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Authors
Ludwig, Kirsten
Kornblum, Harley I

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Molecular Markers in Glioma

Kirsten Ludwig[1] and **Harley I. Kornblum**[1,2]

[1]Semel Institute for Neuroscience and Human Behavior and the Department of Psychiatry and Biobehavioral Research, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

[2]Department of Molecular and Medical Pharmacology and Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

Abstract

Gliomas are the most malignant and aggressive form of brain tumors, and account for the majority of brain cancer related deaths. Malignant gliomas, including glioblastoma are treated with radiation and temozolomide, with only a minor benefit in survival time. A number of advances have been made in understanding glioma biology, including the discovery of cancer stem cells, termed glioma stem cells (GSC). Some of these advances include the delineation of molecular hetereogeneity both between tumors from different patients as well as within tumors from the same patient. Such research highlights the importance of identifying and validating molecular markers in glioma. This review, intended as a practical resource for both clinical and basic investigators, summarizes some of the more well-known molecular markers (MGMT, 1p/19q, IDH, EGFR, p53, PI3K, Rb, and RAF), discusses how they are identified, and what, if any, clinical relevance they many have, in addition to discussing some of the specific biology for these markers. Additionally, we discuss identification methods for studying putative GSC's (CD133, CD15, A2B5, Nestin, ALDH1, Proteasome activity, ABC transporters, and Label-retention). While much research has been done on these markers, there is still a significant amount that we do not yet understand, which may account for some conflicting reports in the literature. Furthermore, it is unlikely that the investigator will be able to utilize one single marker to prospectively identify and isolate GSC from all, or possibly, any gliomas.

Keywords

Glioblastoma; Moledular Markers; Glioma Stem Cell; Pathways; and Mutations

INTRODUCTION

Brain tumors are generally classified using the World Health Organization (WHO) system that is largely based on pathological features. Grade I and II tumors are considered non-malignant, and Grade III and IV tumors are malignant, with Grade IV tumors also termed

Address correspondence to: Harley Kornblum, MD, PhD, Room 341 NRB, 635 Charles E. Young Drive South, Los Angeles, CA 90095, Hkornblum@mednet.ucla.edu.

CONFLICTS OF INTEREST:

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glioblastoma (GBM) [1]. Further subdivisions are based on additional features of the tumor cells, including the predominance of oligodendrocytic or astrocytic characteristics, and the location of the tumor. Glioblastomas are the most common and most malignant brain tumor and carry a dismal prognosis. Current treatments, which include radiation and chemotherapy with temozolimide, provide a survival benefit that can be measured in weeks rather than years. Further understanding of GBM biology and translation of this understanding into treatment is critically needed.

Many recent studies have focused on molecular differences amongst tumors with seemingly similar pathological features. In GBM, several studies have categorized tumors into multiple molecular classes [2–4]. In 2008, the TCGA published a widely used classification of GBM, which identified 4 different subclasses of GBM based upon molecular markers; Classical, Mesenchymal, Neural, and Proneural, with more recent studies eliminating the Neural subgroup [5]. These classifications were better able to predict prognosis, survival time, and response to treatment which opened up a new wave of research into molecular markers of GBM.

Here, we briefly review how molecular markers are being used in the analysis and study of GBM with the goal of synthesizing information for clinical and preclinical investigators. This review will discuss some established markers of glioma, with a focus on GBM. We will also examine some of the emerging markers for GBM stem cells (GSC). Because of its abbreviated nature, this review cannot discuss each potential marker extensively. Table 1, lists many of the known molecular markers for gliomas, with an emphasis on GBM (Table 1). While molecular markers are used extensively to differentiate the individual tumor types, very few provide reliable and reproducible predictive markers. Below, we discuss some of the more well-known molecular markers of brain tumors.

BIOMARKERS FOR GBM

Molecular Markers

MGMT methylation—Temozolimide (TMZ) adds an alkyl group to thymine and guanine, causing DNA damage to initiate apoptosis [6]. O6-methylguanine-DNA methyltransferase (MGMT) is a DNA damage repair protein that removes the guanine-alkyl group and prevents apoptosis [7]. Thus, MGMT mediates resistance to alkylating agents, and its loss makes tumors more sensitive to TMZ treatment [8]. Expression of MGMT is tightly regulated by methylation of its promoter [9], which leads to decreased expression of this protein and ultimately increased response to treatment [7]. Promoter methylation of MGMT is found in about 40% of GBMs, and about 80% of low grade IDH-mutated gliomas. Low MGMT levels correlate with modestly improved survival and response to TMZ [10].

1p/19q co-deletion—Co-deletion of the short arm of chromosome 1 and the long arm of chromosome 19 (1p/19q) is an early genetic event, and is closely associated with tumors of the oligodendroglial lineage, being found in 80% of oligodendrogliomas [11]. Interestingly, this co-deletion is almost never found in any other non-glial malignancy. It is typically associated with mutations in IDH1/2 [12, 13].

Isocitrate Dehydrogenase—Mutations in isocitrate dehydrogenase are found in 70%-80% of stage II and III astrocytomas, oligodendrogliomas, and most secondary GBM, comprising approximately 10% of all GBM [8]. Conversely, IDH mutations are almost never found in primary GBM. IDH mutations often occur in the context of either p53 mutations or 1p/19q co-deletion, rarely both. Mutations in ATRX can also be found in IDH mutant tumors that are not 1p/19q co-deleted. Mutations and amplifications of EGFR and loss of chromosome 10 are rarely found in IDH mutant tumors [13, 14].

The most common mutation of either IDH 1 or 2, which are highly homologous, is a single residue alteration that substitutes a histidine for an arginine, creating an additional function for the enzyme whereby it converts alpha-ketoglutarate (a-KG), the normal product, to D-2-hydroxy-glutarate (D-2HG) [14, 15]. How this promotes tumorigenesis is not currently understood, but is likely related to the effects of D-2HG on DNA demethylases, which promotes DNA and histone methylation.

EGFR—The Epidermal Growth Factor Receptor (EGFR) is a major activator of a variety of signaling pathways and physiological responses including proliferation, survival, migration, and tumorigenesis. EGFR is amplified in about 40% of GBM patients, and is often associated with high-grade Classical tumors. In GBM, there can be tens of additional copies of EGFR [16]. About half of patients with EGFR amplification, but none of those without it, have a constitutively active mutation due to deletion of exons 2-7 (EGFRvIII) [17]. EGFRvIII is expressed by small extrachromosomal pieces of DNA, termed “double minutes”, that are under dynamic regulation via unknown mechanisms [18]. While it is commonly thought that amplification or mutation of EGFR is an indicator of poor survival, several studies have failed to validate this conclusion [8, 19]. Many believe that this molecular marker could serve as a predictor for response to receptor tyrosine kinase (RTK) inhibitors. While EGFR-amplified tumors initially respond to RTK inhibition, data suggests that they often become resistant to this form of treatment [6].

Markers of Molecular Pathways in GBM

p53 pathway—p53 is one of the most well-known tumor suppressor proteins to date, being implicated in almost every cancer, including glioma. p53 deletion can occur, but the pathway is more often modulated by a number of factors, including upstream regulators MDM2, MDM4, and $p14^{ARF}$ as well as downstream effectors such as ATM and ATR. Based upon TCGA data, 78% of GBM have mutations somewhere within this pathway [20]. Found in low grade gliomas, alterations in the p53 pathway are thought to promote progression to high grade**.** Primary GBMs often have a loss of INK4A/ARF (CDKN2A) gene locus along with PTEN mutations and EGFR amplification/loss. Secondary GBMs more often have direct mutations of the p53 gene. However, because the p53 pathway functions in so many different cellular responses such as cell cycle regulation, apoptosis, differentiation, and DNA damage response, what prognostic and predictive response this protein has on the disease is still largely undetermined [19].

PI3K—Phosphoinositide 3-Kinase (PI3K) is responsible for the conversion of PIP2 to PIP3 which activates the downstream target PKB/Akt. The PI3K pathway is normally activated by

the EGFR and other growth factor receptors. [21]. Almost all GBMs show increased activity somewhere in this pathway, although less than 15% of GBM have activating mutations in PI3K itself. Phosphate and Tensin Homolog (PTEN) is a negative regulator of this pathway and thus plays a role in survival, proliferation, and migration through indirect activation of mTOR1/2 activity. Approximately 40% of GBM have mutations in this protein and around 70% show a loss of heterozygosity (LOH) at the PTEN locus [20]. The value of PTEN loss as a prognostic marker has not been validated, and is still somewhat controversial [22].

Rb pathway—The Retinoblastoma (Rb) pathway is commonly de-regulated in brain tumors. Rb is a negative regulator of the cell cycle and was discovered because of its loss in retinoblastoma [20]. While only 20% of GBMs are mutated at the Rb locus, inactivating mutations of the upstream regulator p16INK4a, or activating mutations in the downstream factors CDK4 or cyclin D result in dysregulated control of the E2F1 transcription factor are very common [23]. In addition, promoter methylation of the Rb gene is 43% more prevelant in secondary GBM as compared to primary tumors. This is not, however, found in low grade or anaplastic astrocytomas, suggesting that it may be a late event in astrocytoma progression [24].

Ras/Raf/MAPK—A major event that occurs in a subset of largely Proneural GBM is amplification of Platelet Derived Growth Factor Receptor alpha (PDGFRα) [25]. This receptor primarily activates the Ras/Raf/MAPK pathway utlmately regulating the activity of transcription factors that function in proliferation, survival, differentiation, and apoptosis [26]. Furthermore, the pathway is also activated by EGFR signaling. The pathway can also be directly or indirectly activated through mutations of downstream components. Ras itself is a common form of activation in many tumors, but they are rarely seen in GBM [27, 28]. However, the upstream Ras antagonist, Neurofibromin 1 (NF1) is either deleted or mutated in about 20% of primary GBMs. Additionally, alterations in the NF1 gene are closely associated with the Mesenchymal subtype [26]. BRAF, a downstream target of Ras, is typically activated in pilocytic (Grade I) astrocytoma, through either an activating mutation (V600E), or a fusion oncoprotein with KIAA1549 (KIAA1549:BRAF). Mutations in this pathway have not been associated with reliable effects on survival [28, 29].

MARKERS FOR GLIOMA STEM CELLS

The low survival rate of GBM patients is due in large part to recurrence, following initial response to treatment. One current theory is that recurrence is due to glioma stem cells (GSC), which are thought to be resistant to radiation and chemotherapy [30]. Therefore, identification and targeting of these cells has become a high priority for therapeutic development. While there is still a significant amount of debate regarding the existence of a ‘true’ cancer stem cell in glioma, there is a large body of evidence to corroborate the idea that a small portion of tumor cells are able to promote tumor initiation, propagation, and differentiation [31, 32]. Often times, these cells are also resistant to treatment.

Methods of identification

The identification of most GSC molecular markers, is based upon antibody recognition of specific proteins. However, once those cells have been isolated they need to exhibit both stem cell and tumor initiation properties. Stem cells are capable of self-renewal as well as multilineage differentiation. Rather than requiring multilineage differentiation, which may not occur in glioma, we generally state that the GSC must be capable of giving rise to the different cell types within the given tumor. Ideally, these requirements are best met if a candidate marker labels cells that give rise to and can propagate new tumors with a similar cellular heterogeneity as the parent tumor. Operationally, *in vitro* formation of floating clonal (derived from one cell) colonies, called variably “gliomaspheres” or, incorrectly “neurospheres”, in a very simple medium is often used as an indicator of potential stem cells. These colonies can, themselves give rise to new colonies and can produce the variety of cell types within a tumor [33, 34]. However, it is highly important to note that not all cells that form spheres can be called cancer stem cells. To date, several different putative GSC molecular markers have been identified in the last several years (Table 2). Below, we discuss some of the more researched markers and whether they can reliably detect GSC.

Molecular Markers

CD133—CD133 (also called AC133) is a membrane bound glycoprotein encoded by the PROM1 gene, that may function in cell differentiation, epithelial to mesenchymal transition, and is a marker for human neural stem cells [35, 36]. In 2003 and 2004, the Dirks laboratory demonstrated that a population of CD133+ cells isolated from human brain tumors could repropagate the original tumor at very low cell density in an immuno-compromised mouse, while CD133− cells could not [37, 38]. Since then, investigators have shown that these cells can become a variety of cell types, and that CD133+ cells have high telomerase activity, a possible sign of stem cell activity. CD133+ cells often co-express Nestin, a protein expressed by neural stem and progenitor cells. In many different tumors an increased proportion of CD133+ cells correlate with poorer survival, and the amount of PROM1 mRNA is able to distinguish GBM from low-grade tumors. Furthermore, GBMs that have recurred after radiation and chemotherapy often have a higher percentage of CD133+ cells, as compared to the original tumor [39].

Other studies have called the use of CD133 as a general GSC marker into question. These observations include the findings that in some cases, differentiated cells were CD133+, and that CD133− cells could initiate tumors. It is likely that for some GBM, CD133 will be an informative marker, while in others it will not be. Unfortunately, many studies have equated the percentage of CD133 positive cells in a tumor as being indicative of the number of stem cells [39]. It is evident that such conclusions cannot be drawn unless the case is proven for that tumor.

CD15—Stage-specific embryonic antigen-1 (SSEA-1) or CD15 (also termed LeX) is an antigenic epitope with a carbohydrate structure. CD15 is a known marker for murine, but not human pluripotent stem cells. This protein came to the forefront in GBM when CD133−/CD15+ cells were found to form tumors *in vivo*, prompting the idea that this may be a novel molecular marker. However, since then other papers have found no difference in the ability

of CD15+ and CD15− cells to form tumors in mice. Moreover, a difference in survival and treatment response has not been found with expression of this protein [40, 41].

A2B5—A2B5 is a glycolipid expressed on the cell surface of oligodendrocyte progenitor cells that are, in fact multipotent and, at least in some cases, serve as the cells of origin for gliomas. A2B5+/CD133− human glioma cells were found to form tumors in immunodeficient rats, while A2B5−/CD133− cells were not. A2B5+ cells isolated from gliomas form gliomaspheres, express nestin and can differentiate into cells with charcteristics of neurons, astrocytes, and oligodendrocytes. Additionally, A2B5 is thought to be a marker of poor prognosis and low grade A2B5+ tumors may have a higher rate of recurrence [42]. One advantage to using this marker is that A2B5 positive tumor cells can be compared directly to A2B5 positive glial progenitor cells [43]. It seems likely that, in the majority of gliomas, the A2B5 positive fraction, contains the tumor initiating fraction, but also contains other, more differentiated cells.

Nestin—Nestin is an intermediate filament protein expressed in neural progenitor cells and reactive astrocytes as well as some other cells in the body. Nestin is expressed in many GBMs, and differentiation of GBM cells leads to a downregulation of nestin, prompting researchers to examine its viability as a potential marker for GSC. Nestin positive neural stem cells are competent to give rise to gliomas in murine genetic models when they are transduced with oncogenes [44, 45]. While Nestin positive cells at least in some cases, can form gliomaspheres *in vitro*, no study to date has demonstrated that Nestin positive cells alone can recapitulate a human tumor *in vivo*. Expression studies have found that Nestin expression is correlated with higher grade gliomas and lower patient survival rates at either protein or mRNA expression levels, while other studies have shown that Nestin expression has no effect on prognosis of the patient [40, 46, 47].

Functional Markers

While many of the previously mentioned markers may be valuable tools, they are not consistent, and thus make it very difficult to identify GSC across a broad panel of cells and tumor types. Additionally, molecular markers of GSC do not allow for identification *in vivo.* To get around these issues, researchers have investigated the use of functional markers to identify potential GSC's based upon cellular characteristics that can be identified either *in vitro* or *in vivo.* We have summarized a few of these identification methods below.

ALDH1 activity—Aldehyde dehydrogenase 1 (ALDH1) is a cytosolic protein that oxidizes aldehydes to carboxylic acid, including the transformation from retinol to retinoic acid. Studies suggest that this may play a role in stem cell maintenance, and as such, high ALDH1 activity is theorized to be a functional marker of cancer stem cells [48]. Aldefluor is a substrate that is catalyzed by ALDH1 to a fluorescent product which accumulates and can be detect by FACS for quantification of enzyme activity and used for cell sorting [49]. Initial studies with Aldeflour demonstrated that glioma cells with high ALDH1 activity have a better ability to form spheres *in vitro* and form tumors *in vivo.* However, the validity of using ALDH1 activity as a marker for GSC has not been well validated across a spectrum of

tumors. Furthermore, ALDH1 is a relatively nonspecific marker and is expressed by normal astrocytes [50].

Low Proteasome Activity—Certain stem cells have lower proteasome activity than non-stem cells in the same tumors, which enhances the expression of proteins valuable for stem cell function [51]. The Pajonk group has taken advantage of this and created a novel vector system to identify cells with low proteasome activity based upon accumulation of a fluorescently labeled protein that accumulates in cells with low proteasome activity. This vector appears to identify the cancer stem cell fraction in several types of cancer, including breast, prostate, head and neck, and glioma. When isolated, these cells exhibited increased sphere-forming capacity and expressed CSC markers. *In vivo*, cells with low-proteasome activity are 100-times more tumorigenic than cells with higher proteasome activity, and their numbers increased following radiation [52]. Finally, in patients, low proteasome activity correlates with poorer survival. Thus, low proteasome activity is a promosing functional marker for GSC [53].

ABC Transporters—ATP-binding cassette transporters (ABC) are membrane pumps that export endogenous compounds and a variety of xenobiotics from the cell. This is hypothesized to be a significant reason for cancer stem cell resistance to chemotherapeutic agents [54, 55]. ABC transporters are responsible for the removal of certain fluorescent dyes, such Hoescht 33342, which allow investigators to identify these cells by FACS analysis as a ‘side population’. In GBM, this population of cells has been shown to exhibit cancer stem cell like properties including the ability to self-renew, resistance to chemotherapy, and formation of tumors *in vivo* from low number of cells. Side population analysis has not been well validated across the spectrum of human GBM [56].

Label retention—In general, normal adult tissue-specific stem cells cycle more slowly than other proliferating cells. It has been hypothesized that such is the case in at least some cancer stem cells. This slow division is thought to contribute to resistance against radiation and standard chemotherapeutic drugs which target fast cycling cells [57]. Investigators have used a variety of pulse-chase experiments to identify cells which maintain the expression of a fluorescently labeled marker. These dyes, which label the DNA, protein, or cell membrane permeate cells during the pulse treatment. However, upon removal or activation of the dye during the chase part of the experiment, the dye is diluted by half every time the cell divides. The greater the amount of label remaining, the slower the cell is dividing. One can then either identify the label retaining cells in tissue by fluorescence intensity, or isolate them by FACS. These types of experiments, using CFSE, GFP-labeled proteins, or lipophilic membrane dyes, have found that not only do label-retaining cells form more gliomaspheres, they can differentiate into multiple cell lineages, form tumors *in vivo* with low number of transplanted cells, and are resistant to both radiation and chemotherapy [44, 58, 59].

CONCLUSIONS

While a great deal of information has been gathered on molecular alterations in glioma and glioma stem cells, including the availability of numerous molecular markers, caution must

be taken in drawing broad sweeping conclusions regarding their clinical utility and their place in preclinical studies.

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Table 1

Molecular markers associated with glioma

Marker	Alteration	Tumor type	Comment	Identification Method	Ref
1p/19q	Deletion of short arm Ch. 1 and long arm of Ch. 19	Oligodendrogliomas	Never found in non<glial malignancies, often found with IDH mutations	FISH	[23]
Atrx	Mutation or deletion	Secondary GBM and Low Grade Glioma	Correlates with p53 expression, never found with 1p/19q deletions	PCR, Sequencing, IHC, or WB	[60]
BRAF	V600E or fusion gene KIAA1549:BRAF	Pilocytic Astrocytomas		FISH, Sequencing, IHC, or WB	[10]
CDK4	Amplification	Proneural		FISH	[10]
EGFR/EGFRvIII	Over amplification and mutation	Primary Glioma	Mutually Exclusive of p53 mutations	FISH, IHC, or WB	[23]
HIF1<a	Overexpressed	High Grade Gliomas		IHC	[10]
IDH	Missense mutation at arginine 132 (1) or 172 (2)	Oligodendrogliomas and Secondary GBM	Associated with G<CIMP, precedes 1p/19q deletion or p53 alterations	Sequencing or IHC	[23]
MET	Amplification	Mesenchymal		FISH	[10]
MGMT	Promoter methylation	GBM and Low Grade Gliomas	Beneficial Predictive Response to TMZ	PCR, or IHC	[23]
CDKN2A	Homozygous deletion	GBM and anaplastic glioma		FISH or IHC	[23]
NF1	Mutation or deletion	Mesenchymal and Pilocytic Astrocytoma		FISH, Sequencing, or IHC	[10]
p53	Mutation	Secondary and low grade GBM	Mutually Exclusive of 1p/19q deletions	IHC or Sequencing	[23]
PDGFR	Amplification	Proneural		IHC or FISH	[10]
PI3K	Activation mutation	GBM		Sequencing	[10]
PTEN	Mutation or deletion	GBM		IHC	[23]
RB	Mutation or deletion	GBM		FISH or IHC	[10]
TERT	Promoter methylation	Primary GBM and Oligodendroglioma	Mutually Exclusive of ATRX mutations	Methyl<specific PCR	[23]
VEGF	Overexpressed	Mesenchymal	Increased expression corresponds to increased grade	IHC	[23]
YKL<40	Overexpressed	Mesenchymal		IHC	[61]
ELDT1	Overexpressed	Mesenchymal and High Grade Gliomas		IHC	[23]
H3F3A	Mutation	Pediatric		IHC	[60]

Table 2

Molecular markers associated with glioma stem cells (GSC)

Marker	Non*Glioma Cell Types Often Associated With This Marker	Comments	Identification Method	Ref
Nestin	Neuronal stem cells	Intermediate filament, function in regeneration, and act as reserve of protenitor cells	WB, IHC, or Flow	[46]
SALL4	Embryonic stem cells	Transcription factor, interacts with Oct4 and Nanog	WB or IHC	[46]
Octl4	Embryonic stem cells	Homeodomain transcription factor, involved in selflrenewal of undifferentiated embryonic stem cells. Forms a heterodimer with Sox2	WB or IHC	[46]
SOX2	Embryonic stem cells and nueral tubes	Transcription factor required to make iPSC with Oct4 cIMyc and KLF4	WB or IHC	[46]
STAT3	Embryonic stem cells	Transcription factor active by JAK. Required for mouse embryo development. Implicated in oncogenesis	WB or IHC	[46]
NANOG	Embryonic stem cells	Homeobox transcription factor required for pluripotency. Works with Oct4 and Sox2.	WB or IHC	[46]
cIMyc	Numerous progenitor cells	Transcription factor that regulates 15% of gene expression.	WB or IHC	[46]
KLF4	Embryonic and mesenchymal stem cells	Transcriptoin factor that interacts with CREBI	WB, IHC, or Flow	[46]
CD133	Hematopoetic, endothelial, and neural stem cells	Glycoprotein that localizes to cellular protrusions. Most prolific GSC.	WB, IHC, or Flow	[46]
CD44	Mesenchymal cells	Receptor glycoprotein involed in cellIcell interations, adhesion, and migration.	WB, IHC, or Flow	[46]
GFAP	Astrocytes	Intermediate filament used for astrocyteI neuron communcationand repair.	WB, IHC, or Flow	[46]
Olig2	Oligodendrocyte progenitor cells and motor nuerons	Transcription factor, essential regulator of ventral neuroectodermal progenitor cell fate.	WB or IHC	[62]
Bmi1	Hematopoetic Stem Cell	Polycomb ring finger oncogene. Self reInewal of HSC and inhibits the aging of	WB or IHC	[40]
L1CAM	Neuronal Progenitor Cells	Glycoprotein adhesion molecule, involved in neurite outgrowth and differentiation	WB, IHC, or Flow	[63]
CD15	Mouse Embyonic Stem Cells	Cluster of differentiation antigen and carbohydrate adhesion molecule.	WB, IHC, or Flow	[41]
A2B5	Oligodendrocyte Progenitor Cells	Series of glycolipid antigens including GT3, GT1c, and GQ1c	WB, IHC, or Flow	[41]
Musashi	Glial and Neuronal Progenitor cells	mRNA binding protein that promotes down regulation of 26S proteasome.	WB, or IHC	[64]
Integrin 6!	Neural Stem Cell	Heterodimeric intergrin cell surface receptor that regulates neuronal stem cell growth.	WB, IHC, or Flow	[65]