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STEM CELL SIGNATURES IN GLIOMA

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To my family

ABSTRACT

Gliomas are the most common tumors of the central nervous system in adults. Glioblastoma, the most aggressive form, has a median survival of 15 months regardless of the standard treatment with surgery and temozolomide-based radiochemotherapy. Therefore, it is imperative to improve treatment options for patients with glioblastoma.

It has been suggested that the putative tumor stem cells in brain tumors are responsible for glioma initiation, development and resistance to conventional therapy and therefore may be a potential target for novel therapy.

By searching for stem cell gene expression signatures in human high-grade glioma cultures, two novel subsets (type A and type B) of glioblastoma cultures were identified with gene expression profiles similar to the recently described TCGA proneural/classical/neural and the mesenchymal subsets, respectively. The two subsets of cultures have different capacities in terms of tumorigenic potential, sphere forming ability and distinct tyrosine kinase inhibitor sensitivity profiles. The findings that the resistance of type A cultures to tyrosine kinase inhibitor mono-treatment is SOX2-dependent and can be overcome by a combination of treatments with PDGFR and IGF1-R inhibitors provide a novel strategy for tumor stem cell-targeting therapy.

A set of markers defining neural stem cells and pluripotent embryonic stem cells were tested on a series of human glioma tissues. Results demonstrated that besides deregulated expression of neural and pluripotent stem cell regulatory proteins, high-grade gliomas also show expression of mesodermal- and endodermal-specific transcription factors together with neural proteins. *In vivo* xenograft experiments indicated that the presence of pluripotency markers is dependent on signals from the tumor microenvironment.

An experimental mouse glioma model with human PDGFB overexpressed in astrocyte and astrocyte progenitor cells on a p53 null background was used for studying epigenetic changes in mouse brain tumors and in neural stem/progenitor cells during the pre-neoplastic stage. DNA hypomethylation and elevated Histone3 Lysine9 dimethylation (H3K9Me2) were detected not only in the tumors but also in samples of the adult frontal brain lateral ventricular wall (LVW) and in neurosphere cultures respectively, from the hGFAPpPDGFB/Trp53 null ($B^{+}p53^{-/-}$) brain during the pre-neoplastic stage. Thus, non-neoplastic but epigenetically disturbed LVW cells might be early targets of transformation in the development of glioblastoma.

Taken together, this work provides some new insights into stem cell related mechanisms involved in glioma development. Therefore, targeting brain cancer stem cells presents a new and potentially more effective therapy for glioma.

LIST OF PUBLICATIONS

- I. Daniel Hägerstrand*, **Xiaobing He***, Maja Bradic Lindh*, Saskia Hoefs, Göran Hesselager, Arne Östman and Monica Nistér
Identification of a SOX2-dependent subset of tumor- and sphere-forming glioblastoma cells with a distinct tyrosine kinase inhibitor sensitivity profile.
Neuro Oncol. 2011 Nov;13(11): 1178-91

- II. Johan Holmberg*[#], **Xiaobing He***, Inti Peredo, Abiel Orrego, Göran Hesselager, Christer Ericsson, Outi Hovatta, Sueli Mieko Oba-Shinjo, Suely Kazue Nagahashi Marie, Monica Nistér and Jonas Muhr[#]
Activation of neural and pluripotent stem cell signatures correlates with increased malignancy in human glioma.
PLoS One. 2011 Mar 31; 6(3): e18454

- III. **Xiaobing He**, Mohsen Karimi, Mingqi Qu, Anna Hedrén, Tomas J Ekström and Monica Nistér
Histone3 lysine9 methylation and DNA hypomethylation during the pre-neoplastic stage of experimental mouse glioma.
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LIST OF ABBREVIATIONS

ALV	Avian leucosis virus
BMP4	Bone morphogenetic protein 4
BTSC	Brain tumor stem cell
CDK4	Cyclin dependent kinase4
CIMP	CpG island methylation phenotype
CNS	Central nervous system
CSC	Cancer stem cell
DNMT1	DNA methyltransferase 1
DNMT3a	DNA methyltransferase 3a
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
FGF2	Fibroblast growth factor 2
FISH	Fluorescent <i>in situ</i> hybridization
GBM	Glioblastoma multiforme
GEMM	Genetically engineered mouse model
GFAP	Glial fibrillary acidic protein
HDAC	Histone deacetylase
hESC	Human embryonic stem cell
H3K9Me	Histone3 lysine9 methylation
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
LIF	Leukaemia inhibitory factor
LMW-TKI	Low molecular weight-tyrosine kinase inhibitor
LUMA	Luminometric methylation assay
LVW	Lateral ventricular wall
MADM	Mosaic analysis with double markers
mESC	Mouse embryonic stem cell
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MMLV	Moloney murine leukemia virus

NF1	Neurofibromin-1
NO	Nitric oxide
OPC	Oligodendrocyte precursor cell
PDGF	Platelet-derived growth factor
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma protein
RCAS	Replication competent virus
RTK	Receptor tyrosine kinase
SGZ	Subgranular zone
SOX2	Sex-determining region Y-box 2
SSEA-1	Stage-specific embryonic antigen-1
SVZ	Subventricular zone
TCGA	The Cancer Genome Atlas
TET	Ten-eleven translocation enzyme
TGF β	Transforming growth factor β
TIC	Tumor initiating cell
VEGF	Vascular endothelial growth factor
α -KG	α -ketoglutarate
2-HG	2-hydroxyglutarate
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine

1 INTRODUCTION

1.1 STEM CELLS

In a way, we may say that an organism's life begins with stem cells. In other words, the stem cell could be described as the "origin of life". Stem cells are undifferentiated and self-renewing cells that have the ability to give rise to multiple specialized cell types (differentiated cells) that carry out specific functions in the body. Stem cells play important roles starting from embryonic development (embryonic stem cells) through to adulthood (adult stem cells).

In terms of the differentiation potential, stem cells could be classified into totipotent, pluripotent, and multipotent stem cells (Fig 1). When a sperm and an egg meet, they form a fertilized egg called zygote. The zygote has the potential to form an entire functional organism and also the placenta. This cell is totipotent. When the cell continues to divide, about one week after fertilization, a small ball-like structure with a few hundred cells is formed. This is the blastocyst. The blastocyst has two layers. The outer layer is termed the trophoblast and will form the placenta, while a clump of cells inside the ball called the inner cell mass, will give rise to all the cell types of the body. Embryonic stem cells are derived from the inner cell mass of the blastocyst. As embryonic stem cells can form nearly any tissue of the body, they are described as pluripotent, not totipotent. When the pluripotent cells continue to divide, at around two weeks after fertilization, the cells differentiate into three primary germ layers (ectoderm, mesoderm, endoderm), and each of these layers will give rise to a different set of cell types. Cells from ectoderm will form skin, nervous system and parts of face and neck; cells from mesoderm will form muscles, blood, blood vessels and connective tissues; cells from endoderm will form the digestive and respiratory tracts including pancreas and liver. These less plastic and more differentiated stem cells within a specific tissue type are multipotent stem cells. An adult stem cell is a multipotent stem cell, and plays an important role in the growth, maintenance and repair of the body. Adult stem cells have been found in many different tissues such as blood, blood vessels, brain, skin, muscles, teeth, heart, gut, liver and breast.

Because of this self-renewal capacity and this plasticity and potential to develop into almost any type of cell, stem cells offer a great hope for potential application in regenerative medicine and tissue replacement therapy following injury or disease.

1.1.1 Embryonic stem cells

Embryonic stem cells (ESCs) are cells from the inner cell mass of the blastocyst. Two defining properties of ESCs are pluripotency: the capacity to give rise to cells representing all the three germ layers; and self-renewal capacity: the ability to generate a new pluripotent stem cell when undergoing division.

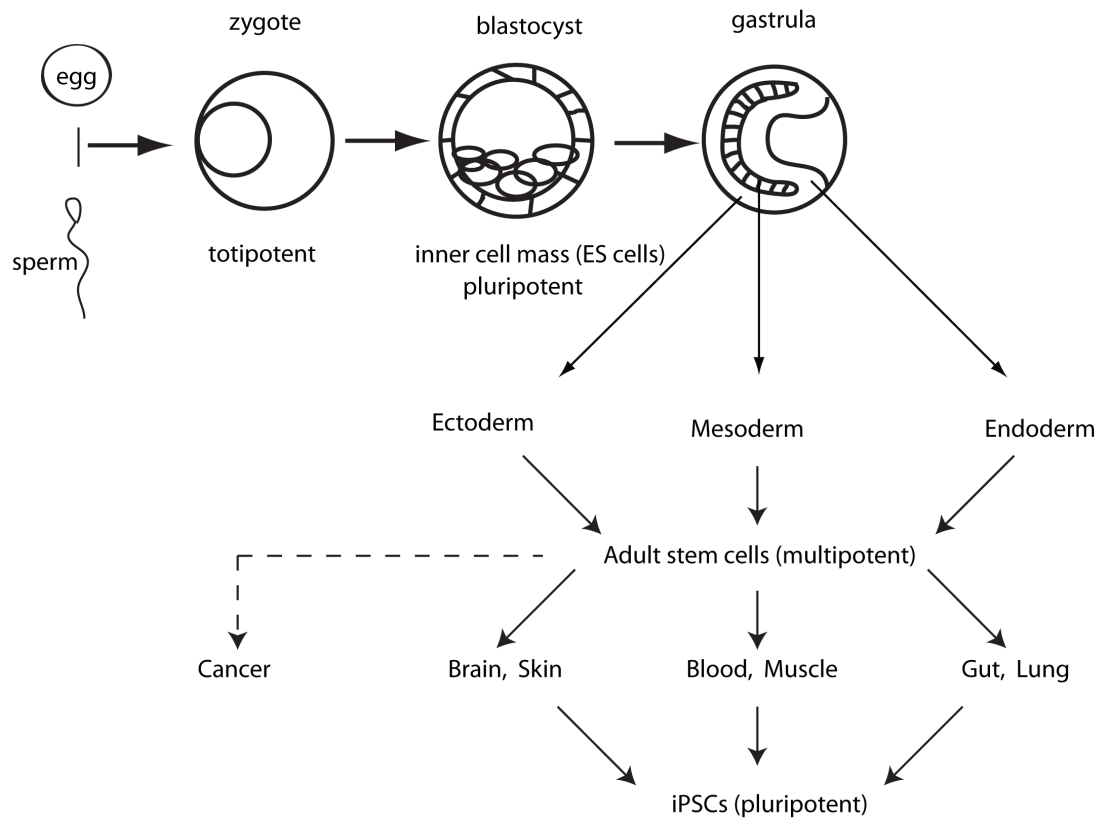


Figure 1. Classification of stem cells [modified from (Banerjee, 2011)]

1.1.1.1 Mouse embryonic stem cells

Mouse ESCs (mESCs) were first isolated from pre-implanted mouse blastocysts in early 1980's (Evans and Kaufman, 1981; Martin, 1981). With the help of leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4), mESCs could be propagated *in vitro*, while retaining pluripotency (Bradley et al., 1984; Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). The application of homologous recombination technology to mESCs makes it possible to manipulate the whole mouse genome and to study gene function *in vivo* in an intact organism (Capecchi, 2005).

1.1.1.2 Human embryonic stem cells

The derivation of human embryonic stem cells (hESCs) from human blastocysts provides a useful system to study early human embryonic development and also a valuable tool for potential application in regenerative medicine (Thomson et al., 1998). Similar to their mouse counterpart, cultured hESCs keep their self-renewal ability during the undifferentiated stage and the pluripotent capacity to differentiate into any cell type in the body. However, hESCs and mESCs differ in their morphology and growth rate. Further, hESCs need the basic fibroblast growth factor (bFGF) and activin/nodal for *in vitro* maintenance (Beattie et al., 2005; Xu et al., 2005). Several

hESC lines have been established and have shown no signs of differentiation, no chromosomal defects, while presenting self-renewal and pluripotent properties, high telomerase activity and the ability to form teratoma in immune deficient mice. These hESC lines are FDA-approved for research and clinical trials (Lu et al., 2009; Sharp et al., 2010).

1.1.1.3 Transcriptional regulatory network in ESCs

In ESCs, the self-renewing and pluripotent state is largely controlled by the core transcription factors Oct4, Sox2 and Nanog (Ng and Surani, 2011; Young, 2011).

Oct4, also known as POU5F1, belongs to the POU transcription factor family (Scholer et al., 1990). It recognizes an 8-bp DNA sequence found in promoter and enhancer regions of many genes. During early embryo development, the expression of Oct4 is mainly restricted to totipotent and pluripotent cell stages, that is, the zygote and the inner cell mass of the blastocyst (Pesce and Scholer, 2001). The essential role of Oct4 in development was first shown by studying the Oct4 knock-out mouse. The Oct4 deficient embryo failed to develop beyond the blastocyst stage due to the lack of a pluripotent inner cell mass (Nichols et al., 1998). Further, by quantitatively manipulating the expression level of Oct4, it was shown that ESCs need a critical level of Oct4 to maintain the stem cell state. A higher level would force ESCs to differentiate to mesoderm and endoderm lineages, while a lower level would lead to trophectodermal cells. Only the “normal” Oct4 level maintains the pluripotent and undifferentiated ESCs state (Niwa et al., 2000). It seems that Oct4 lies at the center of a gene regulatory program that maintains the pluripotency of ESCs.

Nanog is another key transcription factor that is involved in the maintenance of ESCs pluripotency. Nanog was cloned by two independent research groups simultaneously (Chambers et al., 2003; Mitsui et al., 2003). It is a homeobox transcription factor, dominantly expressed in the inner cell mass of the blastocyst. Germline loss of Nanog leads to early embryonic lethality, and down-regulation of Nanog will induce ESCs differentiation.

Sex-determining region Y-box 2 (Sox2) belongs to the family of high mobility group proteins (HMG). Besides its role in neurogenesis, Sox2 also takes part in the regulation of ESCs. Knock-out of Sox2 in mice is embryonic lethal (Avilion et al., 2003). Interestingly, overexpressed Oct4 could rescue the pluripotency of Sox2-null ESCs (Masui et al., 2007), indicating that the function of Sox2 in maintenance of the ESCs pluripotent state is through regulation of Oct4.

By using chromatin immunoprecipitation (CHIP) combined with genome-wide mapping technology, common target sites in the regulatory elements of Oct4, Sox2 and Nanog genes have been found (Boyer et al., 2005; Loh et al., 2006). It was suggested that Oct4, Sox2 and Nanog might form a regulatory circuit that governs pluripotency of ESCs. In this circuit, the three transcription regulators not only regulate each other, but regulate themselves as well. Additional pluripotency-related factors such as Smad1, Stat3 and Klf4 are also linked to this network (Ng and Surani, 2011).

The comparison between tumor cells and ESCs has for a long time been of great interest. The high proliferative capacity and phenotypic plasticity of tumor cells resemble much of the self-renewal capacity and pluripotency of ESCs. Embryogenesis is mirrored in an abnormal way by tumor development. A comprehensive gene set expression analysis suggested that the core ESCs signature (Oct4, Nanog, Sox2) and also their downstream target genes are closely related to histologically poorly differentiated aggressive human tumors, such as high-grade malignant estrogen receptor-negative breast cancer, glioblastoma and high-grade malignant bladder carcinoma (Ben-Porath et al., 2008). Other reports, including our own have also demonstrated that the pluripotent stem cell signature correlates with increased malignancy in human glioma (Holmberg et al., 2011). Respective down-regulation of Sox2, Nanog and Oct4 function could reduce the self-renewal capacity of human glioma cells (Du et al., 2009; Hagerstrand et al., 2011) and limit their tumor initiating potential in mouse brain (Gangemi et al., 2009; Zbinden et al., 2010).

1.1.2 Induced pluripotent stem cells

Knowing the key factors that govern pluripotency of ESCs has enabled the generation of a new type of pluripotent stem cells, the induced pluripotent stem cells (iPSCs). iPSCs are adult somatic cells that have been (genetically) reprogrammed to an embryonic-like pluripotent state by artificial introduction of key genes and factors that sustain the self-renewal and pluripotency of ESCs. The first mouse iPSC was generated in Yamanaka's lab in 2006 (Takahashi and Yamanaka, 2006). One year later and at about the same time, human iPSCs were generated independently by the Yamanaka's group and also by Thomson's group but with different combinations of key factors. Oct4, Sox2, Klf4 and c-Myc were used by the Yamanaka group, while Oct4, Sox2, Nanog and Lin28 were used by the Thomson group (Takahashi et al., 2007; Yu et al., 2007).

The generation of human iPSCs is a milestone in the stem cell research field. It introduced an approach not only for creating a new model system of human diseases but also for potential use in personalized regenerative medicine. Compared to hESCs, human iPSCs have similar phenotypes regarding morphology, gene expression pattern, and differentiation potential to all three germ layers when assayed by embryoid body formation *in vitro* and teratoma formation *in vivo* (Okita et al., 2007). Moreover, iPSCs overcome the ethical obstacles and pave the way for using human cells for custom-tailored cell-based therapy. So far, iPSCs have been generated from different cell types such as dermal fibroblasts, neural stem cells and keratinocytes (Aasen et al., 2008; Kim et al., 2009; Lowry et al., 2008).

Although remarkable progress has been made in reprogramming techniques over the years by using synthetic modified peptides or mRNA instead of retroviral or other genetic vectors for transduction, one should still bear in mind the risk of tumorigenicity of iPSCs when applying them into clinical settings. There are trials exhibiting promising evidence (Hanna et al., 2007; Raya et al., 2009), but iPSCs have the risk of inducing teratocarcinoma and somatic tumors (Ben-David and Benvenisty, 2011). More efforts need to be put into creating safe iPSC lines for future clinical applications.

1.1.3 Adult neural stem cells

Adult stem cells are tissue specific, are less plastic and are in a more differentiated state compared to ESCs. Adult stem cells divide asymmetrically in order to give rise to cells of the particular tissue they reside in and also to conserve the stem cell population, as such are multipotent and self-renewing. Differentiated cells generated from adult tissue stem cells are able to replace damaged cells to recover normal tissue function.

Adult neural stem cells (NSCs) are multipotent cells that reside in the adult mammalian brain. They are capable of long- term expansion and differentiation into neurons and glia. Thanks to pioneering studies decades ago, it is now very well recognized that neurogenesis occurs throughout life in almost all mammals including human (Altman and Das, 1965; Eriksson et al., 1998; Reynolds and Weiss, 1992; Richards et al., 1992).

Two neurogenic regions in the brain have been characterized where adult neural stem cells are located and active neurogenesis takes place. One is the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, where new dentate granule cells are generated; the other is the subventricular zone (SVZ) of the lateral ventricles, where new neurons are created and then migrate to the olfactory bulb through the rostral migratory stream. There are two schools on the identity and lineage relationship of NSCs in the brain. Alvarez-Buylla proposes that glial fibrillary acidic protein (Gfap) positive astrocytes in the SVZ represent the NSCs, also named B cells, from which transit-amplifying cells (C cells) are generated and in turn give rise to neuroblasts (A cells). These neuroblasts migrate along the rostral migratory stream to generate neurons in the olfactory bulb. Gfap⁺ B cells also give rise to oligodendrocytes in the nearby corpus callosum. Dentate gyrus neurons are generated by Gfap⁺ B cells in the SGZ (Alvarez-Buylla and Lim, 2004). Whilst, Gage (Ming and Song, 2011; Suh et al., 2007) proposes that the Sox2 protein expressed in non-radial cells identify active NSCs that give rise to neurons and glia in the adult SGZ, and it was claimed that the Sox2⁺ NSCs favor neuronal differentiation over astrocyte formation. These different views may reflect the coexistence of multiple types of NSCs and their plasticity in the adult brain.

Although it is not unique, Sox2 is a protein marker commonly used to identify NSCs. Sox2 belongs to the SoxB1 group (Sox1, Sox2, Sox3) of HMG transcription factors. Besides being expressed in ES cells, SoxB1 proteins were found in neuroepithelial cells during development and in neurogenic regions in the postnatal and adult CNS (Pevny and Placzek, 2005). *In vitro* gain-of-function analyses have indicated that Sox1-3 could sustain the self-renewal property of NSCs, while a dominant-negative version of these proteins would induce NSCs to exit the cell cycle and differentiate (Bylund et al., 2003; Graham et al., 2003). Further, *in vivo* gene targeting experiments demonstrated defective neurogenesis in the adult brain of Sox2 deficient mice (Ferri et al., 2004). In a very recent paper, Sox2 has been used as a single factor to directly reprogram mouse and human fibroblasts into multipotent NSCs (Ring et al., 2012).

The specialized microenvironments where NSCs reside are termed niches. Stem cell niches are comprised not only of stem cells and their progeny but also include the endothelial cells of the adjacent vessels and the basal lamina cells attached to stem cells. Within the stem cell niches, cells interact with each other, and they are exposed to

a variety of growth factors, and extra-cellular diffusible signals for feedback control of stem cell self-renewal and differentiation (Fuentelba et al., 2012). As suggested in the SVZ niche (Fig 2), the ependymal cells (E cells) that line the lateral ventricle are adjacent to Gfap⁺ stem cells (B cells), transit-amplifying cells (C cells) and neuroblasts (A cells). B and C cells also directly connect to the vasculature. Recent studies are indicating that the vasculature is an important component of the stem cell niche. It has been shown that the dividing neural stem cells and their progeny, transit-amplifying cells are tightly connected with blood vessels both during homeostasis and regeneration (Tavazoie et al., 2008).

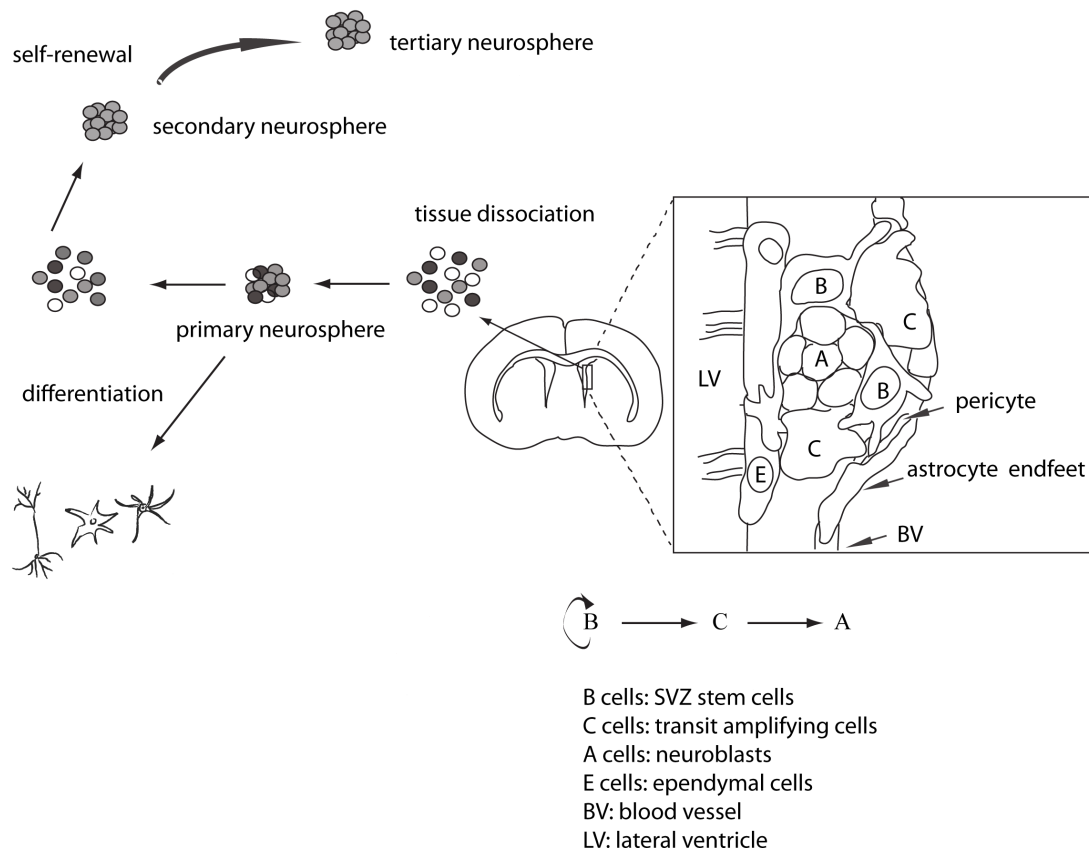


Figure 2. SVZ of the lateral ventricle and neurosphere assay
[modified from (Pastrana et al., 2011)]

Due to the lack of exclusive markers for the identification of stem cells in the brain, the study of the NSCs is usually based on functional assays, using both *in vivo* labeling and *in vitro* culture. In the early 90's, Reynolds and Weiss first cultured cells with stem cells properties as free-floating spheres from the adult brain, named neurospheres (Fig 2) (Reynolds and Weiss, 1992). They microdissected out the lateral wall subventricular zone (SVZ) of the lateral ventricles, dissociated the tissue cells to a single cell suspension, and plated cells in a non-adherent culture dish in serum-free medium supplemented with epidermal growth factor (EGF). After several days, cells formed spheres of proliferating cells. The spheres could be dissociated and cultured again under the same conditions to form secondary and tertiary spheres, which illustrated

their self-renewal property. When plated on an adherent surface in combination with withdrawal of EGF, the cells differentiated into neurons, astrocytes and oligodendrocytes, which showed their multipotency. This neurosphere assay has been widely used in stem cell biology, as it is a simple and effective assay to identify cells displaying the two distinguishing functional characteristics of stem cells, self-renewal and differentiation. However, the individual neurosphere is not a homogeneous sphere, but contains a mixture of stem cells, progenitors and differentiated cells. Practically, some critical points should be taken into consideration when applying the neurosphere assay in research, for example, the cell density, which is a strict parameter for clonality.

1.1.4 Cancer stem cells / tumor initiating cells

The concept of cancer stem cells (CSC) comes from the comparison of normal stem cells and cancer cells. The key to the cancer stem cell hypothesis is that not all the cells in a tumor are equal. Like many normal proliferative tissues, the bulk tumor comprises of self-renewing undifferentiated cells as well as differentiated cells. Furthermore, only a limited population of stem cell-like cells within the tumor may be responsible for tumor initiation, maintenance, resistance to treatment and recurrence. According to the proposed definition from the AACR Workshop on Cancer stem cells 2006 (Clarke et al., 2006), “a cancer stem cell is a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. Cancer stem cells can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor.” Therefore, the terms, CSC and tumor initiating cell (TIC), are interchangeably used.

The putative CSC was first identified in materials from acute myelogenous leukemia (AML) (Bonnet and Dick, 1997; Lapidot et al., 1994). It was demonstrated by Dick's group that only the CD34⁺CD38⁻ fraction of cells could initiate leukemic engraftment in immunodeficient mice. Years later, Clarke and his colleagues applied this idea to solid tumors. They showed for the first time that as few as 100 CD44⁺CD24⁻ cells could form tumors in a xenograft assay (Al-Hajj et al., 2003). Now, more and more evidence has been provided for the existence of CSCs in tumors from brain, colon, prostate, lung, pancreas, and other tissues (Collins et al., 2005; Eramo et al., 2008; Galli et al., 2004; Hermann et al., 2007; Li et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004).

The defined CSCs should be challenged with regard to the two hallmarks of normal stem cells, that is self-renewal and lineage capacity. The best functional assay that meets these criteria is the serial transplantation assay in immune-deficient mice *in vivo*. As a surrogate functional assay to identify CSCs *in vitro*, the tumor sphere assay, which is adopted from the neurosphere assay used for NSC research, has been employed quite often (Al-Hajj et al., 2003; Galli et al., 2004; Singh et al., 2004).

The CSC hypothesis proposes a hierarchical model of tumorigenesis, which challenges the previous stochastic or clonal model of evolution (assuming that the majority of tumor cells can act as TICs). The hypothesis may provide some explanations for the relapse of tumors that showed effective therapy response initially, since the CSCs

might survive through the so-called initially effective treatment phase that only ablated the non-tumorigenic tumor cells. CSCs then remain and initiate tumor formation again. According to this theory, a more effective cancer treatment would aim not merely at reducing the tumor bulk, but rather at targeting the key cells within the tumor, the CSCs.

When the term CSC or TIC is used, it does not relate to the question of cell of origin of cancer. CSC and cell-of-origin are two different concepts. Cell-of-origin is the normal cell that acquired the first cancer promoting mutation (Visvader, 2011). Meanwhile, the CSC or TIC is named after its capability of initiating a tumor when transplanted in animal models. The term initiating is a functional description of the cells in the transplantation assay, which are not necessarily the cell-of-origin of cancer. The cell-of-origin of cancer is still under debate. Any cell with abnormal proliferative property has the possibility to serve as a cell-of-origin of cancer. It could be a mutated tissue stem cell, or progenitor cell and it could be a de-differentiated tissue cell as well. Very recently, one study reported that during *in vitro* oncogenic transformation, a subgroup of human fibroblasts could be reprogrammed to CSC-like cells. These transformed CSCs could initiate and maintain tumor growth (Scaffidi and Misteli, 2011), which implies that the somatic cells have the potential to de-differentiate and acquire CSCs properties.

Cancer treatments and outcomes have improved in past decades, but challenges still remain. The CSC hypothesis provides an attractive new strategy. Targeting CSCs might improve the outcome of cancer treatments. However, the CSC hypothesis is still young, there are lots of challenges to meet, such as the technical limitations, non-specificity of CSC markers (Rahman et al., 2011) and the interconversion of CSCs and non-CSCs within a tumor etc. (Visvader and Lindeman, 2012). An open mind should be kept when considering the still un-matured CSC hypothesis.

1.2 BRAIN TUMORS

According to the most recent population-based incidence statistics in the US, the overall incidence of brain tumors is now 18.71 per 100,000 person/year. Among those, 7.19 per 100,000 person/year are malignant tumors (CBTRUS, 2011; Ostrom and Barnholtz-Sloan, 2011). Malignant brain tumors, account for only 1-2% of all cancers, and are considered rare, but have a high mortality rate.

1.2.1 Histological classification of human brain tumors

The classification of human brain tumors is mostly based on the histological features of each particular tumor type. Malignant gliomas, which account for more than 70% of all brain tumors in adults, are similar to glia. According to the World Health Organization (WHO), astrocytomas are classified into four malignancy grades: pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (GBM) (grade IV) (Louis DN, 2007; Louis et al., 2007).

Anaplastic astrocytomas feature increased cellularity, nuclear atypia, mitotic activity and some microvascular proliferation; Glioblastomas further contain elaborate microvascular proliferations and/or necrosis. Oligodendrogliomas and oligoastrocytomas are divided into two grades: oligodendrogliomas, oligoastrocytomas (grade II) and anaplastic oligodendrogliomas, anaplastic oligoastrocytomas (grade III). Oligodendrogliomas seem to respond better to medical intervention than other types of glioma (van den Bent, 2010). Although the WHO classification is mainly based on the histo-morphological changes, the classification is an indication of cellular differentiation status, malignant potential, response to treatment and patient survival.

1.2.2 Clinical subgroups of glioblastoma

GBM is the most common and most malignant tumor of the central nervous system (CNS) in adults. The overall median survival time for GBM patients is only 15 months (Van Meir et al., 2010; Wen and Kesari, 2008). From a clinical point of view, GBM could be grouped into two categories, primary and secondary. Primary GBMs develop *de novo*, without any evidence of a less-malignant precursor lesion, but this does not necessarily suggest a single-step transformation. Like other tumors, primary GBMs result from an accumulation of genetic and epigenetic alterations. Secondary GBMs develop progressively from low-grade diffuse astrocytoma (grade II) or anaplastic astrocytoma (grade III). The diagnosis of a secondary GBM needs clinical support such as neuroimaging or histological evidence of a progression from a less malignant astrocytoma. Compared to primary GBM, secondary GBM is rare, only represents 5-10% of all the diagnosed GBM cases. Primary and secondary GBMs constitute two distinct disease entities, and they display differences in terms of patients' age at diagnosis, genetic and expression profiles and response to therapy (Dunn et al., 2012). Evidence has accumulated that primary and secondary GBMs develop through different genetic pathways (Dunn et al., 2012). However, from the histo-pathological side, these two subtypes are indistinguishable.

1.2.3 Genomic alterations in glioma

The development of modern molecular technologies provides the opportunities for a detailed description of the molecular characteristics of gliomas. The detailed molecular mapping of malignant gliomas could not only define more accurately the specific subgroups within the same WHO category, but also lay the ground work for more rationally designed and personalized treatment.

The extensive identification of genomic alterations in gliomas has started to refine the classification of gliomas. Analysis of molecular markers in pathology clinics is under way to assist in diagnosis and predict clinical outcome of glioma patients.

1.2.3.1 Chromosome 1p/19q deletions in oligodendroglioma

The combined deletion/translocation of the short arm of chromosome 1(1p) and the long arm of chromosome 19 (19q) has been known for quite some time and linked to a

better prognosis and a better responsiveness of oligodendroglial tumors to certain chemotherapies (Bigner et al., 1999; Cairncross et al., 1998; Smith et al., 2000). Determination of 1p/19q status is recommended for all tumors with an oligodendroglial histo-morphology in routine clinical practice (Robertson et al., 2011).

1.2.3.2 Activation of growth factor pathways in glioma

Two decades of genomic studies of GBMs have demonstrated that both normal growth factor pathways and cell cycle check points are disrupted in malignant glioma. Recently, The Cancer Genome Atlas (TCGA) studies confirmed previously recognized genomic changes and also uncovered some new gene mutations in GBM (TCGA, 2008).

Over-activation of receptor tyrosine kinase (RTK) signaling pathways is one of the most common molecular alterations in GBM, usually caused by the amplification and/or overexpression of genes for both growth factors and their receptors, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGF) and its α -receptor (PDGFRA). Two other RTKs altered in GBM are ERBB2 (mutated in 8% of GBMs) and MET (amplified in 4% of GBMs). When a growth factor binds to its cell surface tyrosine kinase receptor, dimerization and transphosphorylation of receptor molecules occurs. Thus, downstream signaling pathways like PI3K/AKT and RAS/MAPK are activated as well. This occurs in 88% of GBMs, mediating the increased proliferation and survival of cells (TCGA, 2008).

Amplification and over-expression of EGFR is common in primary GBM (40%), but rare in secondary GBM (Ohgaki and Kleihues, 2011). Recent TCGA data showed overall EGFR alteration in 45% of GBMs (TCGA, 2008). All cases with amplification of EGFR also show EGFR overexpression, and 70-90% of GBMs with EGFR overexpression has EGFR gene amplification (Biernat et al., 2004). The most frequent mutant form of the EGFR is variant 3 (EGFRvIII) with deletion of exons 2 to 7. This mutation leads to a constantly active receptor independent of ligand and causes downstream mitogenic effects (Gan et al., 2009).

The PDGFRs are two other RTK receptors that have been found frequently deregulated in GBM. The PDGF family is composed of four ligands, PDGF A, B, C and D, and two receptors, PDGFR α and β . All PDGF ligands and receptors have been found expressed in glial tumors (Lokker et al., 2002; Nister et al., 1982; Nister et al., 1988), suggesting autocrine and paracrine loops stimulating glioma growth (Hermanson et al., 1992). PDGFR deregulation occurs in all grades of glioma (Fleming et al., 1992; Hermanson et al., 1996) and more commonly in secondary GBM compared to primary GBM. Essentially, the PDGFR α is expressed on tumor cells, while the PDGFR β is expressed on tumor vessels. Recent TCGA data confirmed that around 13% of GBMs have PDGFRA gene amplification (TCGA, 2008).

The phosphatidylinositol 3-kinase (PI3K) complex is composed of a catalytic protein encoded by PIK3CA and a regulatory protein encoded by PIK3R1. PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) to PIP3, that activates downstream molecules AKT/PKB (protein kinase B) and the mammalian target of

rapamycin (mTOR), which further stimulates cell growth and proliferation and inhibits apoptosis. Mutations both in the catalytic domain and the regulatory domain of PI3K have been found in glioma. Mutated PIK3CA was found in 15% of GBM (Samuels et al., 2004) and mutated PIK3R1 presents in 10% of GBM (TCGA, 2008). In physiological situations, the PIP3 level is kept relatively low by phosphatases, notably the phosphatase and tensin homolog (PTEN). Inactivation of PTEN would lead to up-regulation of PIP3 and further hyper-activate the PI3K downstream pathway. Indeed, PTEN is frequently mutated in primary GBM (36%), but rarely in secondary GBM (Knobbe et al., 2002; TCGA, 2008). Thus, this suggests two ways by which the PI3K pathway can become de-regulated in GBM, by hyper-activation of PI3K and inactivation of PTEN.

A RAS mutation is rarely found, in only 2% of GBM (TCGA, 2008). RAS is normally activated by RTKs and stimulates the downstream MAPK pathway. Neurofibromin-1 (NF1), a negative regulator of RAS, was recently found to be mutated and deleted in 18% of GBMs, and is recently regarded as a human GBM suppressor gene (TCGA, 2008).

1.2.3.3 Deregulation of the cell cycle in glioma

TP53 is a tumor suppressor gene, widely mutated and/or deleted in about 50% of human cancer (Hollstein et al., 1991). p53, referred to as the “guardian of the genome”, plays crucial roles in cell cycle arrest, apoptosis, senescence and differentiation in response to cell stresses such as hypoxia, DNA damage and oncogenic signaling (Vousden and Lane, 2007). The p53 level in cells is controlled by two critical regulators, MDM2 and p14ARF. MDM2 binds to p53 and blocks the ability of p53 to act as a transcription factor and degrades the protein, while ARF acts as an MDM2 antagonist and increases p53 levels (Soussi and Wiman, 2007). Once a cell has lost ARF activity to block MDM2 function, MDM2 will promote p53 degradation without control. P53 mutations are common in low-grade astrocytoma (Ohgaki and Kleihues, 2007) and were reported more frequent in secondary GBM than in primary GBM (Ohgaki et al., 2004; Ohgaki and Kleihues, 2005). Recent TCGA studies have demonstrated that P53 mutations and deletions are present in 35% of GBMs, amplification of MDM2 in 14% of GBMs and mutations or deletions of p14ARF in 49% of GBMs. The overall frequency of p53/MDM2/p14ARF pathway deregulation in GBM was found to be 87% (TCGA, 2008). MDM2 gene amplification occurs exclusively in those primary GBMs that lack a p53 mutation (Biernat et al., 1997; Reifemberger et al., 1993).

The master governor of the cell cycle clock is retinoblastoma protein (RB). The p16INK4a/CDK4/RB pathway regulates the G1 to S phase transit in the cell cycle. The CDK/cyclin D1 complex phosphorylates RB, hyperphosphorylated RB releases the E2F transcription factor that activates the genes involved in G1/S transition. P16INK4a inhibits the CDK/cyclin D1 complex, thereby inducing hypophosphorylated RB which binds to E2Fs, thus inhibiting the G1/S transition. TCGA has shown that mutations or homozygous deletions in GBM for RB and p16INK4a genes was 11% and 52% respectively, amplification of CDK4 and CCND2 was 18% and 2%, respectively. The overall frequency of alteration of the p16INK4a/CDK4/RB pathway was 78% (TCGA,

2008), including primary and secondary GBM. Deregulation of the p16INK4a/CDK4/RB pathway was also frequent (65%) in anaplastic oligodendroglioma, but rare (4%) in oligodendroglioma (Watanabe et al., 2001).

1.2.3.4 IDH1 mutation

Isocitrate dehydrogenases (IDHs) are a group of enzymes involved in multiple metabolic processes and protect cells from oxidative stress (Weller et al., 2011). There are three IDH variants in humans. IDH1 is present in the cytosol, while IDH2 and IDH3 are located in mitochondria. Only IDH1 and IDH2 mutations are known to be involved in human cancer. IDH1 mutations impair the enzyme's affinity for its substrate and inhibit normal IDH1 activity (Yan et al., 2009).

IDH1 mutations were first identified by a comprehensive genomic analysis of human GBM (Parsons et al., 2008). Mutations in the active site of IDH1 were found in 12% of GBM patients. Follow-up studies have shown that IDH1 mutations are frequent (>80%) in diffuse astrocytoma, anaplastic astrocytoma, oligodendroglioma, anaplastic oligodendroglioma, oligoastrocytoma and secondary GBM (Balss et al., 2008; Watanabe et al., 2009). IDH1 mutations are associated with younger age, secondary GBM and an increase in overall survival. The most common mutation found in IDH1 is the R132H mutation. On the other hand, IDH1 mutations are rare (<5%) or even absent in pilocytic astrocytoma and primary GBM. IDH1 mutations could not be found in ependymoma or other tumors in or outside of the CNS (Yan et al., 2009).

The high mutation frequency in low-grade glioma and secondary GBM suggests IDH1 mutation could be adopted as a useful diagnostic mark to distinguish different types of low-grade gliomas. The majority of low-grade diffuse astrocytomas carry p53 and IDH1 mutations, while the majority of oligodendrogliomas have IDH1 mutation plus 1p/19q loss (Watanabe et al., 2009). Therefore, the molecular classification of glioma by analysis for IDH1 mutation, p53 mutation and 1p/19q loss is becoming more and more accepted in clinical pathology.

1.2.4 Epigenetic alterations in glioma

Epigenetic changes such as histone modifications and DNA hyper-/hypo-methylation changes are known hallmarks of human cancer. The cancer-specific DNA methylation changes include global DNA hypomethylation and gene-specific promoter hypermethylation (Jones and Baylin, 2007) as well as differential methylation of CpG island shores (Irizarry et al., 2009). Global DNA hypomethylation correlates with chromosomal instability (Eden et al., 2003), while promoter CpG islands hypermethylation generally results in transcriptional silencing of the respective genes. Gene-specific promoter hypermethylation has been widely shown to serve as a biomarker for prognosis and prediction of therapy responses (Laird, 2003) in different types of cancer including GBMs and other subtypes of gliomas (Nagarajan and Costello, 2009; Uhlmann et al., 2003). Histone modifications such as changes in histone acetylation and methylation have also been implicated in tumorigenesis (Baylin and Jones, 2011).

1.2.4.1 Global DNA hypomethylation in GBM

Genome-wide reduction in 5-methylcytosine, global DNA hypomethylation, has been documented in many different malignancies compared with matching normal tissues (Ehrlich, 2002; Feinberg and Tycko, 2004) and is associated with the degree of tumor aggressiveness in a mouse skin cancer model (Fraga et al., 2004). In primary GBM, global hypomethylation occurs at a high frequency (80%), but the level of hypomethylation varies between GBM samples. The more severe the global hypomethylation, the more proliferative is the GBM (Cadieux et al., 2006).

The mechanism by which global DNA hypomethylation contributes to tumorigenesis could be through oncogene activation and promotion of chromosome instability (Cadieux et al., 2006). The induced global DNA hypomethylation by loss of DNA methyltransferase 1 (DNMT1) function resulted in increased genomic instability and thus accelerated tumor incidence in a mouse sarcoma model (Eden et al., 2003). The molecular consequences of global DNA hypomethylation in GBM are not yet understood.

1.2.4.2 Gene specific promoter DNA hypermethylation in GBM

Gene-specific DNA hypermethylation mostly occurs at CpG islands of gene promoters. CpG islands (500bp-1kb) are regions with more than 5 times more abundant CpG nucleotides than the rest of the genome. Genes such as TP53, PTEN, RB, CDKN2 that are responsible for cell cycle control and apoptosis have been reported with promoter hypermethylation in many types of tumors including gliomas (Amatya et al., 2005; Baeza et al., 2003; Bello and Rey, 2006; Costello et al., 1996; Nakamura et al., 2001). A study has also shown that in GBM, transforming growth factor β (TGF β) promotes cell proliferation through the epigenetic regulation of the PDGFB gene promoter (Bruna et al., 2007). It has also been shown that secondary GBM has a higher frequency of promoter hypermethylation than primary GBM (Ohgaki and Kleihues, 2007).

1.2.4.3 Silencing of the MGMT gene

Gene-specific promoter hypermethylation can affect the sensitivity of GBM patients to chemo- and radiotherapy. The best example of this is O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation.

MGMT is a DNA repair enzyme that is associated with resistance to alkylating agents used in cancer therapy. MGMT removes alkyl groups from the O⁶ position of guanine. Cells without MGMT allow accumulation of O⁶-alkylguanine in DNA, which triggers mismatch repair and induces DNA damage (Weller et al., 2010). Hypermethylation of the MGMT gene promoter, leading to transcriptional silencing of the gene, occurs in multiple human tumors including glioma (Esteller et al., 1999). GBM patients with MGMT promoter methylation show sensitivity to alkylating agents such as temozolomide, and the methylation status also predicts better prognosis (Hegi et al., 2005). MGMT promoter methylation has now been established as an independent

prognostic marker for GBM patients receiving chemotherapy and radiotherapy (Stupp et al., 2009).

1.2.4.4 CpG methylator phenotype in glioma

DNA hypermethylation in gene promoter regions is regarded as a common feature of cancer. The global pattern of CpG island hypermethylation in human cancer has been described as the CpG island methylator phenotype (CIMP). Cancers with the CIMP⁺ phenotype represent a distinct group with distinct clinical and molecular features (Issa, 2004).

The first CIMP phenotype was found in colorectal cancer (Toyota et al., 1999), and subsequent studies also confirmed CIMPs in other types of cancer (Issa, 2004). Recently, studies on the large collection of GBM samples through TCGA have demonstrated the existence of a glioma CIMP (G-CIMP) subgroup of GBMs (Noushmehr et al., 2010). Furthermore, it was found that the G-CIMP glioma is more prevalent among low-grade gliomas than among high-grade and is tightly associated with IDH1 mutations. Patients with the G-CIMP phenotype are usually diagnosed at a younger age and display significantly improved outcome. Thus, G-CIMP represents a distinct subgroup of human gliomas based on the molecular and clinical characteristics.

1.2.4.5 Histone modification changes in glioma

Histones and DNA comprise the nucleosome, the basic unit of chromatin. The N-terminal tails of histones can be post-translationally modified, by methylation, acetylation and other types of modifications. The histone modification status influences the overall chromatin structure, further having positive or negative effects on gene transcription. Histone acetylation, for example, is usually associated with active gene transcription, while histone methylation could be either activating or repressing gene expression depending on the specific methylation site (Baylin and Jones, 2011). All these histone modifications are governed by a series of enzymes such as histone methyltransferases, demethylases, acetyltransferases, deacetylases (HDAC) etc.

Several studies have shown aberrant HDAC expression and histone modification patterns in gliomas and other types of brain tumors (Campos et al., 2011; Liu et al., 2010; Lucio-Eterovic et al., 2008). Whole genome sequencing revealed that histone-lysine N-methyltransferase genes and the H3F3A gene which encodes histone H3.3 are mutated in childhood glioblastoma and medulloblastoma (Schwartzentruber et al., 2012; Wu et al., 2012). Further, amplification and deletion in genes regulating histone3 lysine9 (H3K9) methylation were identified in medulloblastoma, implying that defective control of the histone code contributes to the pathogenesis of medulloblastoma (Northcott et al., 2009).

1.2.4.6 IDH1 mutation and the altered epigenome of glioma

IDH1 mutations have been identified in about 75% of low grade gliomas and secondary GBM (Yan H, 2009 N Engl J Med), and are increasingly recognized as valuable diagnostic and prognostic markers for gliomas.

Normally IDH1 catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG). IDH1 mutations, however, lead to reduced IDH1 activities, resulting in decreased α -KG and α -KG-dependent prolyl hydroxylase (PHD) activity and further to an increase in HIF-1 α , a PHD substrate (Zhao et al., 2009).

In addition, the mutant IDH1 gains the function to catalyse the α -KG to 2-hydroxyglutarate (2-HG), resulting in accumulation of 2-HG in gliomas. 2-HG is an antagonist of α -KG. It competitively inhibits the α -KG-dependent dioxygenases, including histone demethylases and the ten-eleven translocated (TET) family of oxidizing enzymes, leading to increase of histone methylation and decrease of the 5-hydroxymethylcytosine (5-hmC) level in gliomas (Xu et al., 2011). 5-hmC is generated by conversion from 5-mC by the TET family of oxidizing enzymes, and has been identified as an epigenetic phenomenon occurring during embryonic stem cell differentiation and human cancer (Haffner et al., 2011; Jin et al., 2011; Koh et al., 2011). Therefore, the tumor-derived IDH1 mutations alter the DNA methylation pattern and impair histone demethylation, further blocking the differentiation of non-transformed cells (Lu et al., 2012).

1.2.5 Molecular sub-classification of glioma

The current suggested molecular sub-classification of gliomas is based on transcriptional signatures. These gene expression signatures combined with DNA sequence, gene copy number and methylation data provide rich information concerning the underlying patterns of signaling aberrations, which in turn can be used to predict the clinical prognosis for individual patients.

In recent years, the existence of several subtypes of GBM has been proposed based on their transcription signatures. Two of the studies in particular have received a lot of attention (Phillips et al., 2006; TCGA, 2008).

In 2006, Philips et al., first reported on the identification of three prognostic subgroups of GBM samples, named *Proneural*, *Proliferative* and *Mesenchymal*. The *Proneural* subgroup expresses genes associated with neurogenesis and has a better prognosis, while the *Proliferative* and *Mesenchymal* subgroups are characterized by markers of cell proliferation and mesenchymal phenotype respectively, both with poor prognosis. The *Proliferative* subgroup has an increased MIB-1 labeling index, whereas the *Mesenchymal* subgroup shows increased angiogenesis.

More recently, gene expression data from TCGA suggested four transcriptional subsets: *Proneural*, *Mesenchymal*, *Classical* and *Neural*, three of those are further connected with distinct patterns of genetic alterations (Verhaak et al., 2010). The *Proneural* subset

includes samples with amplification of PDGFRA, IDH1 mutations and TP53 loss/mutation; The *Mesenchymal* subset is associated with loss/mutation of NF1, TP53 and CDKN2A (INK4a/ARF); The *Classical* subset has EGFR amplification and deletion of PTEN, p16. The fourth subset, the *Neural* subset, shows a diversified genotype and has characteristics in common with normal neural developmental lineages.

The proposed sub-classification of gliomas by Philips et al. and the TCGA was based on different sample sets and different methodologies. Both have their prognostic and predictive values. The concordance of the two classification sets is the *Proneural* and *Mesenchymal* subgroups, which reflects the robustness of the *Proneural* and *Mesenchymal* signatures in GBM (Huse et al., 2011). Transcriptional signature sub-classification has not been widely applied in clinics. This could be due to the lack of consensus on the exact nature of the subgroups and also to the lack of effective therapy for any specific subtype (Huse et al., 2011).

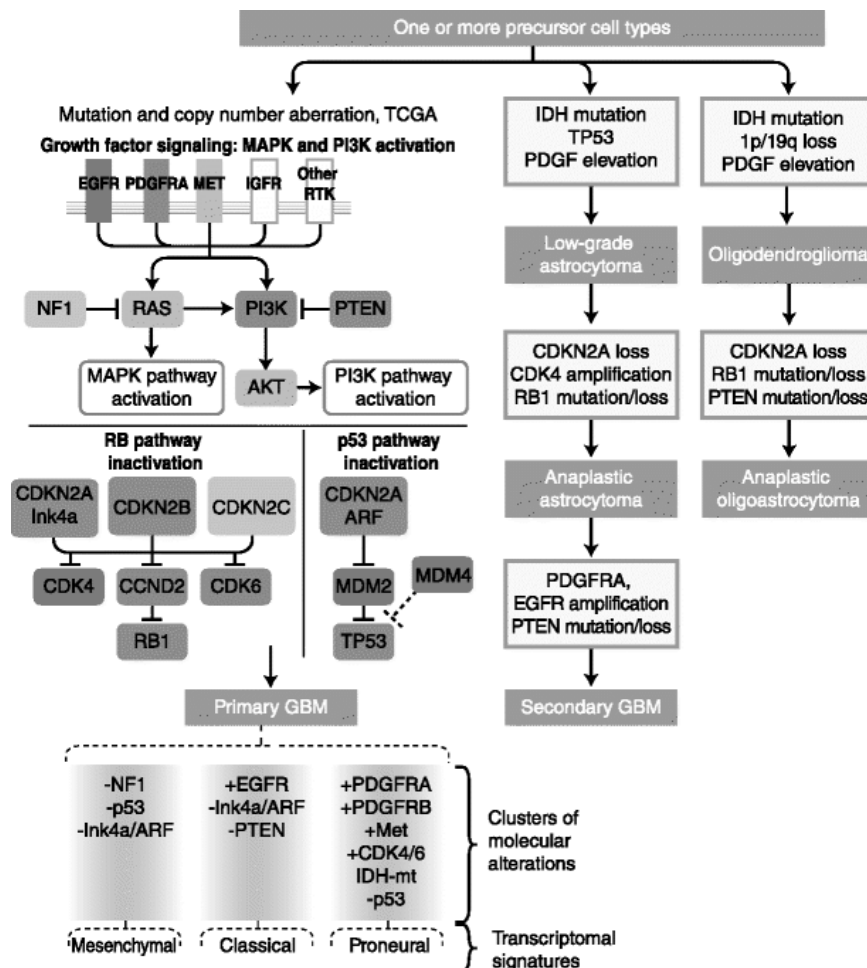


Figure 3. Molecular sub-classification of glioma [copied with permission from (Brennan, 2011)]

1.3 MOUSE MODELS FOR GLIOMA RESEARCH

Experimental mouse models are widely used in cancer research as a useful system to understand basic tumor biology and develop new approaches to conquer cancer. Basically, these *in vivo* mouse model systems fall into two categories: implanting human tumor cells into mice and creating tumors in mice *de novo*.

1.3.1 Implantation models

In the implantation model, tumor cells can be injected back to the same species (allografts) or implanted to a different species (xenografts). Xenograft models can also use orthotopic xenografts (in the original site) or heterotopic xenografts (in a site different from the original tumor location, often subcutaneously) in immunodeficient mice. Orthotopic xenograft GBM models have been used in many studies concerning GBM cancer stem cells and their microenvironment (Inda et al., 2010; Singh et al., 2003; Singh et al., 2004). It has been shown that tumors induced from orthotopic injections with the cells freshly isolated from patient samples propagated in conditioned media without serum but with growth factors were more close to the phenotype and genotype of the primary tumors when compared with the tumors generated from cells maintained in regular serum containing media (Lee et al., 2006).

There are some drawbacks with xenograft models. Most of the intracranial injections are done in immunocompromised mice, thus this *in vivo* assay will neglect the importance of immune surveillance in tumor development. In addition, the tumor initiation process in xenograft models differs considerably from spontaneous tumor development.

However, brain tumors generated from implanted tumor cells are highly reproducible, some with high growth rate. Serial transplantation of brain tumor cells *in vivo* has been taken as a golden standard for the functional definition of brain tumor stem cells (BTSCs) in GBM (Vescovi et al., 2006) and yields valuable information regarding cancer stem cell biology in GBM.

1.3.2 Genetically engineered mouse models of brain tumors

Another approach to modeling brain tumors in animals is to use genetically engineered mouse models (GEMMs), which mimic genetic changes in patients.

GEMMs can be generated by germline modifications, such as by transgenic techniques (gain of function) and gene targeting (loss of function). Transgenic mouse models of glioma usually use a cell type-specific promoter to drive expression of an oncogene. The cDNA of the gene of interest, which is introduced into an expression vector under the GFAP or Nestin promoter, is microinjected into a fertilized oocyte and subsequently randomly incorporated into the host genome. Gene targeting, on the other hand, is using homologous recombination technique to manipulate the gene of interest, by “knock-in” or “knock-out”. With the development of the Cre-loxP system,

conditional deletion of a gene of interest can be made tissue-specific. By cross-breeding the transgenic and gene-targeted mouse strains, the co-operative effects of oncogenes and tumor suppressor genes can be determined. Many transgenic or gene targeting malignant glioma mouse models have been generated and have provided exciting results (Ding et al., 2001; Reilly et al., 2000; Wang et al., 2009; Zhu et al., 2005).

GEMMs can also be achieved with somatic cell gene transfer techniques by using retroviral transduction. One technique uses the replication competent Moloney Murine Leukemia Virus (MMLV) in combination with the wild-type MMLV helper virus to deliver the targeted gene to proliferating cells in newborn mouse brains. However, the origin of the infected cell is unknown (Uhrbom et al., 1998). Another approach is using an avian leucosis virus (ALV)-derived replication competent virus (RCAS) together with its receptor tumor virus-A (tv-a) to transfer genes to selected cell types. Normal mammalian cells do not express the tv-a receptor. Transgenic tv-a mice have been designed to be controlled by different cell type-specific promoters such as the Gfap or Nestin promoter. By injection of RCAS virus with the gene of interest to the tv-a mouse brain, the RCAS infection will be restricted to tv-a expressing cells (Holland et al., 1998; Holland and Varmus, 1998). The RCAS-tva model could also be combined with the Cre-loxP system or gene knock out mouse lines to allow postnatal over expression of oncogenes and/or deletion of tumor suppresser genes in a cell type-specific manner (Hu et al., 2005; Uhrbom et al., 2002).

Recently, using other viral transduction systems like adenoviral (Alcantara Llaguno et al., 2009; Zhu et al., 2009) and lentiviral vectors (Marumoto et al., 2009) combined with the Cre-loxP system to express the genes of interest, more region- and cell type-specific glioma mouse models could be established (Marumoto et al., 2009).

1.4 BRAIN TUMOR STEM CELLS – POSSIBLE TARGETS FOR GLIOMA THERAPY

1.4.1 Brain tumor stem cells

Many brain tumors, especially GBMs, are heterogeneous. They often contain undifferentiated cells as well as differentiated cells. Putative BTSC, CSCs in brain tumors, might be responsible for tumor initiation, formation and maintenance of heterogeneity of the tumor.

1.4.1.1 Identification of BTSCs in brain tumors

The BTSCs in brain tumors were first identified based on the neurosphere assay that was adopted from the studies on NSCs. Isolated brain tumor cells were cultured in serum free media, in the presence of EGF and bFGF growth factors, forming floating spheres from single cells (Galli et al., 2004; Hemmati et al., 2003; Singh et al., 2004). These tumor sphere cells express NSC/progenitor cell markers such as CD133, SSEA1, Nestin, Sox2, BMI1 and Musashi (Dahlstrand et al., 1992; Favaro et al., 2009; Stojnik

et al., 2007), but not differentiated neural cell markers. They can be partially differentiated to neuronal and glial cell lineages in the presence of serum.

Subsequent studies used CD133 (Prominin 1) as a marker to isolate BTSCs from GBMs (Galli et al., 2004; Singh et al., 2004). It was found that only the fractions of CD133⁺ cells could give rise to tumor xenografts in mice. However, these results have been challenged by other studies showing that also CD133⁻ cells can be tumorigenic (Beier et al., 2007; Wang et al., 2008). Several groups reported that only around 60% of primary GBM samples contain CD133⁺ cells, meaning that CD133 may not be a universal enrichment marker for BTSCs (Beier et al., 2007; Joo et al., 2008; Ogden et al., 2008). In search of an alternative marker, stage-specific embryonic antigen-1 (SSEA1) was proposed as a more general enrichment marker for BTSCs in GBM after demonstrating that SSEA1⁺ cells fulfilled the criteria for BTSCs and were present in almost all tumor samples analyzed (Son et al., 2009).

1.4.1.2 Cell of origin of BTSCs

The cell-of-origin of BTSCs is a matter of debate although there is accumulating evidence for the existence of BTSCs in brain tumors. BTSCs are defined as such because they share some properties with adult neural stem cells in the brain and have self-renewal and *in vivo* tumorigenic properties. Nevertheless, NSCs are attractive candidates as the cell-of-origin of BTSCs. NSCs are slowly dividing cells and have a long life span, which probably allow them to accumulate enough mutations during the life time of an individual for neoplastic transformation to occur (Chen et al., 2012). There is a considerable similarity between NSCs and BTSCs in terms of the signaling pathways, cell surface markers, invasive ability and self-renewal capacity (Varghese et al., 2008).

Many human malignant gliomas locate very close to the lateral ventricles (Barami et al., 2009), which supports the idea that the SVZ is a sensitive site for tumorigenesis. However, there are also many tumors arising in locations distant to the SVZ (Behin et al., 2003). This could be explained by the asymmetric division of BTSCs into two non-identical daughter cells, where one may remain in the SVZ, and the other may migrate to a distant site and form a tumor mass there (Vescovi et al., 2006).

The use of animal models has greatly facilitated cell-of-origin studies. Increased proliferative activities of neurospheres from p53^{-/-} mice suggested an expansion capacity of the NSC pool in the SVZ, making it more vulnerable to malignant transformation (Gil-Perotin et al., 2006; Meletis et al., 2006). Further studies showed that exogenously added PDGF could induce aberrant proliferation of PDGFR α ⁺ SVZ cells to form hyperplastic but reversible lesions with glioma features (Jackson et al., 2006). Furthermore, direct introduction of initiating mutations into specific cell types using different promoters active during embryonic or early postnatal stage, such as Gfap and Nestin promoters, results in obvious glioma formation (Holland et al., 2000; Zheng et al., 2008; Zhu et al., 2005). Conditional deletion of Nf1 in the Gfap⁺ cell compartment on a p53 null background leads to 100% penetration of GBM in mice. Interestingly, early neoplastic changes were observed in the SVZ before tumors appeared, implying that transformed cells come from Gfap⁺ stem cells/progenitors in

the SVZ (Zhu et al., 2005). Recently, by using a stereotactic viral delivery method, it was possible to specifically target adult NSCs to address their capacity to initiate glioma formation. Malignant glioma formation was observed upon introduction of Nf1, p53 and/or Pten tumor suppressor mutations in neural stem/progenitor cells through a tamoxifen-inducible Nestin-driven Cre transgene (Alcantara Llaguno et al., 2009). Moreover, stereotactic delivery of Cre-expressing virus into the SVZ of adult mice with different combinations of mutated tumor suppressor genes such as Rb/p53/Pten results in tumor development. Interestingly, when virus was transduced into more differentiated brain regions such as the cerebral cortex, no tumors formed (Jacques et al., 2010). These data suggested that malignant gliomas more likely originate from neural stem/progenitor cells rather than from differentiated cells.

Although an increasing amount of evidence show that SVZ neural stem/progenitor cells are the most likely cell-of-origin of many malignant gliomas, the possibility that de-differentiation of mature glial cells generates more immature stem-like cells or restricted progenitor cells serving as the origin can not be excluded. More differentiated brain cells can acquire not only stem-like features but also the capacity to induce tumor formation (Zaidi et al., 2009). In a recent MADM (Mosaic analysis with double markers) mouse model, it was shown that malignant transformation only occurred in the oligodendrocyte precursor cells (OPCs) when p53 and NF1 were homozygously mutated in NSCs (Liu et al., 2011).

1.4.1.3 The microenvironment of BTSCs

One of the hallmarks of malignant behavior of glioma is the microvascular proliferation, providing nutrition to the tumor and contributing to tumor progression and invasion. Just like the normal stem cell compartment, the distribution of the putative BTSCs in the tumor mass is not random. It has been shown that BTSCs are often located in a special environment called the tumor perivascular niche, a microvascular proliferation zone that mimics the normal NSC niche (Calabrese et al., 2007; Charles et al., 2010).

The brain tumor perivascular niche is heterogeneous. It may be composed of different cell types including astrocytes, endothelial cells, pericytes, fibroblasts, microglia, differentiated tumor cells and BTSCs. The BTSCs secrete high levels of vascular endothelial growth factor (VEGF) to build up a surrounding microvasculature (Bao et al., 2006). Endothelial cells closely interact with BTSCs and secrete factors maintaining BTSCs properties (Calabrese et al., 2007). One of the endothelial cell-derived factors could be nitric oxide (NO) (Charles et al., 2010). In a PDGF-induced mouse glioma model, NO secreted by endothelial cells promoted stem cell like characteristics in cells present in the perivascular niche, enhancing this neurosphere forming capacity in primary cultures and their *in vivo* tumorigenic capability. This effect was mediated through the cGMP-dependent protein kinase (PKG) (cGMP/PKG) and the Notch pathway. When tumors grow and invade the surrounding tissues, they usually recruit external vessels for blood supply. More recently, two groups simultaneously found that BTSCs could give rise to endothelial cells that line the tumor vasculature (Ricci-Vitiani et al., 2010; Wang et al., 2010). Moreover, the tumor-derived endothelial cells were found to lack VEGF receptors and were therefore resistant to anti-VEGF therapy of the

experimental GBM tumors (Sodaa Y and Sandra Pastorinod, 2011). These findings describe a new cross talk between BTSCs and endothelium in the perivascular niche, a new way of tumor vascularization, and a potential mechanism of resistance to anti-VEGF therapy in GBM, and provide significant implications for future strategic therapy of GBMs.

Oxygen is also a critical component of the perivascular niche in brain tumors (Mohyeldin et al., 2010). Oxygen tension is strictly regulated in physiological situations. Hypoxia (low oxygen tension) is important in maintaining stem cells in an undifferentiated state. Hypoxia maintains the self-renewal capacity of ES cells and prevents NSCs from differentiating (Clarke and van der Kooy, 2009; Simon and Keith, 2008). Apart from the microvascular proliferation, pseudopallisading necrosis is another major feature of GBM, which is promoted by hypoxia. This hypoxic necrotic region within the tumor, usually located distal from the vasculature, is due to a rapid growth of the tumor mass and lack of an adequate vascular supply. Recently, hypoxia was proposed to define a secondary niche for BTSCs (Li et al., 2009; Seidel et al., 2010). The BTSCs in the hypoxic niche were found to be mainly regulated by HIF-2 α . Targeting the HIF-2 α pathway inhibited BTSCs self-renewal, proliferation and survival *in vitro* and decreased tumorigenic potential *in vivo*.

Interestingly, both hypoxia and NO from the perivascular niche not only affect cancer stem cell self-renewal and tumor growth, but was also shown to reprogram non-stem cancer cells towards a stem-like phenotype (Charles et al., 2010; Li et al., 2009), suggesting that tumor cells have a higher degree of plasticity compared to normal tissue cells. Tumor cells can dramatically change their phenotype depending on the microenvironment. Therefore, tumor targeting may not only focus on cancer stem cells, but the BTSC niche and the non-BTSC cancer cells should be targeted as well.

1.4.1.4 Epigenetic origin of BTSCs

Given the idea that CSCs give rise to other differentiated non-CSCs within the tumor, this type of cellular heterogeneity of tumors might be initiated by epigenetic reprogramming (Vincent and Van Seuning, 2012). It would, for example, be interesting to compare the epigenetic profile between CSCs and non-CSCs to see if differences exist. There are some initial reports showing disturbed level of DNA methylation and H3K27me3 repressive histone marks in putative breast CSCs compared to their non-CSCs (Yasuda et al., 2010). Also, a genome-scale histone reprogramming profile during epithelial-to-mesenchymal transition (EMT) was recently presented (McDonald et al., 2011). A research goal of many groups is to discriminate tumorigenic CSCs from their differentiated non-tumorigenic counterparts based on their epigenetic landscapes, so that the epigenetic modifications of CSCs could be taken as potential targets for therapy.

1.4.2 Treatment of glioblastoma

1.4.2.1 *Conventional treatment*

Standard treatment for newly diagnosed malignant glioma is surgery plus radiotherapy and chemotherapy. Surgical resection is the first line of treatment. However, due to the infiltrative nature of GBM, residual tumor cells usually remain. The addition of radiotherapy can increase survival of GBM patients from 3-4 months to 7-12 months (Stupp et al., 2005). Unfortunately, after standard radiotherapy, 90% of the tumors recur at the original site (Wen and Kesari, 2008). Chemotherapy is now playing an increasingly important role in the treatment of GBMs. The combination of radiotherapy and temozolomide improves the median survival by 2.5 months compared with radiotherapy alone (Stupp et al., 2009). Current temozolomide-based radiochemotherapy has become the standard treatment (Stupp and Weller, 2010). For the recurrent GBM, Bevacizumab, a monoclonal antibody neutralizing VEGF, is widely used in clinical settings, regrettably the clinical responses are merely transient (Mellinghoff et al., 2011). Despite optimal treatment and some beneficial effect of systematic combination of temozolomide and radiotherapy, the median survival is around 15 months for GBM patients (Asklund T, 2012).

1.4.2.2 *Targeted therapy of GBM*

A better understanding of the molecular changes involved in the development of malignant glioma provides better chances to develop more effective personalized targeted therapy for patients. Relevant growth factor pathways in malignant gliomas include EGF, PDGF, VEGF and insulin-like growth factor (IGF). Targeting ligands and receptors in these signaling pathways would hopefully increase efficacy and minimize toxicity, provided individual patient's tumor could be analyzed before choices of treatment and the substances could be designed to reach their targets in the brain. Low molecular weight-tyrosine kinase inhibitors (LMW-TKI) and monoclonal antibodies are two types of agents being developed for glioma targeted therapy.

Approximately half of GBMs have EGFR gene amplification, often accompanied by overexpression of the EGFRvIII mutant. Two kinase inhibitors of EGFR, erlotinib and gefitinib have been used in clinical trials. Disappointingly, there was no significant effect on overall survival or progression-free survival at 6 months in GBM patients (Mellinghoff et al., 2011; Sathornsumetee and Rich, 2008). Drug response and tumor genotype are usually closely related. One reason for the trial failure might be that the trial was not conducted on a molecularly defined patient population. Another explanation could be that GBMs have co-activation of multiple tyrosine kinases and redundant signaling pathways. A single EGFR inhibitor might thus not have enough activity.

PDGF signaling is also critical for glioma development. Imatinib (Gleevec), a PDGFR inhibitor, has failed to show effects in some phase I/II clinical trials (Wen et al., 2006). However, a combination of Imatinib and hydroxyurea has demonstrated promising but not statistically significant effects in patients (Dresemann, 2005; Reardon et al., 2005).

The rich vasculature within GBMs presents attractive targets of angiogenesis inhibitors (Norden et al., 2009). VEGF is a key regulator of tumor angiogenesis. Targeting the VEGF pathway has become one focus of malignant glioma treatment. Bevacizumab (Avastin), a monoclonal antibody towards VEGF, is the first FDA approved antiangiogenic agent in cancer treatment. Bevacizumab may reduce tumor vascularity, and increase the uptake of chemotherapeutic agents (Sathornsumetee and Rich, 2008), but may facilitate tumor cell invasion (Keunen et al., 2011). In the clinical setting, Bevacizumab may not affect overall survival, but controls peritumoral edema and prolongs progression-free survival (Norden et al., 2009). Therefore, there is still a big challenge in finding a better way of targeting the VEGF pathway and the vasculature for therapy in GBM.

1.4.2.3 Clinical implications and complexity of BTSCs in glioma treatment

The identification of BTSCs in glioma presents a new insight to treatment. The BTSCs could survive through conventional chemo- and radiotherapy and initiate recurrence. Targeting BTSCs represents a platform for discovering new strategies to treat GBM patients.

Therapeutic strategies to target BTSCs could for example be: targeting BTSC-dependable signaling pathways (Bar et al., 2007); differentiating BTSCs to more committed cells losing their capacity to self-renewal and to give rise to other tumor cells by using bone morphogenetic protein (BMP) (Piccirillo et al., 2006) or through epigenetic drugs, such as HDAC or DNMT inhibitors (Vincent and Van Seuning, 2012); destroying the perivascular niche for BTSCs (Norden et al., 2009); and immunotherapeutic targeting of BTSCs (Li et al., 2012).

Although the CSC concept has relevance in some clinical settings, more complexities and challenges have been highlighted in the application of CSC targeting therapy. Recent research has shown that CSCs can be phenotypically very flexible both within tumors and between tumors and also non-CSCs can be reprogramed into CSCs under certain circumstances (Gupta et al., 2009). Therefore, new treatment strategies should include both CSC targeted therapy and conventional chemotherapy destroying the tumor mass (target non-CSCs).

2 AIM OF THE STUDY

The general aim of this study was to characterize the stem cell-like features of glioma and to start to apply them for experimental cancer stem cell-targeting therapies. This was achieved by using both human glioma materials and an experimental mouse glioma model in the following ways.

- Paper I: By analyzing gene expression signatures of high-grade human glioma cultures, we aimed to identify different subsets of glioblastoma stem cells and explore their sensitivities to tyrosine kinase inhibitors.
- Paper II: By examining human glioma tissues of various malignancy grades for the expression of transcription factors normally controlling self-renewal and pluripotency of embryonic stem cells and neural stem cells, we investigated if these ESC and NSC key regulators also are present and may have similar functions in human gliomas.
- Paper III: By utilizing a recently established mouse glioma model with overexpression of human PDGFB in astrocytic brain cells in combination with a p53 null background, we aimed to explore the epigenetic changes occurring in the tumor-prone mouse brain before overt tumor formation.

3 RESULTS AND DISCUSSION

3.1 PAPER I:

IDENTIFICATION OF A SOX2-DEPENDENT SUBSET OF TUMOR- AND SPHERE-FORMING GLIOBLASTOMA CELLS WITH A DISTINCT TYROSINE KINASE INHIBITOR SENSITIVITY PROFILE

By analyzing the gene expression data of 11 high-grade glioma cultures for their sensitivities to the LMW-TKIs mono-treatments, imatinib and NVP-AEW541, we identified two subsets of glioma, designated type A and type B cultures.

A significance analysis of microarrays (SAM) revealed that type B cultures had high expression of genes encoding extracellular matrix proteins, suggesting that the subset had mesenchymal features. Type A cultures were characterized by high expression of GFAP, an intermediate filament of normal SVZ neuroepithelial stem cells in mice, early glia progenitors, and astrocytes. Moreover, gene set enrichment analysis (GSEA) suggested that genes characterizing type A and type B cells were regulated by different mechanisms involving transcription factors and microRNAs.

As there were not so many known markers of adult neuroepithelial stem cells and their progeny within the microarray data set, we performed on type A and type B cultures a quantitative PCR (qPCR) analysis to measure the expression of a set of 17 genes. The 17 genes selected had been previously implicated as markers for neuroepithelial stem cells and their progeny. Results showed that 8 out of the 17 genes had a different expression pattern between type A and type B cultures. SOX2, chemokine C-X-C receptor 4 (CXCR4), excitatory amino acid transporter 1 (EAAT1), and GFAP were more highly expressed in type A cultures, whereas type B cultures showed higher expression of genes associated with microglial and perivascular cells [laminin gamma 1 (LAMC1) and PDGFRB] and a gene associated with the oligodendrocyte lineage [2', 3'-cyclic nucleotide 3' phosphodiesterase (CNP)]. Chemokine C-X-C motif ligand 12 (CXCL12) was also more highly expressed in type B cultures. NESTIN and BMI1, two established neuroepithelial stem cell markers, however, showed no difference between the two culture types.

Next, we investigated the cancer stem cell properties of these cultures by analyzing their tumorigenic potential *in vivo* and self-renewal capacities *in vitro*. Tumor tissue from the patients that was used to establish the original cultures was minced and subcutaneously xenografted in severe combined immunodeficiency (SCID) mice. All 8 tissue preparations representing the type A cultures formed tumors. In contrast, tumor formation was only observed with 4 out of the 12 tumor tissue preparations representing type B cultures. In addition, we prepared xenografts from two cell cultures of each type. Both the type A cultures induced tumors, but not the type B cultures.

The self-renewal capacity of the glioma cultures was examined using both a neurosphere assay and a limiting diluting assay. Results showed that type A cultures generally formed higher number of larger spheres and displayed increased self-renewal

capacity compared to the type B cultures. Thus, the stem cell features of type A were more pronounced than for the type B cultures with regard to tumor- and sphere-forming capacities.

The qPCR analyses suggested that high GFAP and SOX2 expression were features of type A cultures, and this was confirmed by immunofluorescence staining. The presence of SOX2 in most of the type A cell cultures indicates a tissue stem/progenitor cell phenotype in the majority of these cells. By knocking down SOX2 expression using the siRNA technique, we further demonstrated that the sphere-forming phenotype and the defining gene expression signature of type A cultures was SOX2 dependent.

Previous studies have shown that type A cultures have reduced sensitivities to monotherapy with two tyrosine-kinase inhibitors, imatinib, the PDGFR inhibitor and NVP-AEW541, the IGF-1R inhibitor. Interestingly, when SOX2 expression was down-regulated, the sensitivity of type A cultures to imatinib and to NVP-AEW541 increased. More excitingly, all three type A cultures tested displayed a significant growth inhibition when treated with a combination of imatinib and NVP-AEW541.

Finally, the clinical relevance of the two cultures was investigated by analyzing and comparing the publicly available gene expression data sets generated from human glioblastoma samples. Results demonstrated that the type A- and type B-defining gene signatures were expressed in different sets of human glioblastoma rather than coexpressed in the same tumors. Classification of glioblastoma with type A- and type B-defining genes generated two groups of tumors composed of the proneural/classical/neural subsets and the mesenchymal subset, respectively. Moreover, tumors with EGFR mutations were enriched in the type A group.

Taken together, we have identified a SOX2-dependent subset of tumor- and neurosphere-forming glioblastoma cultures characterized by a gene expression signature similar to that of recently described proneural/classical/neural subsets of glioblastoma. The findings that resistance to PDGF- and IGF-1-receptor inhibitors is related to SOX2 expression and can be overcome by combination treatment should be considered in ongoing efforts to develop novel stem cell-targeting therapies.

3.2 PAPER II:

ACTIVATION OF NEURAL AND PLURIPOTENT STEM CELL SIGNATURES CORRELATES WITH INCREASED MALIGNANCY IN HUMAN GLIOMA

It has been suggested that mechanisms promoting the maintenance and self-renewal of stem cells have a similar function in glioma tumor cells. However, little is known about the expression pattern of these neural and pluripotency-associated transcriptional network proteins in gliomas, and if these proteins are important for glioma tumorigenesis. Furthermore, independently of genetic alterations, would these proteins constitute relevant targets for therapeutic strategies aiming to prevent growth and recurrence of the tumor?

Therefore, we examined human glioma tissues for the expression of transcription factors with key roles in controlling the self-renewing undifferentiated state of NSCs and ESCs, such as SoxB1 (Sox1, Sox2 and Sox3), Oct4, Nanog and Klf4.

Previous studies have shown that SoxB1 genes are co-expressed by the majority of NSCs both in the developing and adult CNS, and constitute important regulators of NSC maintenance. By using *in situ* hybridization and immunofluorescence staining, we investigated expression at both the mRNA and protein level, and we found that undifferentiated proliferating cells in high grade gliomas co-express Sox1-3 transcription factors which was similar to stem cells of a healthy CNS. Further, an *in vitro* gain-of-function assay demonstrated that as in the developing CNS, Sox3 has the capacity to maintain glioma cells in an undifferentiated and proliferating state, whereas active repression of Sox3 causes cells to exit the cell cycle.

The expression of ESC regulatory genes in different grades of glioma was examined by qRT-PCR and immunostaining. Results showed that the combined expression of Sox2, Oct4, Nanog and Klf4 was associated to an increased malignancy grade of glioma. Moreover, an array of mesodermal and endodermal markers was used to analyze the glioma samples. It was found that apart from neural markers, many cells in grade IV tumors had activated gene expression representative of the mesodermal and endodermal cell developmental lineages, indicating that gene expression programs normally present in other than neural lineages are active in high-grade glioma.

Finally, the influence of the microenvironment on the expression of stem cell properties was assayed by measuring the Sox2, Oct4 and Nanog protein level in primary glioblastoma cells after intracranial xenografting, serial subcutaneous transplantation and *in vitro* culturing. The resulting xenograft tumors from both intracranial transplantation and serial subcutaneous transplantation contained comparable amounts of Sox2, Oct4 and Nanog expressing cells as the original human tumors, whereas glioma cells cultured *in vitro* initially expressed high levels of Sox2, Oct4 and Nanog, but the expression of Oct4 and Nanog declined upon continuous propagation *in vitro* either as monolayer or as spheres. In contrast, Sox2 expression remained unaltered in cultures. These data suggested that while the NSC gene Sox2 remains unchanged in cultures, the expression of Oct4 and Nanog is dependent on extra-cellular regulatory cues present *in vivo* but not *in vitro*.

In conclusion, this paper demonstrated a generally deregulated expression of neural and pluripotent stem cell gene signatures in human malignant gliomas, which might allow for the pluripotent features presented by glioblastoma cells. Independently of any genetic alterations, the stem cell regulatory program, when aberrantly present in tumor cells, may constitute a significant target for therapeutic strategies.

3.3 PAPER III:

HISTONE3 LYSINE9 METHYLATION AND DNA HYPOMETHYLATION DURING THE PRE-NEOPLASTIC STAGE OF EXPERIMENTAL MOUSE GLIOMA

As an alternative to the human tumors (used in the previous two papers), we used an established mouse glioma model to systematically study epigenetic alterations in mouse brain tumors and in the morphologically normal mouse brains before tumor formation.

We have previously generated a transgenic mouse in which the human GFAP promoter directs expression of human PDGFB to mouse astrocyte and astrocyte progenitor/neural stem cells. This hGFAPpPDGFB (B^+) mouse was then crossed on to a p53 null background, mimicking the genotype of human secondary GBM. These hGFAPpPDGFB/p53 null ($B^+p53^{-/-}$) mice do not develop visible brain tumors until 3-6 months postnatally making it possible to follow the initial stages of tumor development in the mouse brain. In this study, we showed that histone3 lysine9 methylation (H3K9Me) and global DNA hypomethylation were present in mouse glioma samples and had occurred in the mouse neural stem/progenitor cells before tumor formation.

We examined the epigenetic status of the mouse glioma including the global DNA methylation level and global histone3 methylation status by immunohistochemistry. We found a profound reduction of DNA methylation in brain tumor tissue compared to adjacent normal brain tissue as visualized by both 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) stainings. On the contrary, the H3K9Me2 level was increased in the tumor cells compared to normal brain cells. It was also interesting to see that the Ki67 positive, proliferating tumor cells were almost devoid of 5-hmC staining.

NSCs are one of several cell types to be the putative origin of glioma. Do NSCs from the tumor permissive ($B^+p53^{-/-}$) brain exhibit proliferative advantages and are they subject to epigenetic modifications before tumor formation? The sphere formation assay and the limiting dilution assay demonstrated a higher self-renewal capacity and proliferative advantage of NSCs from the tumor permissive $B^+p53^{-/-}$ mouse brain than wild type (WT) NSCs.

We also monitored the global DNA methylation levels in neurospheres by 5-mC and 5-hmC immunostainings on cryosectioned spheres, by antibody based dot blot analysis and also by Luminometric Methylation Assay (LUMA), a methylation-sensitive restriction enzyme based assay followed by pyrosequencing. Although we could not demonstrate obvious global DNA methylation differences among neurospheres from four different genotypes of mice, there was a trend towards decreased DNA methylation in tumor spheres compared to neurospheres derived from the same $B^+p53^{-/-}$ mouse.

The DNA methylation status could be very much influenced by the *in vitro* and *in vivo* environment. Therefore, we analyzed the global DNA methylation status in mouse brain tissues. Previous analyses of clinical samples and mouse models provide evidence

pointing to the SVZ of the lateral ventricular wall (LVW) as a region of origin for malignant glioma. Therefore, we dissected out the LVW of different mouse brains and examined the global DNA methylation status of this particular region by LUMA assay. We found that the global DNA methylation level was significantly lower in the LVW from B⁺p53^{-/-} mice compared to WT mice. There was a trend of Dnmt3a (*de novo* DNA methyltransferase) RNA down-regulation in the same LVW tissue but not in WT.

Finally we investigated the H3K9Me2 level in neurospheres from the same four different genotypes of mice by immuno-blot. Results showed that there was almost no H3K9me2 in WT and p53^{-/-} neurospheres, while a strong H3K9Me2 signal was observed in B⁺p53^{-/-} and a low signal in B⁺ neurospheres. An up-regulated level of H3K9Me2 was also observed in tumor spheres relative to “normal” neurospheres generated from the same (B⁺p53^{-/-}) animal.

In summary, this work suggests major epigenetic disturbances in stem/progenitor cells of the pre-neoplastic mouse brain. The findings point to the possibility that these non-neoplastic but epigenetically disturbed stem/progenitor cells might be the cell-of-origin of brain tumors and mechanisms regulating these cells could be crucial targets for therapy. The current model is a useful tool to study this possibility further.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The concept of putative cancer stem cells in malignant glioma has inspired many studies on stem cell signatures in glioma, the comparison of glioma tumor stem cells with normal stem cells and the development of stem cell-based experimental glioma therapy. For this purpose, both human tumor tissues and a mouse glioma model have been used in this thesis.

The first paper defines two novel phenotypically distinct subsets of glioblastoma cultures with different stem-like properties. In the paper, SOX2 is identified as a key regulator of the Type A cultures, which have a more profound capacity for tumor formation *in vivo* and self-renewal *in vitro*. Type A cultures are also characterized by a gene expression signature similar to that of the recently described proneural/classical/neural subsets of glioblastoma, whereas type B cultures are more related to the mesenchymal subset. Additionally, the findings that the resistance of type A cultures to mono-treatment with PDGF- and IGF-1-receptor inhibitors could be overcome by combination treatment with the two inhibitors, are highly relevant to ongoing efforts to develop cancer stem cell-targeting therapies. It is highly tempting to design a future clinical study based on combined gene expression analysis, selected gene mutation profiles and selective treatment regimens. The mesenchymal subset (similar to type B cultures) would be assigned to either PDGF- or IGF-1-receptor inhibitor, whilst the rest (resembling type A cultures) would be assigned to combination treatment. At the same time, it would be highly interesting to learn the effect of the combination vs. mono-treatment on the intra-cellular signaling pathways in the glioma cells.

In the second paper, deregulated expression of neural and pluripotent stem cell regulatory proteins Sox2, Oct4, Nanog and Klf4 was demonstrated in human malignant gliomas. The presence of the four factors correlated with increased malignancy of human glioma. Results also showed that the expression of this pluripotency gene signature in brain cancer cells is largely dependent on signals in the microenvironment. Further studies are needed to address the exact role of these stem cell regulators in gliomas and the environmental signals regulating them. Are these pluripotency factors important for glioma tumorigenesis? If so, these proteins might constitute relevant targets for therapeutic strategies aiming to prevent growth and recurrence of tumors.

The third paper describes a significantly elevated level of histone3 lysine9 methylation and global DNA hypomethylation in the pre-neoplastic stage of a mouse brain tumor model. This model was generated by astrocyte and astrocyte progenitor/stem cell-specific over expression of PDGFB combined with a p53 null background. The DNA hypomethylation was seen in the pre-neoplastic brain especially in the morphologically normal LVW of the lateral ventricles where neural stem/progenitor cells are known to be located, as well as in mouse glioma samples. This is the first work describing epigenetic changes of precursor cells during the pre-neoplastic stage using an animal model. The combinatorial effect of PDGFB expression and p53 loss of function seems to lead to epigenetic alterations of the neural stem/progenitor cell niche and may also set the stage for later transformation to cancer cells. The exact mechanism of this

epigenetic change is still unknown. By analyzing for potential genetic changes, such as gene copy number changes and whole epi-genome analyses of the tumor material and the NSC region, we hope to gain valuable data leading to a better understanding of the underlining mechanisms and new potentials for future glioma targeted therapy.

In summary, this thesis has presented connections between stem cells and glioma in different aspects such as gene expression signatures, stem cell features as a potential resistance mechanism to targeted therapies and epigenetic alterations in a stem cell niche of the pre-neoplastic mouse brain. A better understanding of different self-renewing stem cell populations within gliomas is lending more and more insights into the mechanisms of gliomagenesis and may ultimately lead to new approaches for more personalized stem cell based glioma treatment.

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