoxic agents in order to protect cells from xenobiotics (15). When incubated with the fluorescent Hoechst 33342 dye that binds to the AT-rich regions of DNA, most cells accumulate the dye and correspond to the "main-population" (MP) on FACS analysis plot. However, there exists a small population of cells that effectively efflux the dye via ABC transporters and form the "side population" (SP). The SP cells can be isolated, quantified, and further studied. A significant fraction of hematopoietic stem cells was found in the SP fraction, and the majority of the cells in this fraction were identified as stem cells (42). Similarly, this technique was used to isolate BTICs from both mouse and human cells. In PDGF-driven gliomas, ABCG2 activity was shown to contribute to the SP phenotype and be used as a marker to isolate stem-like cells. When human GBM neurospheres sorted into SP and MP were injected into SCID mice, those injected with SP cells exhibited shorter tumor latency compared to those injected with MP cells (40).

More recently, the cell morphology as well as intrinsic autofluorescence properties exhibited by unique cellular metabolic profiles were used to isolate BTICs using FACS. As few as 10^3 FL1+ cells gave rise to tumors when injected into SCID mice. The self-renewal capacity of this subset of cells was confirmed via serial transplantation assay. A clonal FL1+ cells gave rise to both FL1+ and FL1- cells, although both populations showed similar differentiation markers when they were placed in serum culture condition (31). Taken altogether, present studies suggest that BTICs cannot be defined by a sole marker, but rather by a combination of markers and functional assays. The integrity of CSCs may not be as strictly defined as in normal stem/progenitor cells, and these cells can be better studied in a context-dependent manner using various markers.

4.3. Specialized microenvironments required for BTICs

Available data suggests that BTICs may require a specialized microenvironment in order to maintain their stem-like properties. In normal brains, stem cells reside in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus near the capillaries (43, 44). In recent years, normal neural stem cells as well as BTICs were shown to be localized to the perivascular niches. These are specialized microenvironments where endothelial cells have been shown to secrete soluble factors that support stem cell renewal (45-47). One of the pathologic hallmarks of GBM is the pressure of microvascular proliferating structures, and the perivascular niche is believed to surround these structures to house BTICs. When BTICs from human tumors were isolated and cultured in a co-culture experiment, these cells migrated towards endothelial cells. The importance of the microenvironment and factors released from this niche is highlighted by the fact that tumor formation is enhanced following co-injection of endothelial cells with BTICs (47).

Recently, nitric oxide (NO) was identified as one of these factors produced from eNOS in nearby endothelial cells of the perivascular niche in PDGF-driven gliomas (48). NO is produced by Nitric Oxide Synthases (NOSs) that use L-arginine, NADPH, and oxygen as substrates. Elevated levels of eNOS correlate with increasing grade of gliomas, and eNOS has been shown to promote a migratory and invasive phenotype by aiding the blood flow and vascular permeability in various tumors (49, 50). NO signals by binding to its receptor, soluble guanylate cyclase (sGC), on neighboring cells, which upon activation converts GTP to cGMP for the activation of downstream effectors such as cGMP dependent protein kinase (PKG) (50). Using the RCAS-tv-a system, the ability of NO to drive SP phenotype via the NO/sGC/PKG pathway and to activate the Notch signaling pathway was demonstrated, and this might mediate the maintenance of stem-like properties in a subset of cells found in the perivascular niche of PDGF-induced gliomas (48).

Data suggests the importance of the Notch pathway in maintaining self-renewal ability and undifferentiated state of neural stem cells. Mice with the deletion of Notch-1 show reduced levels of neural stem cells (51). Notch-1 and its ligands, Deltalike-1 and Jagged-1, are overexpressed in several glioma cell lines as well as in human primary gliomas where they play crucial roles in cell survival and proliferation (52). Increasing Notch-1 expression level correlates with higher-grade human gliomas (53). Moreover, Notch-1 can target the Nestin promoter, and the activation of both *Kras* and *Notch-1* results in the expansion of Nestin-expressing cells in the mouse SVZ (54). Nonetheless, its potential tumor-initiating role in the perivascular niche remains elusive and requires further investigation.

Furthermore, other developmental pathways such as Wnt and Hedgehog have been implicated in BTIC biology. Components involved in Sonic Hedgehog (SHH) signaling such as *Ptch* and *Gli* have been reported to be overexpressed in grade IV astrocyoma cell lines (55). In another study, the expression of *Ptch* was shown to be colocalized with the proliferation marker Ki67 as well as stem-like cell marker Bmi-1 (56). In addition, the blockade of SHH signaling by pharmacologic as well as genetic approaches reduces tumor cell proliferation and self-renewal capability (57, 58).

The expression of bone morphogenetic proteins (*BMP*) receptors and ligands has also been correlated with increasing glioma grade (59). Recently, BMP4/SMAD signaling was shown to strongly suppress the growth of CD133+ BTICs. Intracellular signaling transduction was mediated by SMAD signaling in this subset of cells (60). In a similar study, a multikinase inhibitor, sorafenib, was shown to promote cell growth arrest and apoptosis in glioma cell lines as well as in primary GBM cultures. The Janus-activated kinases (*JAK*) and signal transducers and activators of transcription 3 (*STAT3*) were inhibited in response to sorafenib treatment (61). In addition, GBM has been shown to express higher levels of interleukin 6 (*IL6*) protein than normal control brains (62), and increased *IL6* transcript levels have been correlated with poor survival in GBM patients (63). More recently, BTICs were shown to express two IL6 receptors - IL6 receptor alpha and glycoprotein 130. Knockdown of these receptors reduced cell growth as well as neurosphere forming ability while increasing apoptosis and reducing the level of STAT3 activation. Targeting IL6 receptor and its ligand conferred survival benefits in mice bearing human glioma xenografts (64).

An interactive role between BTICs and the perivascular niche in tumor progression was shown using CD133 as a marker for BTICs. CD133+ cells were shown to promote angiogenesis by secreting vascular endothelial growth factor (VEGF) (65). Moreover, the localization of BTICs to the perivascular niche was demonstrated by a novel marker, integrin alpha-6. Integrin alpha-6+ cells were found near the vasculature and co-localized with CD133+ and Nestin+ cells. Integrin alpha-6 in combination with CD133 enriches for BTICs, and SCID mice injected with Integrin alpha-6-high cells showed shorter tumor latency than those injected with Integrin alpha-6-low cells (66).

5. ANIMAL MODELS TO STUDY GBMs & BTICs

Early animal models have used DNA alkylating reagents such as ethylnitrosourea and nitrosoureas to induce brain tumors in rat brains (67, 68). These models have been shown to give rise to various CNS tumors and be useful in studying the events that lead to propagation of tumors. Nonetheless, they are not widely used due to their limited breadth in representing the tumor pathology. For instance, the cell affected by the alkylating reagents may not be the same as the origin of CNS tumors as in humans. Since then, several animal models have been generated that utilize transplantation, transgenic, and somatic gene transfer techniques.

5.1. Transplantation models

Transplantation models involve the use of xenograft or allograft models. These models are widely

Table 1. Summary of murine somatic cell gene transfer models

Transgenics	Virus/Receptor	Latency (Incidence)	Tumor Type	Ref.
PDGF-B	MMLV	by 10 mo. (40%)	Oligodendroglial	(73)
kRAS; Akt	RCAS/Ntv-a	by 3 mo. (25%)	Astrocytic	(75)
kRAS; Akt; Ink4a/Arf-/-	RCAS/Ntv-a	by 3 mo. (20-50%)	Astrocytic	(86)
kRAS; Pten-/-	RCAS/Ntv-a	by 3 mo. (60%)	Astrocytic	(87)
PDGF-B	RCAS/Ntv-a	by 3 mo. (60-100%)	Oligodendroglial	(88)
PDGF-B; Ink4a/Arf-/-	RCAS/Ntv-a	by 3 mo. (60-100%)	Oligodendroglial	(88)
Nf1+/-; p53+/-; Pten-/-	Adenovirus	by 6 mo. (100%)	Astrocytic	(89)
EGFRvIII; Ink4a/Arf-/-; Pten-/-	Adenovirus	by 13 weeks (100%)	Astrocytic	(90)

Table 2. Summary of murine transgenic models.

Transgenics	Latency (Incidence)	Tumor type	Ref.
Nf1+/-; p53+/-	by 6-8 mo. (~100%)	Astrocytic	(91)
Nf1+/-; p53+/-	by 5-10 mo. (100%)	Astrocytic	(93)
Nf1+/-; p53+/-; Pten-/-	by 5-8 mo. (100%)	Astrocytic	(94)
GFAP-V^{12} Ras	by .5-3 mo. (100%)	Astrocytic	(21)
GFAP-V^{12} Ras; Pten-/-	by 1.5 mo. (100%)	Astrocytic	(95)
S100-v-erbB	by 12 mo. (60%)	Oligodendroglial	(22)
S100-v-erbB; Ink4a/Arf-/-	by 12 mo. (100%)	Oligodendroglial	(22)
S100-v-erbB; p53+/-	by 12 mo. (100%)	Oligodendroglial	(22)
GFAP-T_{121}	by 12 mo. (100%)	Astrocytic	(96)
GFAP-T_{121}; Pten-/-	by 6 mo. (100%)	Astrocytic	(24)

used and are generated by either subcutaneously or orthotopically transplanting primary or cultured human or murine cells into immunocompromised animals (69, 70). Tumors generated are highly reproducible with high growth rates and incidence. Traditionally, serum-cultured, established cell lines such as U87MG have been used to generate tumors in transplatation models. However, these tumors do not accurately mimic histological features of human gliomas. In addition, they lack immunological interactions between the host and the tumor. Tumors arising from serum-cultured cell line were shown to lack migratory capacity, remaining rather as a mass at the site of injection (13). In addition, there are discrepancies in karyotypic characteristics between cells cultured in serum condition and primary tumors from which the cells were derived (71). The genetics of the original tumor are likely altered by selective pressure from extended cell culture.

Nonetheless, testing tumor-initiating potential and self-renewing capability of cells isolated using markers and functional assays in xenografts has emerged as a gold standard in CSC biology. In addition, recent advancement in stem-cell culture conditions has provided ways to generate tumors that resemble original patients' tumors in transplatation models. After surgical resection, tumor-initiating cells of the primary tumor can be maintained in serum-free medium supplemented with EGF and FGF2 as spheres. When reinjected into mouse brains, the resulting tumors exhibit similar histological features and heterogeneity as the parental tumors (12, 13). Human primary tumor samples that have been grafted into mice also retain the characteristics of their original tumors despite *in vivo* selection pressure (72). Taking advantage of the fact that xenografts replicate original patient tumors and are relatively inexpensive and easy to use, these models are widely utilized in rapid screening of novel therapeutics as well as in drug response correlative studies.

5.2. Somatic cell gene transfer models

Somatic cell gene transfer techniques utilize viral vectors to transfer genes to a specific subset of somatic cells postnatally. Tumors developed by viral transduction more closely reflect the acquisition of mutations in somatic cells that occur in sporadic cancers (Table 1). One of the systems that utilizes this approach involves the replication competent Moloney Murine Leukemia Virus (MMLV). The wild type MMLV helper virus help deliver the targeted gene to proliferating cells *in vivo* (73). Tumors that arise with this system histologically mimic those features observed in human gliomas. However, as MMLV infect most proliferating cells nonspecifically, the cell of origin of tumors that arise in this system is unknown. Based on the number of cells successfully infected, secondary genetic alterations may occur that lead to tumor formation.

The second type of somatic cell gene transfer technique utilizes an avian leukosis virus (ALV)-based replication competent virus (RCAS) and its receptor tumor virus-A (tv-a), to transfer genes to specific cell types (74, 75). This system is comprised of two key parts: 1) a retroviral vector that, upon infection, delivers a genetic payload that induces either oncogene expression or tumor suppressor gene loss, and 2) mice with genetically engineered cell type specific sensitivity to retroviral infection (Figure 4). The retroviral vector in this system is derived from the avian sarcoma and leukosis virus subgroup-A (ASLV-A). The oncogene, v-*src*, normally carried in the ASLV retrovirus is replaced with a cloning site allowing customized insertion of oncogenes of interest, such as an activated form of *Akt* (76). The genetically modified ASLV-A thus serves as a delivery device of genetic material, which incorporates into the genome of infected cells. Retained *gag*, *pol*, and *env* genes allow the virus to replicate in avian cells, as ASLV is an avian virus. This vector is called replication competent ASLV long terminal repeat with splice acceptor (RCAS) (77).

However, mammalian cells are not normally sensitive to infection by ASLV-A (and by extension RCAS). Infectability requires expression of the receptor for the ASLV subgroup, tumor virus-A (tv-a) (78, 79). Avian cells naturally express tv-a; however, mammalian cells do not. By genetically engineering mice to express tv-a under the control of a promoter of interest, only cells in

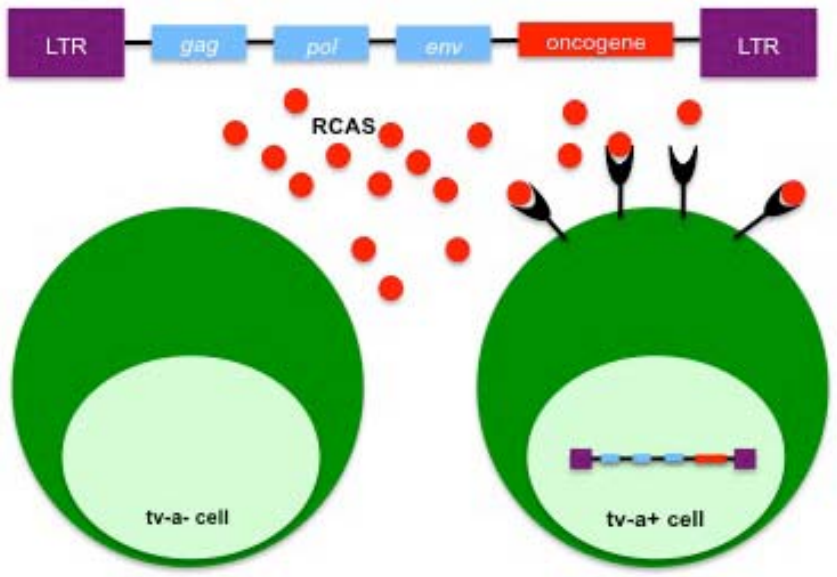

Figure 4. Schematic representation of RCAS-tv-a delivery system

which the promoter is active are susceptible to infection by RCAS. For example, glial cells expressing glial GFAP are susceptible to RCAS infection in mice transgenic for tv-a expression under control of the GFAP promoter (Gtv-a transgenic mice) (74). Similarly, neuro-epithelial stem cells express the intermediate filament protein Nestin. Therefore, glial precursors are susceptible to RCAS infection in mice transgenic for tv-a expression under control of the Nestin promoter (Ntv-a transgenic mice) (75). In addition, tv-a expressing cells are sensitive to infection by multiple RCAS vectors, allowing the delivery of multiple oncogenes from more than one genetically engineered vector. Finally, although RCAS is able to replicate normally in avian cells, inefficient expression of *gag, pol,* and *env* in murine cells prevents synthesis of additional RCAS virions and spreading of the infection to adjacent tv-a expressing cells in Gtv-a or Ntv-a mice (80). The threshold for transformation is high because tumors form only as a consequence of experimentally induced oncogenic abnormalities following RCAS-infection. Moreover, inefficient infection leads to a limited population of infected cells because RCAS virions cannot replicate in murine cells. Therefore, one advantage of RCAS/tv-a modeling is the ability to conclude that the experimentally induced oncogenic abnormalities are highly transforming.

However, this is also disadvantageous for detecting mutations that contribute to tumor formation but are otherwise insufficient. To address this problem, the RCAS/tv-a system can also be combined with additional modeling techniques to allow even more powerful investigation of tumor biology. For example, proliferation control normally exerted by the tumor suppressor inhibitor of *CDK4A* (*INK4A*) or the protein encoded by its alternate reading frame (*ARF*) is lost or functionally disrupted in most human gliomas. Crossing *Ink4a/Arf* knockout mice with Gtv-a or Ntv-a mice yields *Ink4a/Arf* null animals susceptible to RCAS infection. Therefore, oncogenes encoded by one or multiple RCAS vectors are transferrable in a cell-type specific manner to GFAP or Nestin expressing cells in *Ink4a/Arf* null animals, allowing investigation of the role of *Ink4a/Arf* loss in tumor formation and latency. The loss of some tumor suppressor genes is embryonically lethal making it difficult to determine the importance of germline deletions in tumor biology. The RCAS/tv-a modeling system can be adapted to targeted deletion of tumor suppressors through combination with the Cre-lox system. Crossing mice transgenic for a floxed sequence with tv-a mice allows cell-type specific gene removal following infection by an RCAS vector engineered to carry Cre recombinase (RCAS-*Cre*). For example, *PTEN* is lost or otherwise disrupted in most human GBMs (81, 82). However, homozygous *Pten* loss in mice is embryonically lethal (83-85), but Cre-lox system combined with RCAS/tv-a technology allows postnatal deletion of the tumor suppressor in a cell-type specific manner.

One particularly distinguishing feature of the RCAS/tv-a system is the ability to accurately recapitulate histological features observed in human gliomas such as microvascular proliferating structures and foci of pseudopalisading necrosis. When constitutively active forms of both RCAS-*kRas* and RCAS-*Akt* were delivered to Ntv-a mice, ~25% of mice developed astrocytic tumors by 12 weeks (75). Additional homozygous deletion of *Ink4a/Arf* increases the incidence of tumor formation, and some of these tumors show microvascular proliferation and pseudopalisading regions, histological hallmarks of high-grade gliomas (86). RCAS-*Cre* infection of mice transgenic for both tv-a and floxed *Pten* allows the interrogation of the role *Pten* plays in tumor formation and latency. The delivery of RCAS-*kRas* in combination with *Pten* deletion in Ntv-a mice was shown to give rise to astrocytic tumors within 3 months (87). RCAS-*PDGF-B* vectors have further underscored the advantage of using this model and

Table 2. Summary of murine transgenic models.

Transgenics	Latency (Incidence)	Tumor type	Ref.
Nf1+/-; p53+/-	by 6-8 mo. (~100%)	Astrocytic	(91)
Nf1+/-; p53+/-	by 5-10 mo. (100%)	Astrocytic	(93)
Nf1+/-; p53+/-; Pten-/-	by 5-8 mo. (100%)	Astrocytic	(94)
GFAP-V^{12}Ras	by .5-3 mo. (100%)	Astrocytic	(21)
GFAP-V^{12}Ras; Pten-/-	by 1.5 mo. (100%)	Astrocytic	(95)
S100-v-erbB	by 12 mo. (60%)	Oligodendroglial	(22)
S100-v-erbB; Ink4a/Arf-/-	by 12 mo. (100%)	Oligodendroglial	(22)
S100-v-erbB; p53+/-	by 12 mo. (100%)	Oligodendroglial	(22)
GFAP-T_{121}	by 12 mo. (100%)	Astrocytic	(96)
GFAP-T_{121}; Pten-/-	by 6 mo. (100%)	Astrocytic	(24)

obtaining a subclass of high-grade tumors in a convenient, timely manner. When Ntv-a or Gtv-a mice were injected with RCAS-*PDGF-B*, oligodendroglial tumors develop by 12 weeks, and additional deletion of *Ink4a/Arf* increased the incidence of tumor formation (88). Combination of techniques is also possible, for example, by crossing Ntv-a, *Ink4a/Arf* null, and *Pten* floxed mice and infecting them with RCAS-*Cre*.

Recently, another viral transduction system was used to achieve the localized deletion of tumor suppressors. Replication-incompetent adenovirus that infect both dividing and non-dividing cells were engineered to carry Cre recombinase (Ad-*Cre*), and these vectors were stereotactically injected into various regions of *Nf1*, *p53* and/or *Pten* floxed mouse brains. Mice developed high-grade astrocytomas by 6 months in selective regions that will be further discussed below (89). Similarly, the stereotactic injection of Ad-*Cre* to activate the expression of EGFRvIII and to simultaneously delete *Pten* in a region-specific manner was achieved by another group using mice with *Ink4a/Arf* null background. In this model, the activation of EGFRvIII and *Ink4a/Arf* and *Pten* deletions gave rise to astrocytomas by 13 weeks (90).

5.3. Transgenic models

Transgenic modeling involves the genetic manipulation of signal transduction pathways considered to play roles in the development of gliomas as discussed above. Transgenic models are useful in exploring the causal role of certain genetic abnormalities in the formation of gliomas. The establishment of transgenic models is achieved through germline genetic modification strategies, and these models recapitulate the mechanism through which human gliomas arise more closely than transplantation models do (Table 2). Germline genetic modification strategies utilize gain or loss of function mutations that are integrated into the germline in a Mendelian fashion. Mice generated by this modification are predisposed to develop tumors through the overexpression of oncogenes or the loss of tumor suppressors in combination with secondary mutations. To investigate the function of a specific gene in a certain cell type, oncogenes are driven by a tissue specific promoter for an overexpression in transgenic mice. For example, *Nf1* and *p53* combined mutant was established by crossing *Nf1+/-* with *p53+/-* to create double heterozygotes. These mice developed astrocytic tumors by 6-8 months, and the age of mice tended to correlate with the grade of tumors (91).

Alternatively, conditional knockouts can be engineered by flanking the gene targeted for deletion between two loxP sites. Following recombination by Cre expression (Cre catalyzes the recombination between two loxP sequences), the target gene in all cells or cells with targeted expression of Cre can be deleted (92). Utilizing this technology, another *Nf1* and *p53* combined mutant was developed by pairing *p53* mutant allele with *Nf1* floxed allele flanked by two loxP sites. By crossing these mice with those expressing Cre under control of GFAP promoter (GFAP-*Cre*), *Nf1* was deleted specifically in GFAP-expressing cells. This powerful system led to the region-specific loss of heterozygosity and high-grade astrocytic tumor development by 5-10 months (93). Additional deletion of *Pten* allele was shown to shorten the tumor latency by about 2 months while increasing the tumor grade (94).

Combining transgenic and gene-targeted mutations through the crossing of animals allows determination of the cooperative effects between oncogenes and tumor suppressor genes. Gliomas induced by the loss of tumor suppressors arise in a subset of mutant mice despite the alteration of genes in a large population of cells, suggesting secondary mutations are required for the actual tumor initiation. Transgenic mice overexpressing a constitutively active Ras (V^{12}Ras) under control of GFAP promoter were shown to develop high-grade astrocytic tumors depending on the dosage of the transgene given (21). Additional deletion of tumor supressor *Pten* shortens the tumor latency and give rise to high-grade astrocytomas (95). Similarly, transgenic mice overexpressing a conditionally active EGFR, *v-erbB*, under control of S100-beta promoter was developed. These mice gave rise to low-grade oligodendrogliomas by 12 months with 60% incidence. Additional *Ink4a/Arf* loss and heterozygotic loss of *p53* were shown to cooperate with *v-erbB* to increase the tumor incidence up to 100% in both cases (22).

Some transgenic models have taken advantage of the RB pathway. Components of the pathway such as *pRb*, *p107*, and *p130* were inactivated in astrocytes using a truncated SV40 T antigen (T_{121}) under control of GFAP promoter. The expression of the viral antigen in astrocyte precursors as well as in mature astrocytes resulted in astrocytomas by 10-12 months with 100% penetrance (96). Conditional deletion of *Pten* by Cre expression in *Pten* floxed animals shortened tumor latency in half while enhancing the tumor grade (24).

Germline modification strategies help identify mutations that contribute but are by themselves not sufficient for tumorigenesis. These models recapitulate human gliomas in various aspects and are applicable to testing therapeutic interventions. However, these strategies more accurately reflect inherited genetics disorders like retinoblastoma (RB mutation) and Li-Fraumani (p53 mutation) than the majority of brain tumors that arise from spontaneous mutations in somatic cells. Thus, molecular transformations that take place during gliomagenesis in humans may be better represented by a region-specific tumor formation via viral transduction system.

5.4. Animal modeling to study BTICs

The importance of neural progenitor cells that self-renew and accumulate oncogenic mutations in gliomagenesis is paramount. Several studies provide data to support this model. For instance, the modification of extracellular signals in committed oligodendroglial progenitors were demonstrated to revert them back to a precursor state with a multipotential neural stem-like phenotype, which resulted in chromatin remodeling and reactivation of Sox2, a primitive neural epithelial marker (97). It is also possible that gliomas arise from self-renewing normal stem/progenitor cells that accumulate mutations and are transformed by altering cell proliferative pathways.

Several reports have suggested a stem cell origin of gliomas. Abnormal hyperplasia were found in mice with excessive PDGF signaling in neural stem cells in the SVZ, thus suggesting a potential stem cell of origin in gliomagenesis (98). In addition, the SVZ was larger and more dense in mice bearing gliomas, and the expansion of this area was accompanied by cell migration via the olfactory bulb and the rostal migratory stream (99). Indeed, a sequential loss of *p53* and *Nf1* in mice resulted in high-grade astrocytomas localized specifically to the SVZ, the region of the brain where putative neural stem cells reside, but little or no tumor incidence in the cortex or striatum (93). This suggests that stem-like cells within the SVZ are more prone to developing astrocytomas induced by a deficiency of *p53* and *Nf1* or more susceptible to tumorigenesis by the microenvironment. In another study, when the SVZ cells were grown in serum-free condition from tumor-prone mice, these cells showed increased proliferation and self-renewal capabilities than cells taken from the wild type control (89). Furthermore, the forced expression of *PDGF* in GFAP- or Nestin-positive progenitors within the brain predisposed mice to a higher incidence of tumor formation when stem-like Nestin positive populations were targeted (88).

In contrast to what is seen with *p53* and *Nf1* mutant mice, gliomas induced by RCAS-*PDGF* occurred from the SVZ or the cortex at the same frequency. Stereotactic injection into the cerebellum generated tumors in most mice (100). Another study showed that the *EGFRvIII* expression with *Ink4a/Arf* and *Pten* loss in the mouse striatum can give rise to high-grade astrocytomas, complicating the matter involving the cell of origins for gliomas (90). Tumors from different locations in the brain have been shown to exhibit unique gene expression profiles even with identical histologies, implicating the involvement of region-specific progenitor cells during the development of these tumors (101-103). In addition, some subsets of gliomas can arise from both differentiated glial cells as well as differentiated or undifferentiated multipotent cells, which begs the question of whether the cell of origin defines the disease. It is still possible that the cell of origin may dictate the type of glioma whether it is of a primary/*de novo* type or progression from a secondary glioma. Therefore, further animal modeling is critical in dissecting these possibilities and investigating the involvement of the microenvironment and molecular and cellular abnormalities in the formation of gliomas.

6. CONCLUSIONS: IMPLICATIONS FOR PRECLINICAL TESTING

Despite the increasing amount of attention given to the field of CSCs, the clinical benefit from targeting this subset of cells remains elusive. The mainstays of the treatment include surgical resection followed by chemotherapy and radiation, but more rational, individualized therapeutic regimens are urgently needed. Recent success in targeting GBMs exhibiting *EGFRvIII* mutation and intact *PTEN* with erlotinib is leading the field into more targeted approaches in treating the disease (104). Currently, there is no single functional assay or a marker to define cells driving tumor recurrence after therapies or murine models to evaluate and project clinical outcomes of targeting these cells. Recently, the survival benefit for human GBM-bearing mice treated with BMPs was demonstrated (60). BMPs promote neural precursor cell differentiation into mature astrocytes. Interestingly, BMPs had differentiation-promoting effect on CD133+ cells, implying intact functional hierarchy in a population of heterogeneous tumor cells. This study further implies potential benefits in developing therapeutics geared towards promoting stem cell differentiation in efforts to eradicate CSCs that are believed to drive tumor recurrence.

Moreover, potential clinical benefits targeting Notch pathway have been strengthened by two recent studies. The blockade of the Notch pathway can eradicate CD133+ cells, resulting in the inhibition of neurosphere growth and clonogenicity as well as tumor growth in GBM xenografts. Local gamma-secretase inhibitor (GSI) treatment conferred survival benefits in mice bearing intracranial GBMs (105). Furthermore, GSI treatment can render BTICs more sensitive to clinically relevant doses of radiation implicating the role of Notch signaling in radioresistance of BTICs (106). Although there are numerous questions and challenges that remain to be answered, there seems to be an ongoing progress in the characterization of genetic abnormalities found in GBMs. Nevertheless, the better understanding of CSCs and the development of animal models that closely recapitulate the biology of this subset of cells will be critical in the development of potential therapeutics for the disease.

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Abbreviations: GBM: glioblastoma multiforme; CSC: cancer stem-like cell; BTIC: brain tumor-initiating cell; GFAP: glial fibrilary acidic protein; RB: retinoblastoma; RTK: receptor tyrosin kinase; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; CDK4: cyclin-dependent kinase 4; HGFR: hepatocyte growth factor receptor; PTEN: phosphatase tensin homolog; NF1: neurofibromatosis type-1; MMLV: moloney murine leukemia virus; ALV: avian leukosis

virus; RCAS: ALV-based replication competent virus; tv-a: tumor virus-A; ASLV-A: avian sarcoma and leukosis virus subgroup-A; HSC: hematopoietic stem cell; FACS: fluorescence-activate cell sorting; AML: acute myelogenous leukemia; NOD/SCID: non-obese diabetic, severe combined immunodeficient; SSEA-1: stage-specific embryonic antigen 1; ABC transporter: ATP-binding cassette transporter; MP: main population; SP: side population; SVZ: subventricular zone; SGZ: subgranular zone; VEGF: vascular endothelial growth factor; NO: nitric oxide; NOS: nitric oxide synthase; sGC: soluble guanylate cyclase; PKG: cGMP dependent protein kinase; SHH: sonic hedgehog; BMP: bone morphogenic protein; JAK: Janus-activated kinases; STAT3: signal transducers and activators of transcription 3; Interleukin 6: IL6; GSI: gamma-secretase inhibitor

Key Words: Glioblastoma Multiforme, Genetic Alterations, Murine Model, Cancer Stem Cell, Brain Tumor-Initiating Cell, Microenvironment, Review

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