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Glioma Stem Cells

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1. Introduction

Gliomas are the most frequent primary brain tumors and are classified as grade I to IV according to their degree of malignancy (Daumas-Duport et al., 1988). Grade I and II gliomas are clinically benign or semi-benign with relatively long-term survival, while grade III and IV are malignant and lethal within several years. In particular, glioblastoma multiforme (GBM), the most malignant glioma as grade IV often relapse even after radical surgical resection and standard chemo/radiation therapies, because of their diffuse infiltration into the surrounding brain parenchyma and high degree of chemo/radioresistance. Despite extensive efforts, the overall survival of GBM patients remains still short and has not yet been dramatically improved for more than several decades.

GBMs are often composed of various types of cells with distinct morphology and clinical phenotypes. Their histological and biological multiformity has been classically explained by the stochastic clonal evolution model (Nowell, 1976). According to this model, all tumor cells should have low but inheritable ability to form tumors. However, recent evidence has suggested another concept, the cancer stem cell (CSC) hierarchy model, which shows that only a rare stem cell population has high ability to proliferate (Jordan et al., 2006). CSCs have similar characters to normal stem cells in the way of having high ability to self-renew and differentiate into multiple types of progenies to organize tissue architectures. Exclusively, CSCs can proliferate uncontrollably to propagate tumor cells. CSC model have direct relevance with tumor replenishment, disease recurrence and metastatic activity, suggesting that CSCs should be the target to eradicate the tumors.

The aim of this chapter is to provide our insights into how CSCs in human glioma; i.e. glioma stem cells (GSCs) should be attacked. In the first parts, we summarize the general basis of CSC concept inclusive of the current knowledge on how a CSC is defined, how CSCs should be technically prepared and modeled, and what characteristics CSCs possess. In the following part, we highlight what abnormal signaling pathways regulate CSCs as the potential therapeutic targets. Understanding the framework of a GSC research field could help us to think of novel treatment strategy. Most importantly, however, CSCs have enhanced resistance to conventional chemotherapies. Considering this fact, we finally propose that it could be most promising strategy to disrupt the extracellular environments

supporting GSCs, which is a newly emerging concept "niche therapy". We present possible cellular and molecular components of GSC niche and discuss their potentiality as innovative therapeutic targets.

2. General outlines of cancer stem cell

2.1 Heterogeniety of cancer tissue

Over the past several decades, the idea that cancer tissues are heterogeneous and composed of multiple subpopulation of cells differing in morphology, proliferation rate, metastatic potential, marker expression, and sensitivity to chemo/radiation therapies, has been highly appreciated by many investigators. This tumor heterogeneity has been classically explained by the following two models (Figure 1).

Clonal evolution (stochastic) model: Cells may acquire various combinations of genetic and epigenetic mutations asynchronously and once such mutations confer selective advantages of tumor malignancy, more aggressive and adaptive clones can dominate the whole tumor population.

In 1976, Nowell proposed the evolutionary view into tumor progression as a stochastic process of genetic instability and natural selection (Nowell, 1976). According to this model, tumor initiation occurs in the individual cells receiving multiple mutations, and cells having an advantageous mutation of growth can clonally proliferate and selectively occupy an entire tumor. During tumor progression, genetic instability yields the additional mutations by chance, resulting in a diversity of genome and cell characteristics, such as being invasive, metastatic and therapy-resistant. The adaptation to surrounding microenvironment is also a determinant of the selection. Standing on this concept, all the tumor cells should have low but inheritable ability to form tumors, therefore, the rational targets for cancer therapy is most or all of tumor cells. After this proposal, researchers have confirmed clonal expansions and genetic heterogeneity within various types of human neoplasms.

Hierarchy (cancer stem cell) model: Cells may acquire various genetic and epigenetic mutations that confer stem-like characteristics, and divide to produce its identical copy (self-renewal) and progenies terminally growth-arrested (differentiation). Such a hierarchy in a stem cell division manner results in cellular diversity of a rare stem cell and a majority of multiple types of progenies.

The CSC model is not a newly emerging concept indeed. The efforts to gain insights into tumor's malignant potential, especially their differential sensitivity to anticancer drugs were made on this concept with specimens from patients with myeloma and with ovarian cancer (Salmon et al., 1978). The technological advances of flow cytometry and discovery of stem cell surface antigens accelerated investigators to ascertain this concept, starting in human leukaemia (Bonnet and Dick, 1997) along with the subsequent identification in breast (Al-Hajj et al., 2003) and brain tumor (Singh et al., 2003).

Normal stem cells replenish normal tissues by fresh cells continuously throughout life in individual organs. Stem cells are characterized by two main hallmarks; those are multipotency and self-renewal capacity. The former is the ability to produce multiple types of functional progenies, which is associated with terminal growth-arrest. Self-renewal is the ability to produce new stem cells identical to original one with retaining the intact two main hallmarks, and thereby maintains the stem cell pool. Likewise, tumors are also organized as a hierarchy where rare CSCs stand at the top of pyramid with extensive potential to proliferate and self-renew and are responsible for maintaining the homeostasis of the tumor bulk, except that CSCs disorderly propagate tumor cells and increase cell number.

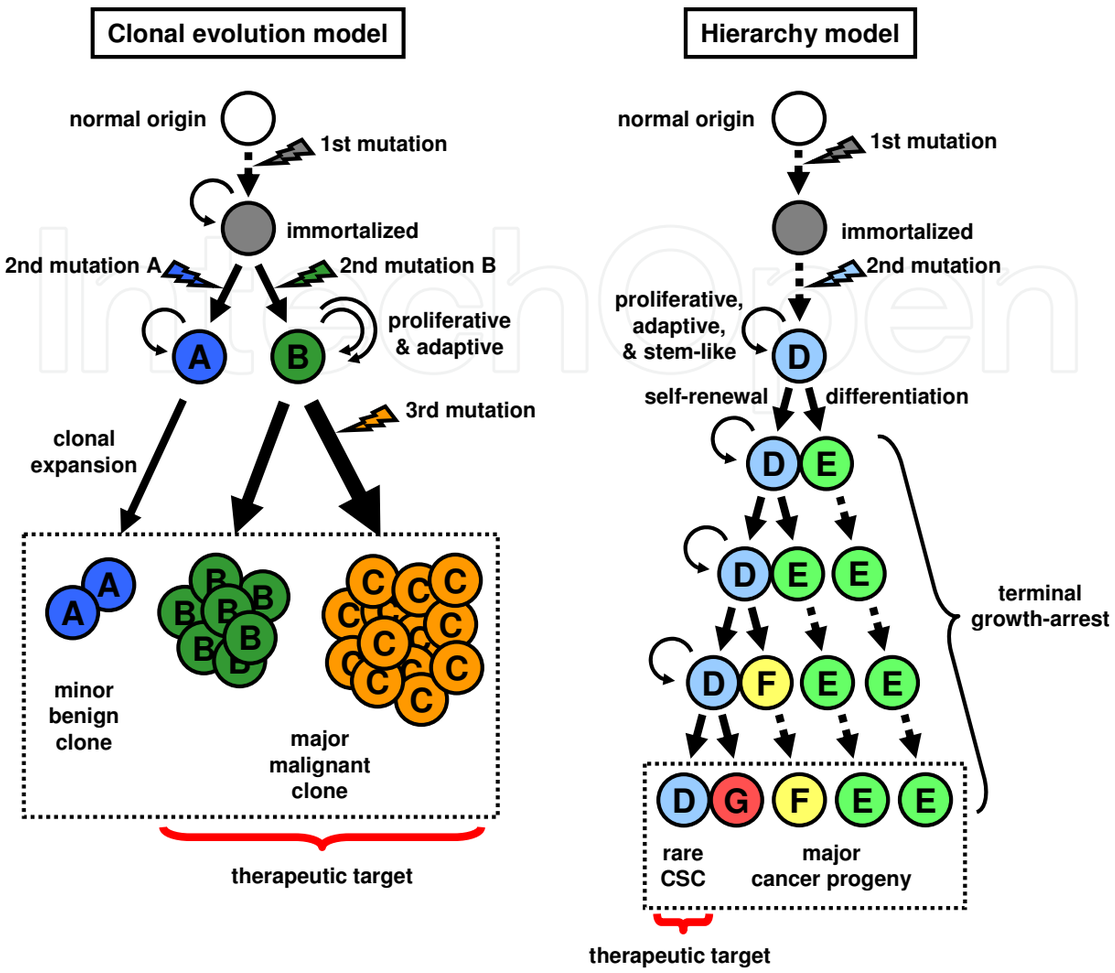

Fig. 1. The color of the arrow for 2nd mutation in Hierarchy model is changed from pale yellow to light blue.

In this concept, cancer heterogeneity means the different characteristics between CSC and differentiated cancer progenies. However, the clonal evolution model and the CSC model are not necessarily mutually exclusive. Despite types of primary cells to suffering the first set of mutations, CSCs also have genetic instability, which increases the probability of acquiring additional mutations. Potentially, CSCs might expand clonally by a symmetric division. The adaptation to surrounding microenvironment has also been recognized as important factors on normal stem cell maintenance, which is called stem cell niche. Therefore, we should consider the CSC model as a revised version by taking the idea of asymmetric division. However, we must note again that only rare CSCs are tumorigenic by definition and specific targets for cancer eradication is in a minority population within a tumor bulk.

2.2 Definition of cancer stem cell

CSCs are re-defined in the AACR Workshop 2006 on Cancer Stem Cells as "a small subset of malignant cells that constitute a pool of self-sustaining cells with the exclusive ability to

maintain the tumor" (Clarke et al., 2006), and in part share the fundamental properties with normal stem cells specific to their original organs; e.g. neural stem cell for glioma.

2.3 Current methods to isolate cancer stem cell

In current studies, CSCs from cancer tissues and cell lines are firstly isolated by using diverse experimental techniques as mentioned below.

Sphere formation assay: Floating spherical cell clusters are formed *in vitro* when mitotic cells are cultured in serum-free media supplemented with mitogens (epidermal growth factor and fibroblast growth factor for neural stem cells) (Figure 2), which is originally developed to characterize the behaviour of neural stem cells (Reynolds and Weiss, 1992).

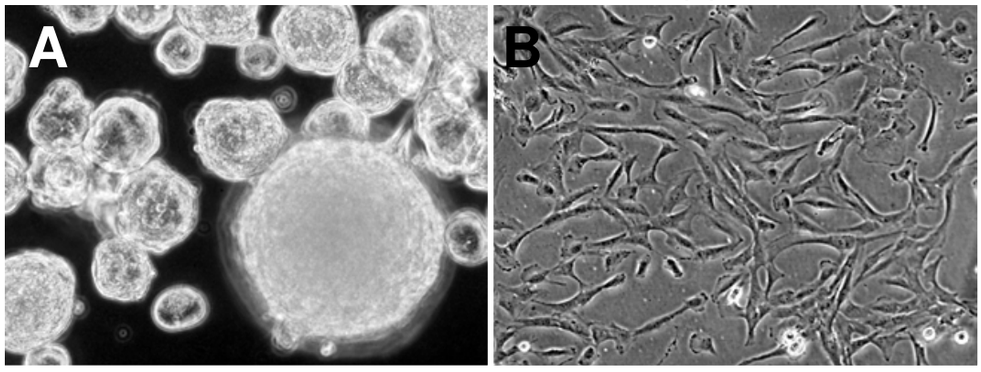

Fig. 2. Glioma spheres generated from oncogenic Ras-transformed human astrocytes (A) and differentiation of dissociated sphere cells by culturing in 10%FBS media for 1 week (B).

When cells are derived from brain, breast and others, each sphere is called neurosphere, mammosphere, and others, respectively. The number and diameter of spheres are thought to reflect the frequency of stem cells and mitotic activity of a single stem cell within the examined population, respectively. When a 1st sphere is picked up, re-dissociated into single cells, and re-cultured, the generated secondary spheres imply the sustained self-renewal potential of a true stem cell. For a while, this method has been utilized to expand cancer stem cell population by serial passage, but Bexell et al. recently showed that sphere formation is not prerequisite for enrichment of CSCs, and pointed out that glioma cells growing as monolayers are also tumorigenic (Bexell et al., 2009), like in the case of neural stem cells that can be maintained in monolayer cultures (Johe et al., 1996). In addition, it is virtually impossible to form spheres of CSC that have arisen from a tissue in which the condition of sphere assay is unestablished for normal stem cells, and even if spheres are formed, it is uncertain if they have significance for stem cell activity in the tissues. Therefore, we must note that sphere formation assay has limited utility for isolation of some types of CSCs.

Hoechst 33342 dye effluxing side population (SP): Hoechst 33342 is a fluorescent dye that binds to the AT-rich regions of DNA, and most cells are stained with this dye. In most cases, stem cells are not stained due to the high expression of ABC transporter family genes to efflux this dye. Human fetal neural stem/progenitor cells express high levels of multidrug resistance 1 (MDR1, also known as ABCB1, P-gp) gene and ATP-binding cassette sub-family G member 2 (ABCG2, also known as Bcrp), and these transporters have important roles in normal physiology on the active efflux of xenobiotics from cell body, protecting cells from

cytotoxic agents. Cells with the capacity to efflux the dye were first identified in the mouse bone marrow (Goodell et al., 1996). After staining with Hoechst 33342 dye and exposure to UV laser during FACS, a population is referred to as side population (SP) cells because it appears as a "side" relative to the positively stained "main" population (MP) in FACS plots (Figure 3). Since this discovery, SP technique is applied to enrich putative stem cells and progenitors in a number of normal tissues and malignant tumors, including glioma. SP cells preferentially express high levels of stemness genes and are capable of differentiating into multiple lineages, and thus are considered to function as stem cells in the original tissues and tumors (Dean et al., 2005). Importantly, this method itself may affect the tumorigenic potential because Hoechst dye is toxic for cells. Minimally toxic concentrations of Hoechst 33342 should be also determined in the individual experiments. Rhodamine 123, which is a mitochondrial dye reflecting mitochondrial content and kinetics, has been used as a less toxic dye, by which quiescent stem cells display a low fluorescence in the flow cytometry plots. Human bone marrow hematopoietic stem cells (HSCs) have been demonstrated to be isolated by the combination of Hoechst 33342 and Rhodamine 123 (Leemhuis et al., 1996). In addition, some investigators have reported that MP cells in human GBM biopsies are also tumorigenic in mice, although transplantation of SP cells result in a shorter survival of mice than MP (Bleau et al., 2009).

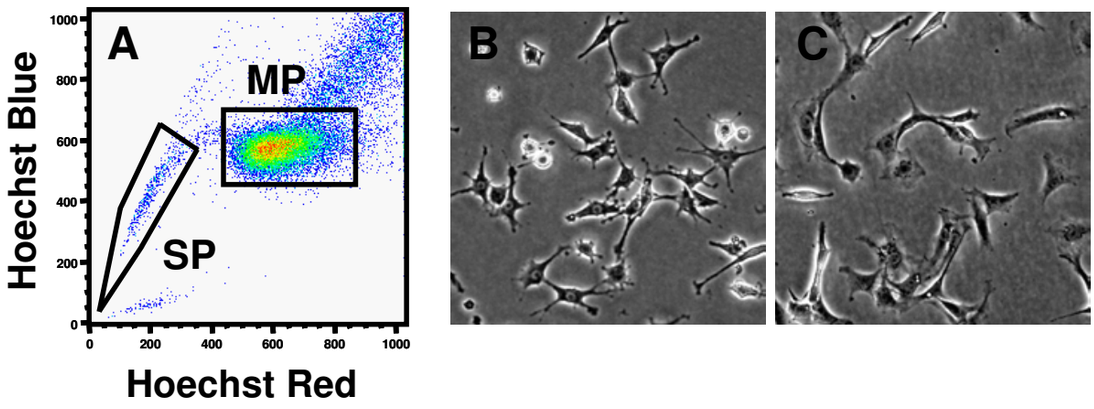

Fig. 3. FACS plots expanded by side population analysis (A) and representative images of SP (B) and MP (C) cells in the C6 glioma cell line.

Stem cell surface antigens: Potential CSCs can be identified by striking variety of cell surface markers or their combinations; e.g. CD34(+)CD38(−) for AML (Bonnet and Dick, 1997), CD44(+)CD24(−/low) for breast tumor (Al-Hajj et al., 2003) and CD133(+) for brain tumor (Singh et al., 2003). However, these methods based on the surface molecules have increased some contradiction. For instance, different stem cell markers identify distinct subpopulations of cells in one tumor, in all of which population CSC properties may sometimes confirmed. In melanoma, at least four subpopulations, CD20(+), CD133(+), BrdU label-retaining cells, and SP cells has been separately identified, all of which possess stem cell features (Zabierowski and Herlyn, 2008). This diversity of CSC markers suggests that one population isolated by certain surface molecules is still heterogeneous, and that complete enrichment of CSCs has not been achieved at least in some particular tumor types. Furthermore, the frequency of CSCs is highly variable even among the same type of tumors. In colorectal tumors, the frequency of tumor initiating cells assessed in NOD/SCID mice

broadly ranges from 1.8 to 24.5% (O'Brien et al., 2007), pointing out the requirement for more refined markers (or their combinations) and assay systems. Nevertheless, it is evident that these surface markers and their roles are important keys to unveil the regulatory pathway to maintain CSCs. Actually, in many studies on human brain tumors, GSCs are isolated by the elevated expression of CD133, and the CD133(+) subpopulation has the higher ability than the other populations to regenerate the original tumors in immunodeficient mice (Singh et al., 2004).

ALDEFLUOR (aldehyde dehydrogenase activity): Measurement of ALDH activity is a recent approach for isolation of putative CSCs. The main function of ALDH enzyme is the oxidation of intracellular aldehydes. Several isoforms of ALDH family are identified in human, and ALDH1 is the primary isoform isolated from human hematopoietic progenitors. Recent studies have shown that human and murine hematopoietic progenitors can be isolated using the bodipy-aminoacetaldehyde (BAAA, commercially available in a diethyl acetate form as ALDEFLUOR), a fluorescent labeled substrate specific for ALDH activity (Ginestier et al., 2007). ALDH1 has a role in early differentiation of hematopoietic stem cells (HSCs) through conversion of retinol to retinoic acid (Chute et al., 2006). Increased ALDH activity is also found in normal neural stem cells and CSC in acute myeloid leukemia (AML) (Pearce et al., 2005) and breast carcinomas (Korkaya et al., 2008).

Xenotransplantation into immunodeficient mice: The first evidence of CSCs was obtained by transplantation experiments in the severe combined immunodeficient (SCID) mouse (Lapidot et al., 1994), and it was later re-demonstrated in the nonobese diabetic (NOD)/SCID mouse (Bonnet and Dick, 1997). However, the use of NOD/SCID mice, which T cells and B cells are lacked, has the risk to underestimate the frequency of tumorigenic human cancer cells due to the immune response of natural killer cells against the xenobiotic cells. Actually, the percentage of melanoma stem cells that form tumors in NOD/SCID/ interleukin-2 receptor γ chain (IL2Rγ)-null (NOG) mice, which lack not only T and B but natural killer cells, is higher than that in NOD/SCID mice; one in 1000 melanoma cells form tumors in NOD/SCID mice, but 1 in 4 can form tumors in NOG mice (Quintana et al, 2008), suggesting that the reported frequency of CSCs should be reevaluated in some cancers by using the more highly immunocompromised model. Thus current gold standard assay for testing whether each cell population fulfils the criteria of CSC (self-renewal, differentiation and tumorigenicity) is the orthotopic xenograft experiment where cells are transplanted at limiting dilutions into NOD/Shi-scid/IL2Rγ-null (NOG) mice and the frequency of CSCs is determined. Especially, serial transplantation into NOG mice is regarded as the best functional assay for testing the long-term self-renewal capacity. However, even the use of NOG mouse might underestimate the frequency of CSCs due to different tissue environment between mouse and human. If mouse ligands such as growth factors and adhesion molecules do not have the compatibility with human receptors, tumorigenic human cells might lose the ability to proliferate and survive in mice. Also, even being compatible, rodent normal tissues could not precisely recapitulate the patients' tumor microenvironment. Therefore, the challenges to establish humanized mouse models in which human microenvironment is recapitulated in an animal are recently highlighted.

2.4 Clinical implications of cancer stem cell

CSC model has important implications for clinical treatment of tumors, because some features of CSCs make them particularly difficult to kill. CSCs reside in relatively quiescent

state on cell cycle referred as in the state of "dormancy" and this allows them to evade from typical chemotherapeutic regimens that target actively proliferating cells. Many investigators attempt to kill tumor cells independently of the cell cycle state or to selectively prompt them to enter the cycling state. Moreover, CSCs express the high level of multidrug resistant proteins and transporters associated with detoxification, and they are consequently conferred multidrug resistance by expelling chemotherapeutic reagents (Dean et al., 2005). In addition, CSCs are frequently resistant to standard radiotherapy regimens owing to their elevated expression of reactive oxygen species (ROS) scavenging (Diehn et al., 2009) and DNA damage response (Bao et al., 2006) genes. Unexpectedly, conventional chemo/radiotherapy eventuates in an increased proportion of CSC fraction and in rapid relapse of original tumor. Long-term treatment of PTEN$^{-/-}$ neurospheres isolated from PDGF-induced gliomas with temozolomide, a standard therapy for GBM patients, induces an increase in the amount of SP cells (Bleau et al., 2009). An effective therapeutic approach to CSCs enhancing the sensitivity of chemotherapeutic drugs and radiation is therefore imperative to develop.

2.5 Origin of cancer initiating cell

The revival of CSC concept triggers a debate on whether cancer origin is stem cells or terminally differentiated cells. Since 1970s, a large corpus of studies on direct introduction of viral oncogenes and mutated genes isolated from patients' tumors has demonstrated that most of normal cells are susceptible to genetic mutations leading to neoplastic transformation for the initiation and expansion of tumors. In addition to these findings, the observation that a large proportion of tumor cells retain the features of the surrounding differentiated epithelium has assured our common interpretation that oncogenic gene mutations occur in terminally differentiated cells. However, there are several controversial examples that are still difficult to explain standing on this concept for tumor cell origin. For example, malignant tumors histologically contain some degree of undifferentiated components, but differentiation of cells had been regarded as a unidirectional process under normal physiological conditions, and nuclear reprogramming had remained to be clarified until recent achievement of nuclear transfer, ES cell-fusion, or creating the induced pluripotent stem (iPS) cells (Yamanaka and Blau, 2010). In addition, terminally differentiated cells cannot normally divide to proliferate *in vivo* and thus the possibility is low that such sleeping cells have in sequence the opportunities to acquire oncogenic gene mutations, although they often undergo spontaneous immortalization *in vitro* and proliferate infinitely as cell lines. In fact, cancers prone to occur in specific tissues with the high rate of turnover in cell division, such as skin and gut but not heart. It is conceivable that this is a consequence of genetic instability during the frequent divisions of stem cells.

3. Glioma stem cell

3.1 Discovery and isolation

Subsequent to the identification in human AML and breast tumor, CSCs in human glioma i.e. glioma stem cells (GSCs) were initially identified in 2004 according to the cell surface expression of CD133 protein (Singh et al., 2004). CD133 is one of two members of pentaspan transmembrane glycoprotein prominin family and a human homolog of originally isolated mouse PROM1 selectively localizing at the apical surface of murine neuroepithelial stem cells (Weigmann et al., 1997) and HSCs from human fetal liver, bone marrow and cord

blood (Yin et al., 1997). Notably, 100 CD133(+) cells recapitulated the original tumor identical to original patients' tumors in histopathological features, whereas 100,000 CD133(−) cells could not form any tumors in NOD/SCID mouse brains (Singh et al., 2004). Therefore, it was postulated that like normal neural stem-cells (NSCs), stem-like glioma cells may compose a glioma hierarchy upon differentiation and that their differentiation lineages determine tumor subtypes, i.e. astrocytomas or oligodendrogliomas. On the same year, Kondo et al. also succeeded in concentrating GSCs as stem-like SP cells from a rat C6 glioma cell line (Kondo et al., 2004). Since these discoveries, many investigators have confirmed the major utility of CD133, SP technique, and others for GSCs isolation (Table 1).

Parameters	Details of assay	Observed GSC phenotypes	References
Neurosphere culture	Culturing in serum-free media containing EGF and bFGF	self-renewal differentiation tumor formation	Singh et al. 2003
CD133 (AC133 antigen)	Magnetic sorting or flow cytometry sorting by CD133 expression	self-renewal differentiation tumor formation	Singh et al. 2004
Side population	Staining and flow cytometry sorting based on Hoechst 33342 dye efflux	self-renewal differentiation tumor formation	Kondo et al. 2004
A2B5 antigen	Flow cytometry sorting by the expression of a glial progenitor marker A2B5	tumor formation	Ogden et al. 2008
SSEA-1	Flow cytometry sorting by the expression of an embryonic marker SSEA-1 (stage-specific embryonic antigen 1)	self-renewal differentiation tumor formation	Son et al. 2009
Laminin-coated flask	Culturing in serum-free media containing EGF and bFGF on laminin-coated flask	self-renewal differentiation tumor formation	Pollard et al. 2009
Integrin alpha 6	Magnetic sorting or flow cytometry sorting by integrin alpha 6 expression alone or in combination with CD133 expression	self-renewal tumor formation	Lathia et al. 2010
Autofluorescence	Flow cytometry sorting by intrinsic autofluorescence emission around 520 nm upon laser excitation at 488 nm	self-renewal differentiation tumor formation	Clement et al. 2010

Table 1. Cell surface markers and functional assays for the isolation of glioma stem cells

However, these methods indeed produce some contradictory. First, accumulating reports have demonstrated the presence of CD133(-) GSCs (Ogden et al., 2008). Some of fresh GBM specimens and commonly used glioma cell lines do not express CD133, but nevertheless they can form tumors *in vivo* (Beier et al., 2007). Second, CD133(+) population in human gliomas are generally found at a very low frequency in flow cytometry and sometimes barely detectable, which lead to the concept that CSCs are a rare subpopulation in solid tumors, but immunohistochemical analysis by several groups and us have demonstrated that some of human GBMs contain high CD133(+) fractions (Figure 4B). This discrepancy has been thought to be partially caused by the limitation of FACS analysis; i.e. trypsin digestion during preparation of cell suspensions is predicted to cleave the 865-amino acids long-CD133 sequences at 79 different sites. And many of cleavage sites by trypsin are within the glycosylated extracellular loops containing AC133 or AC141 epitopes, although the majority of studies on CD133 have ever made use of antibodies against AC133 or AC141 glycosylated epitopes. Third, CD133 expression was found to be retained in differentiated

cancer cells when a glucosylation-independent CD133 antibody was used (Florek et al., 2005). Other studies also have demonstrated that the expression of AC133 and AC141 epitopes is down-regulated independently of CD133 mRNA (Corbeil et al., 2000), and that the distribution of CD133 mRNA in adult tissues is much wider than that of immunoreactivity to the AC133 epitope (Miraglia et al., 1997). Furthermore, human CD133 transcripts have alternatively spliced variants devoid of AC133 or AC141 epitopes (Fargeas et al., 2007), and cytoplasmic localization of CD133 is also observed in human glioma sections (Sakariassen et al., 2007). In addition, we also found in human GBM sections the broadly ranged frequency of CD133(+) cells using an antibody against C-terminal intracellular region of CD133 (Figure 4A and B) and distinct discordance on the expression levels between CD133 and another neural stem cell marker Nestin (Figure 4C and D).

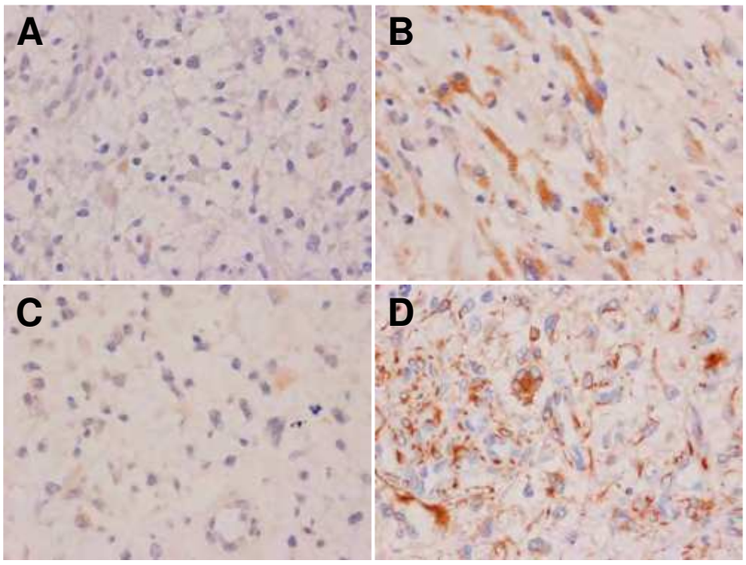

Fig. 4. (A and B) Different levels of CD133 expression in two human GBM tissues. Each tissue was stained with an anti-CD133 antibody. Relatively high frequency of CD133(+) cells are observed in B, but rarely detected in A. (C and D) The expression levels of two neural stem cell markers CD133 and Nestin does not coincide in the same GBM tissue. A GBM tissue was stained with an anti-CD133 (C) and nestin (D) antibody.

Given these unresolved problems might lead to our underestimation of the size of true GSCs, it must be again deliberated whether CD133 could be utilized as a marker for GSC isolation. It may be one goal in future studies to find other markers (or their combination with CD133) to accurately define GSC population.

In contrast, SP method can compensate the disadvantage of cell surface marker-dependent isolation that fails when stem cell markers are unknown. Subsequently to the study with the C6 glioma cell line, SP cells have been detected in not only human glioma cell lines U87MG, T98G, U251 and U373, but also two primary gliomas in transgenic mouse models; Ntv-a/Ink4a$^{-/-}$Arf$^{-/-}$PTEN$^{flox/flox}$ mice (Bleau et al., 2009) and S100β-verbB:p53$^{-/-}$ mice (Harris et al., 2008), which are reported to be more self-renewable and tumorigenic than MP cells and able to reconstitute cellular heterogeneity. However, the isolation of SP cells from primary human GBMs and relevant correlation between SP and glioma malignancy have

been little reported. Interestingly, Pollard and colleagues have proposed a protocol for the efficient establishment of GSC lines as culturing on laminin-coated flask, in which GSCs could be expanded in adherent culture for at least 1 year (>20 passages) with retaining stem cell properties and tumor initiation capacity (Pollard et al., 2009). They pointed out some problems on the use of the traditional neurosphere culture in GSCs isolation and highthroughput assays i.e. drug screenings. First, they claimed that the efficiency to successfully establish GSC lines from primary tumors is very low (from 1 to 30%). Second, spheroid cells tend to spontaneously undergo differentiation and apoptosis, and therefore it is complicated to identify the precise molecular targets against the true GSCs. Finally, the non-adherent aggregates make real-time observation of cellular behaviors more challenging, compared to adherent cells. Actually, they provided a proof-of-principle chemical screen using a live-cell imaging system to monitor the effects of FDA (Food and Drug Administration)-approved 450 compounds for potential anti-GSC activity, and identified 23 compounds having cytotoxic activity against all tested GSCs. This approach could greatly improve the work to probe GSC biology and to identify agents that selectively and directly target GSCs.

3.2 Signalling to regulate GSC (Therapeutic candidate)

GSC now attracts much attention with expectations that targeting GSCs could induce effective eradication of glioma. Currently, remarkable insights have been gained regarding intracellular and extracellular signalling pathways that are crucial in GSC malignancies (Figure 5), all of which are potential candidates for anti-GSC therapy.

3.2.1 Membrane-bound receptor-mediated signalling

Receptor tyrosine kinases (RTKs)

RTKs are the cell surface receptors for many growth factors, cytokines, and hormones, and have been known as not only key regulators of normal cellular processes but also critical stimuli in the development of many cancers.

The most frequent genetic alteration associated with human GBMs is amplification of EGF receptor (EGFR) gene, and its overexpression is observed in approximately 50–60% of GBM patients. The most common EGFR mutant is EGFRvIII, which is an in frame deletion variant that has a truncated extracellular domain with ligand-independent constitutive activity. The Ink4a/Arf$^{-/-}$ astrocytes dedifferentiate in response to EGF. Moreover, transduction of EGFRvIII into Ink4a/Arf$^{-/-}$ neural stem cells (NSCs)/astrocytes induces tumors displaying a common high-grade glioma phenotype (Bachoo et al., 2002). Soeda et al. (2008) have demonstrated that human GBM CSCs retain their self-renewal ability in the presence of EGF and that the tyrosine kinase inhibitors of EGF signaling, AG1478 and gefitinib suppress the proliferation and self-renewal of these cells. Mazzoleni et al. (2010) also have showed that EGFR expression is heterogenous in human GBMs and its high expression can identify tumor initiating cells. Interestingly, genomic heterogeniety of EGFR gene links to mutual communication between tumor cells, in which IL-6 and LIF proteins secreted from EGFRvIII-expressing GBM cells enhance wild-type EGFR-expressing GBM cell growth (Inda et al., 2010). From these data, targeting of EGFR seems reasonable for eradication of GSCs. However, despite several inhibitors of EGFR are now available in the glioma clinic, their efficacy is limited, suggesting that some combinatorial approach could improve clinical outcome.

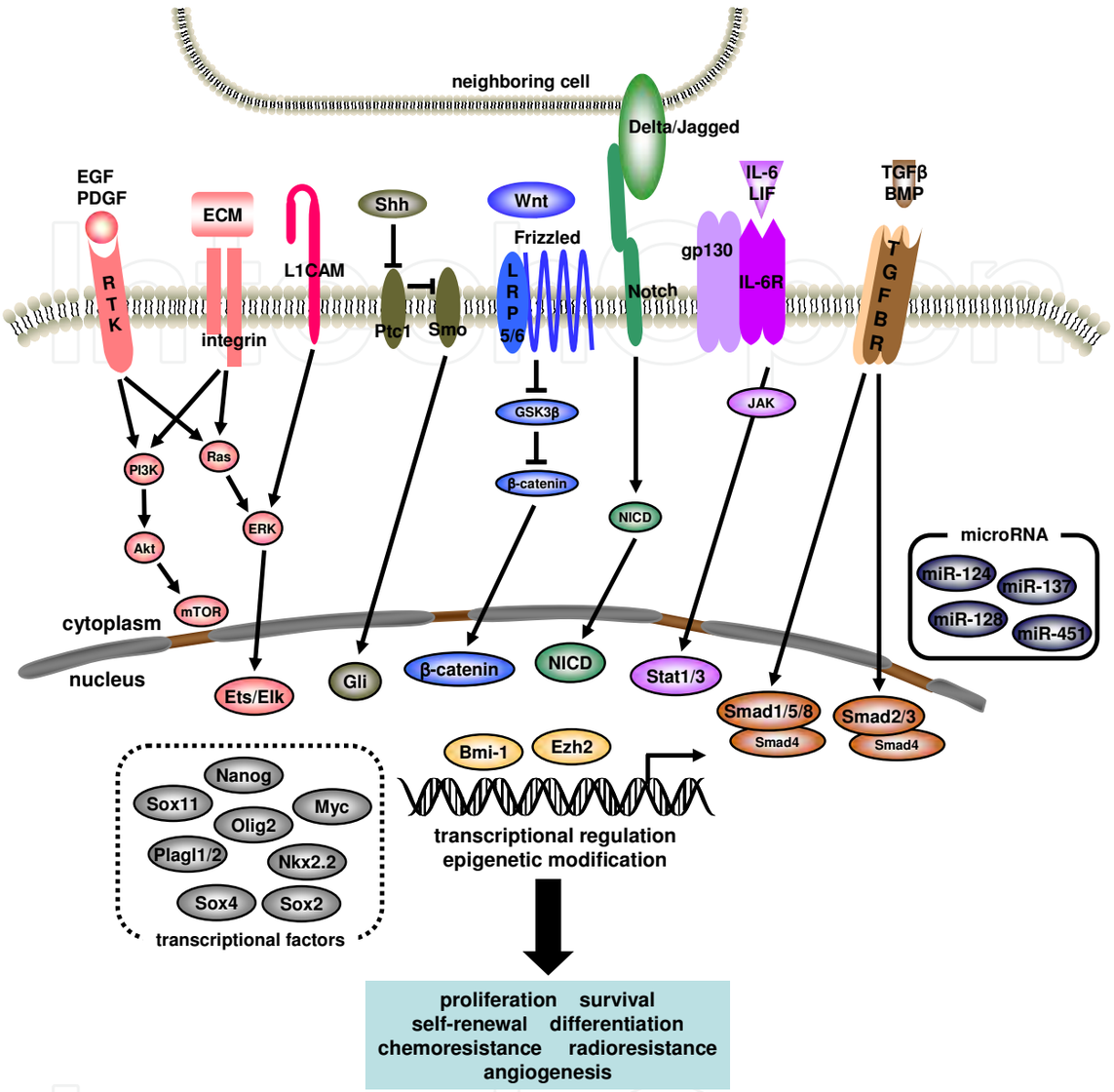

Fig. 5. Extracellular signaling and intracellular factors regulating glioma stemness.

PDGF functions in the later period in development as a potent mitogen of oligodendrocyte precursor cells (OPCs). OPCs express PDGF receptor (PDGFR), and PDGF is important for regulating OPC number and oligodendrocyte production. Additionally, PDGF signaling has been closely associated with the formation of glioma. Activation of PDGF pathway has been found in more than 80% of human oligodendrogliomas and in 50-100% of human astrocytomas. Daii et al. have reported that PDGF-B overexpression causes the proliferation of Nestin+ neural progenitors to form human oligodendroglioma-like tumors and also induces the dedifferentiation of GFAP(+) astrocyte into glial precursor-like cells (Dai et al., 2001). Recently, PDGFRα-expressing cells in the adult subventricular zone (SVZ) have been proposed as adult NSCs, and they are known to exhibit hyperplastic proliferation in response to excessive PDGFR activation (Jackson et al., 2006). These data suggest that PDGF/PDGFR pathway may contribute to not only glioma progression but also glioma initiation originated from immature stem-like cells.

RTK signallings are transmitted into nucleus by intracellular molecules, such as Ras/ERK and PI3K/Akt. Given that the combined activation of Ras and Akt in nestin(+) neural progenitors induces GBM in mice (Holland et al., 2000) and that the activity of drug transporter ABCG2 is regulated by PI3K/Akt pathway (Bleau et al., 2009), RTK signalling might control multiple behaviors of GSC in glioma progression and therapy-resistance.

Transforming growth factor-beta (TGF-β)

TGF-β superfamily proteins play a critical role on morphogenesis and cell lineage specification during brain development. In response to TGF-β, Smad2/3 becomes phosphorylated and forms a heterodimeric complex with Smad4, and then its complex is translocated into the nucleus to transactivate downstream target genes. Bone morphogenetic proteins (BMPs) belonging to a subset of the TGF-β superfamily is known to maintain self-renewal and multi-lineage differentiation potential of NSCs within the SVZ of the adult brain. In human GBMs, enhanced expression of the TGF-2 and TGF receptor I/II are detected. Two groups have reported that TGF-β enhances human GBM stemness and tumorigenic activity; one is through Sox4-mediated Sox2 induction (Ikushima et al., 2009) and another is through the induction of LIF secretion in an autocrine/paracrine fashion (Peñuelas et al., 2009). Consistent with these data, TGF-β receptor I inhibitor LY2109761 and SB431542 decrease the $CD44^{high}/Id1^{high}$ GBM CSCs population through the repression of Id1 and Id3 levels (Anido et al., 2010). However, TGF-β family proteins can function as both oncogenes and tumor suppressors. Lee et al. (2008) have reported that the expression of BMP receptor 1B is downregulated in human GBM CSCs through EZH2-dependent epigenetic silencing and thereby tumor stemness is maintained with impaired astroglial differentiation. Although these opposite roles of TGF-β on differentiation has remained to be resolved, these data indicate that TGF-β family proteins and their downstream targets have fundamental roles in glioma stemness and suggest that control of them could yield glioma eradication.

Interleukin-6 (IL-6)

IL-6 family of cytokines stimulate cells by forming the receptor complex composed of the each cytokine-specific receptor and the common component gp130 glycoprotein, leading to phosphorylation of signal transducers and activators of transcription (STAT3 and, to a less extent, STAT1) by gp130-associated janus kinases (JAKs). The phosphorylated STAT3 and 1 homodimerize and translocate into the nucleus where downstream target genes are transactivated. Several reports have shown that GBM tissues contain significantly higher levels of IL-6 protein, and higher IL-6 mRNA correlates with poor survival of GBM patients. In addition, phosphorylation and nuclear translocation of STAT3 positively correlate with the histopathological grade in human glioma samples (Weissenberger et al., 2004). Recent papers have demonstrated that targeting of either IL-6 receptor alpha or IL-6 by using lentiviral transduced shRNA or IL-6 antibody decrease tumor growth and survival in mice bearing intracranially xenografted human glioma (Wang et al., 2009). Likewise, STAT3 inhbitors (S3I-201 and STA-21) and RNAi-mediated knockdown of STAT3 prevent growth of human primary GBM CSCs (Sherry et al., 2009). Leukemia inhibitory factor (LIF), one of the cytokines of the IL-6 family which is known to be an essential factor for mouse embryonic stem cell self-renewal and maintenance, induces astrocytic differentiation of NSCs in cooperation with BMP2. As described above, transactivation of the LIF gene in glioma spheres by TGF-β enhances glioma stemness and then results in significantly shorter survival in mice when inoculated (Peñuelas et al., 2009). These effects of LIF on GSCs show

important contrast to those on NSCs and therefore LIF inhibition might be one potential approach whose side-effects are minimized during treatment.

Sonic hedgehog (Shh), Notch, and Wnt

Because GSCs share common properties with NSCs, molecular mechanisms underlying self-renewal and differentiation are usually overlapped among them. The Shh, Notch and Wnt pathways play a major role in normal stem cell regulation and also there is much evidence that aberration of these pathways cause tumorigenesis.

Sonic hedgehog binds to the Patched-1 (Ptc1) receptor and in turn activates the transcription factors of GLI family by cancelling the Patched-mediated inactivation of Smoothened (Smo). Shh signalling regulates neural progenitor proliferation and self-renewal in adult cerebellum, and it is well known that deregulation of Shh pathway in cerebellum neural progenitors induces medulloblastoma in mouse models. In human GBMs, amplification and overexpression of Gli-1 is reported in several types of gliomas. Hsieh et al. (2011) have reported that Shh/Gli1 signaling regulates insulin-like growth factor (IGF)-1-dependent GSC proliferation and chemo/radioresistance through insulin receptor substrate 1 (IRS1) gene transactivation. Xu et al. (2008) have identified Shh-dependent CSCs in some cases of human GBMs and found that activity of Shh signaling in PTEN-positive GBMs correlates with reduced patients' survival.

Notch is a membrane-bound receptor, and its intracellular domain is proteolytically released by binding of membrane-associated ligands Delta and Jagged and in turn binds to promoters of genes encoding bHLH transcription factors. Notch activation is involved in the regulation of NSC self-renewal, but it promotes glial differentiation in some conditions. Abnormal activation of Notch signaling has been found in a wide range of human cancers, including glioma. Fan et al. (2010) have reported that a γ-secretase inhibitor GSI-18, which prevents Notch secretion, suppresses neurosphere clonogenicity *in vitro* and growth of intracranial CD133-positive GBM cells through loss of AKT and STAT3 phosphorylation. Importantly, the combination of γ-secretase inhibitors and irradiation can attenuate cell growth and clonogenic survival of GBM CSCs (Wang et al., 2010a).

Wnt signaling pathway also controls the size of the NSC population through β-catenin activation. Wnt binds to a transmembrane receptors Frizzled (Fzd), leading to the phosphorylation of the Dishevelled, which prevents glycogen synthase kinase 3β (GSK3β)-dependent degradation of β-catenin. β-catenin translocates into the nucleus where it binds to TCF/LEF and displaces corepressors to induce expression of target genes. Although Wnt is mutated in a subgroup of medulloblastomas, its role in gliomas is little known. Zheng et al. (2010) have recently proposed Wnt signaling as promising targets for GBM treatment. They identified PLAGL2 as a protooncogene amplified and/or overexpressed in human GBMs. Enhanced PLAGL2 expression promotes GSC self-renewal and contributes to gliomagenesis. They showed that PLAGL2 modulates the transcription of Wnt6 and Fzd9 and Fzd2 receptors, but in this paper transcriptome analysis is examined in only $p53^{-/-}$ NSCs, and therefore it remains still unclear whether Wnt contributes to the malignant behaviors of GSCs.

L1CAM

The neural cell adhesion molecule, L1CAM (CD171) regulates neural cell growth, survival, migration during brain development. Although the role of L1CAM in the normal adult brain is not well defined, L1CAM is overexpressed in human gliomas and associated with

invasive phenotype. L1CAM is highly expressed in CD133(+) glioma cells and that its knockdown suppresses glioma growth *in vivo* and increases survival of xenografted mice with downregulation of NSC transcription factor Olig2 and upregulation of cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Bao et al., 2008). These data suggest that L1CAM is a functional marker of GSC and its inhibition might be useful as a CSC–directed therapy.

3.2.2 Nuclear transcriptional factors

Signals and stresses from outside of the cells are transduced into nucleus, where expression of downstream targets is regulated by transcriptional and/or epigenetic factors, and lead to a variety of cellular phenotypes. Some nuclear factors are currently suggested to be essential for GSC behaviors and most of them regulate the self-renewal and differentiation.

Myc

Zheng et al. (2008) have performed wide-scale transcriptome/promoter studies and found strong enrichment of Myc binding elements in human primary GBMs. They revealed that the increase of Myc expression by the defect of p53 and PTEN in GBM CSCs withstands the differentiation and then enhances tumorigenic potential. These results strongly support pro-differentiation as a potential strategy against GSCs and encourage the identification and screening of agents targeting these differentiation pathways.

Sox11

Hide et al. (2009) have identified Sox11 as a candidate to induce differentiation in GSCs. Sox11 is exclusively overexpressed in non-tumorigenic glioma cells, and overexpression of Sox11 inhibits tumorigenesis of GSCs by inducing their neuronal differentiation. Sox11 is expressed in the committed neuronal precursor cells but not in NSCs, and negatively regulates the expression of PLAGL1, which is expressed in developing neuroepithelial cells. Knockdown of PLAGL1 significantly improves overall survival of xenotransplanted mice. Considering that PLAGL2 also regulates GSC self-renewal (Zheng et al., 2010), these PLAG family genes of transcriptional factors might play essential oncogenic roles. As both PLAGL1 and PLAGL2 are known to activate IGF2 promoters, the relevance between PLAG genes, Shh, IGF, and Wnt suggest that highly complicated cross-talk of multiple signaling regulates GSC features.

Nkx2.2

Muraguchi et al. (2011) have recently identified the homeodomain transcription factor Nkx2.2 as an inducer of GSCs toward oligodendroglial differentiation by using a sophisticated mouse models developing glioma at 100% penetrance. During normal development of brain, Nkx2.2 cooperates with Olig2 to promote oligodendrocyte maturation. Overexpression of Nkx2.2 inhibits self-renewal of human GSCs by induction of oligodendroglial differentiation, and therefore reactivation of Nkx2.2 expression in glioma is suggested as a novel therapeutic strategy.

Nanog

The puluripotent protein Nanog promotes not only embryonic stem (ES) cell expansion but also GSC tumorigenicity. GBMs show ES-like stemness signature that includes the expression of Nanog, OCT4, SOX2 and c-Myc (Ben-Porath et al., 2008). Knockdown of Nanog1 or Nanog2 suppress tumor growth after orthotopic xenotransplantation of GBM spheres and this effect is mediated by a positive-feedback loop of Nanog with Gli-1, and

repression of Nanog by p53, which acts in a negative-regulatory loop with Gli-1, suggesting functional crosstalk among transcriptional factors in regulating the stem cell behavior and glioma growth (Zbinden et al., 2010).

3.2.3 DNA-damage response (DDR)

The large-scale genomic analyses have revealed genomic loss of at least one allele of ATR, ATM, CHEK1, or CHEK2 in 36% of human GBM samples, which are genes encoding key components of DDR required for cell-cycle checkpoint. Approximately 50% of GBM patients with CHEK2 alterations carry defects in the p53 signaling pathway (Squatrio et al., 2010). As the defect of DNA repair factors leads cells to genomic instability, genomic loss of DDR components might contribute to glioma initiation. However, Bao et al. (2008) have reported that $CD133^+$ primary GBM cells, not $CD133^-$ cells, have high activity of DDR response and thereby can repair ionizing radiation-dependent DNA damages. Wang et al., (2010a) showed that Notch signaling promotes radioresistance through regulation of the PI3K/Akt pathway and myeloid cell leukemia 1 (Mcl-1), but it remains unelucidated why only GSCs acquire and/or retain the high activity of DDR response.

3.2.4 Epigenetic factors

The DNA-methylation and histone-modification patterns are associated with the development and progression of cancers. Hundreds of genes are subject to DNA hypermethylation at their CpG island promoters. On the contrary, DNA hypomethylation is also detected by locus-specific and genome-wide. Histone modifications are also likely important in the pathology of GBM. However, the molecular aspects of them are not yet fully determined. One important example of DNA hypermethylation is the silencing of O6-methylguanine-DNA methyltransferase (*MGMT*) gene in human GBMs (Hegi et al., 2005). *MGMT* gene encodes a DNA repair protein that removes alkyl at the O6 position of guanine, which protect glioma cells from chemotherapeutic alkylating agents, such as temozolomide. MGMT hypermethylation has been considered as a predictor of outcome after treatment, but it is unrevealed if longer patients survival is directly due to reduced MGMT expression. As epigenetic modification can alter the differentiation properties of normal stem cells, some epigenetic factors have been examined their roles in GSC maintenance and tumor development.

EZH2

Enhancer of zeste homologue 2 (EZH2) is a component of the polycomb repressive complexes (PRC) 2 that participates in transcriptional repression of specific genes by mainly trimethylation of lysine 27 of histone H3. EZH2 is upregulated in a broad range of human neoplasms and its overexpression is correlated with poor prognosis. Suvà et al. (2009) have reported that pharmacologic depletion of EZH2 by the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A (DZNep) reduces the self-renewal and tumor-initiating properties of GBM GSCs. Interestingly, their effects are mediated by c-myc downregulation, even though EZH2 belongs to a repressive complex.

Bmi-1

B lymphoma mouse Moloney leukemia virus insertion region (Bmi)-1 is a component of PRC1 complex which allows stable silencing of gene expression. PRC1 and PRC2 cooperatively modify histone status. Abdouh et al. (2009) have reported that Bmi-1, as well as EZH2, is highly enriched in CD133(+) GBM cells and that knockdown of Bmi-1 can deplete the

CD133(+) cell population and inhibit intracranial tumor formation. Bmi-1 directly represses $p21^{Cip}$ expression. It may also directly repress FOXOA3 expression, a potent inducer of apoptosis, and thereby supports cell-cycle progression and survival (Abdouh et al. 2009). Polycomb group proteins seem to regulate important aspects of GSC biology.

3.2.5 microRNA (miRNA)

microRNAs are non-coding RNAs of 22 nucleotides in length and regulate the translation and degradation of mRNAs by base-pairing to untranslated regions (UTRs) of targets. Approximately 1000 species of microRNA are thought to exist in the human genome. One microRNA has hundreds of targets, and one gene is targeted by multiple microRNAs (Krek et al., 2005). Increasing evidence strongly suggest that microRNAs are involved in neural development. The most highly expressed microRNAs in brain are miR-124 and miR-128, both of which are preferentially expressed in neurons. Some groups have investigated the direct roles of miRNAs in GSC features. SMAD3/4-controlled miR-451 expression has been shown to increase neurosphere formation of CD133(+) GBM cells (Gal et al., 2008). Interestingly, another group has reported that miR-451 regulates the expression of MDR1, which links to chemoresistant feature in cancer cells (Zhu et al., 2008). Conversely, the expression of miR-124 and miR-137 are significantly reduced in grade III and IV malignant gliomas and overexpression of these two microRNAs promotes differentiation of human GBM stem cells, indicating their tumor suppressive roles (Silber et al., 2008). miR-128 targeting to the Bmi-1 gene expression is known to be down-regulated in human GBMs, and its restoration specifically blocks proliferation of glioma spheres (Godlewski et al., 2008).

3.3 GSC niche (Therapeutic strategy)

As well as normal NSCs, GSCs are believed to be physically situated in the specialized microenvironment called "stem cell niche". Niche cells provide extrinsic cues as described above, including soluble factors, membrane-bound ligands, and extracellular matrix (ECM) proteins. Cell-to-cell communication between stem cells and the niche regulates self-renewal, differentiation, and maintenance of proper stem cell numbers and functions. Tumor niche is thought to be composed of tumor vascular endothelial cells, stromal fibroblasts, and immune cells. Considering that CSCs display enhanced resistance to conventional chemotherapies due to their increased expression of membrane pumps, cancer treatment should be aimed at not the conventional elimination of the proliferating cancer cell population but the disruption of cellular niches to induce stem cell differentiation. The stromal components that function as niche would therefore be most promising targets to eradicate GSCs (Figure 6).

Vascular niche

Enhanced neovascularization is one of the characters of malignant gliomas and it significantly correlates with tumor aggressiveness and poor clinical prognosis. An initial report on GSC niche was proposed as a perivascular niche, where GSCs closely interact with vascular endothelium (Calabrese et al., 2007). It was verified by subsequent studies showing that glioma cells express high levels of laminin receptor integrin α6β1 in the perivascular niche and CD133(+)/integrinα6 (high) glioma cells exhibit stem cell properties (Lathia et al., 2010). In addition, secreted nitric oxide (NO) from the vascular endothelium regulates adjacent Nestin(+) glioma cells (Charles et al., 2010). GSCs are themselves tightly involved in tumor angiogenesis. Folkins et al. (2009) have demonstrated that GSCs increase

endothelial cell proliferation and tube formation *in vitro*. In addition, GSCs in xenografted mice increase mobilization of bone marrow–derived endothelial progenitor cells and recruit them to the tumor through amplified secretion of vascular endothelial growth factor (VEGF) and stromal-derived factor 1 (SDF-1, also known as CXCL12). Surprisingly, two groups have provided strong evidence that a proportion of the vascular endothelial cells in human GBMs are originated from the tumor cell itself, which are differentiated ones from GSCs (Wang et al., 2010b; Ricci-Vitiani et al., 2010). DAPT, a γ-secretase inhibitor that effectively inhibits Notch signalling was found to induce significant suppression of the transition from CD133(+)/CD144(−) GSCs into CD133(+)/CD144(+) endothelial progenitors. In addition, bevacizumab, a VEGFA-binding antibody, blocked further maturation from endothelial progenitors into CD105(+) endothelial cells. These data suggested again that GSCs are critical targets in glioma biology and treatment.

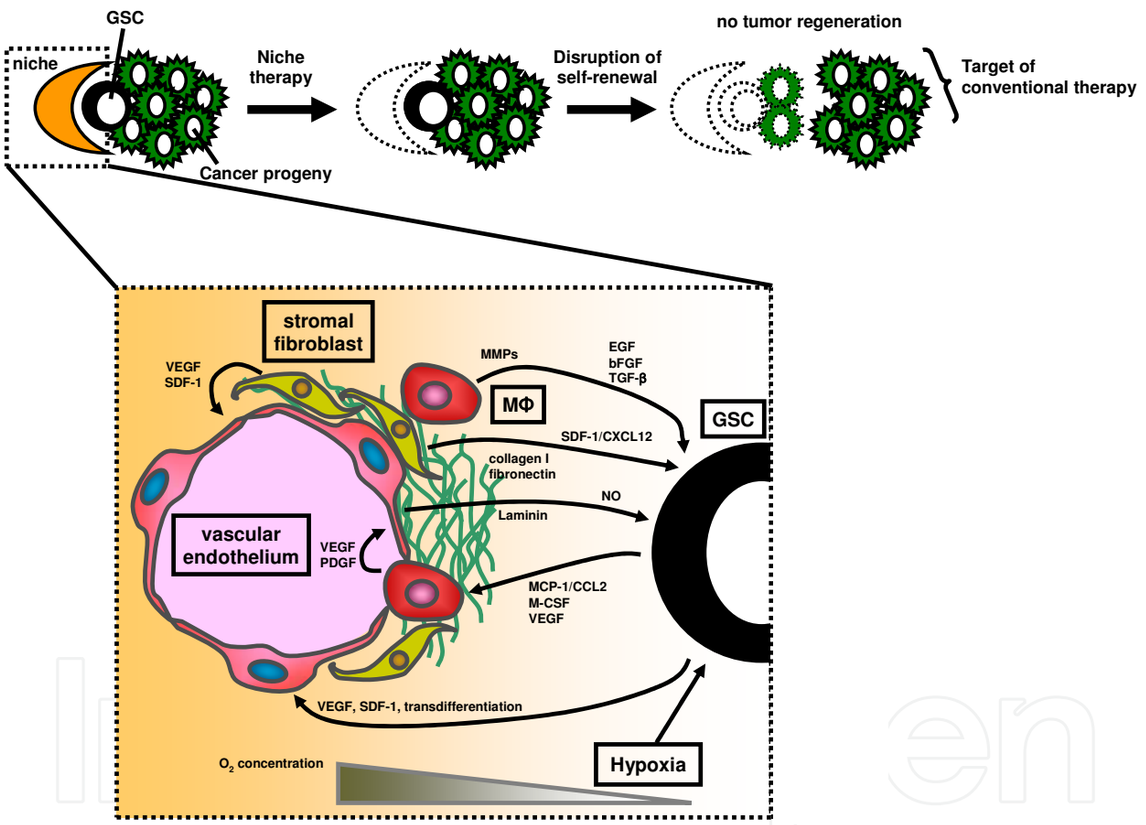

Fig. 6. The concept of niche therapy and components of GSC niche.

Hypoxic niche

Although the perivascular niche is well accepted, low oxygen areas away from tumor vessels are also recognized as secondary niches that play a role in GSC regulation. CD133 expression increases by approximately two-fold when primary glioma spheres are incubated in physiological concentrations of oxygen (7%) (McCord et al., 2009). In addition, hypoxia (1%) increases the self-renewal capacity of CD133(+) human GSCs independent of PI3K/Akt or ERK pathways (Soeda et al., 2009). The hypoxia-inducible factors (HIFs) are transcription factors that help cells to adapt to changes in oxygen concentrations. HIFs have

more than 60 putative downstream genes that are involved in angiogenesis, glucose transport, glycolysis, cell survival, and metastasis. Li et al. (2009) have reported that knockdown of either HIF-1α or HIF-2α in GSCs decreases tumorigenic potential and increases the survival of xenografted mice and that elevated HIF-2α expression is correlated with poor survival of human glioma patients. Méndez et al. (2010) have also demonstrated that HIF-1α plays a role in the survival and self-renewal potential of GSCs. Some chemotherapeutic agents which target RTKs, such as gefitinib (Iressa, an inhibitor of EGFR/ERBB1) and imatinib (Glivec, an inhibitor of Bcr-Abl and PDGFRβ), have been shown to inhibit HIF-1 activity (Pore et al., 2006; Kimura et al., 2007), and further evidence has shown that hypoxia mediates resistance to chemotherapy and radiation (Teicher, 1994). The formation of necrosis, which is a resultant of increasing cellularity and poorly organized tumor vasculature, is a biologic hallmark in a highly aggressvie gliomas. Therefore, understanding of stem cell biology in hypoxic environment could also provide useful clues for glioma biology and treatment.

Cancer-associated fibroblast

Stromal fibroblasts, which are potentially originated from bone marrow-derived mesenchymal stem cells (MSCs), are observed as a cellular component in the tumor microenvironment and have important roles in the tumor progression. Especially, during tumor-induced angiogenesis, fibroblasts actively contribute to neovascularization through secretion of VEGF. They also produce ECM proteins such as fibronectin and type I collagen, both of which are involved in the initiation of tumor angiogenesis. Additionally, fibroblast-derived SDF-1 is responsible for recruiting endothelial progenitor cells into a tumor mass, thereby boosting tumor angiogenesis (Orimo et al., 2005). In parallel, the SDF-1 protein enhances tumor growth in a direct paracrine fashion via the CXCR4 receptor. Although whether stromal fibroblasts could affect GSCs behaviors is not yet so clear, it is worth noting that some MSCs are exactly detected as a tumor-stimulating component around the blood vessels and closely associated with endothelial cells/pericytes in tumor xenograft models (Spaeth et al., 2009).

Tumor-associated macrophages

Tumor-associated macrophages (TAMs) represent the major inflammatory component of the stroma in many tumors. TAMs are derived from circulating blood monocytes and recruited into the tumor region toward monocyte chemotactic protein-1 (MCP-1, also known as CCL2) and macrophage colony stimulating factor (M-CSF). TAMs display several pro-tumoral functions, including tumor growth (growth factors), neo-angiogenesis (VEGF and PDGF), matrix remodeling (MMPs), and anti-tumor immune suppression (prostaglandins, IL-10 and TGF-β) (Sica et al., 2006). There is no evidence on the exact roles of TAMs against GSCs, but ablation of TAMs might provide some therapeutic benefits against GSCs beyond disrupting vascular niche.

4. Conclusion

The existence of CSCs has altered our view on glioma biology, which prompts our re-evaluation of conventional strategies in therapies for glioma. Although some controversy is still unresolved regarding the definition, isolation methods, characteristics, and their exact roles *in vivo*, it is assured that gliomas have a cellular hierarchy and that certain subpopulation within tumors has extraordinary potential for tumor progression, therapeutic

resistance, and tumor recurrence. Importantly, GSCs are highly resistant to chemo/radiotherapies, which are convinced by the currently poor record of clinical remission with conventional treatments. Therefore the development of novel therapies directed against GSCs is our urgent task. Remarkable insights into the molecular mechanisms or signaling pathways that are differentially regulated in GSCs and non-GSCs have accumulated in virtue of recent advance in this field. In this chapter, we have provided several key signaling pathways that are potentially useful for the future development of anti-GSC therapeutics, but at this point most of them are still far away from the clinical application. As an achievement, anti-vascular niche therapy with a humanized anti-VEGF-A monoclonal antibody bevacizumab, has displayed beneficial effects in phase II clinical trials, and approved for recurrent GBM patients in 2009. For relapsed or progressed GBMs, tumor responses were observed in 22 of 85 patients (26%) treated with bevacizumab alone (Friedman et al., 2009). This is the first major advance in glioma treatment over a decade. Nonetheless, our goal of glioma eradication has not been achieved. Additional basic and translational research must be carried out to exploit this promising concept for clinical advantage.

5. Acknowledgment

Our work is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to KT, TT, and ST. We wish to thank all members in Dept. of Stem Cell Regulation and Lab. of Cancer Research for their technical advice and helpful discussions.

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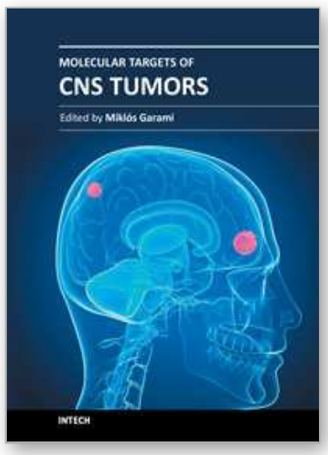

Molecular Targets of CNS Tumors

Edited by Dr. Miklos Garami

ISBN 978-953-307-736-9
Hard cover, 674 pages
Publisher InTech
Published online 22, September, 2011
Published in print edition September, 2011

Molecular Targets of CNS Tumors is a selected review of Central Nervous System (CNS) tumors with particular emphasis on signaling pathway of the most common CNS tumor types. To develop drugs which specifically attack the cancer cells requires an understanding of the distinct characteristics of those cells. Additional detailed information is provided on selected signal pathways in CNS tumors.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Kouichi Tabu, Tetsuya Taga and Shinya Tanaka (2011). Glioma Stem Cells, Molecular Targets of CNS Tumors, Dr. Miklos Garami (Ed.), ISBN: 978-953-307-736-9, InTech, Available from: http://www.intechopen.com/books/molecular-targets-of-cns-tumors/glioma-stem-cell

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