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The Pivotal Roles of GSK3β in Glioma Biology

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1. Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults and one of the most lethal of all human cancers. Despite substantial advances in surgical intervention and combining radiotherapy regimens with new generation chemotherapies, the median survival for these patients is still about 15 months (Nagane, 2011). In recent years, cancer treatments using molecularly targeted drugs that act against the respective molecules have been successful, although successful molecular targeted therapies for glioma have yet to be established. Because the mechanisms underlying glioma formation and progression are complex, many candidate target molecules are identified as potential therapeutic targets (Nakada M et al., 2011). To date, the benefit of molecularly targeted therapies is limited, although there are many completed and on-going clinical trials (Quant & Wen, 2010). Thus, promising molecular targets should be identified to establish innovative molecularly targeted therapies against GBM.

Glycogen synthase kinase 3β (GSK3β) is a serine-threonine protein kinase originally identified for its inhibitory role in the conversion of glucose to glycogen via phosphorylation and inactivation of glycogen synthase. Recent studies suggest a conflicting role of GSK3β in various human cancers, either as a tumor suppressor or tumor promoter. Emerging evidence suggests that GSK3β is a tumor promoter in glioma, acting to regulate and link key players that control proliferation, resistance to radiochemotherapy, activation of invasion, and anti-apoptosis (Kotliarova et al., 2008; Miyashita et al., 2009a; Nowicki et al., 2008). The combined anti-proliferative and anti-invasive properties of small molecule GSK3β inhibitors make them an attractive treatment modality for controlling GBM.

The aim of this chapter is to highlight important aspects of the biology of GSK3β, focusing on the pathological role, signal transduction, and possibility of being a molecular target for GBM.

2. General knowledge of GSK3β

GSK3 was discovered in 1980 as a kinase that phosphorylates glycogen synthase, a key enzyme involved in glycogen synthesis (Embi et al., 1980). GSK3 is evolutionarily conserved and consists of 2 distinct isoforms encoded by 2 different genes, GSK3α and GSK3β, in mammals (Woodgett, 1991). GSK3 is ubiquitously expressed, and is highly enriched in the
brain. Compared with GSK3α, the GSK3β protein lacks 60 amino acid residues at the N terminus, resulting in a lower molecular weight for GSK3β (46.7 kDa). The 2 isoforms share extensive homology, most notably in the kinase domain (ATP binding site), which shares 97% homology. Initially, the primary function attributed to GSK3 was negative regulation of glycogen synthesis through phosphorylation and inactivation of glycogen synthase. However, further studies have uncovered additional functions of GSK3, such as cell cycle regulation, proliferation, differentiation, apoptosis, and migration. Despite high homology, the 2 isoforms are not functionally redundant, as demonstrated by gene knockout studies (Hoeflich et al., 2000).

2.1 Biological characteristics of GSK3β

Although the differences in functional roles of the 2 isoforms are not fully elucidated, research thus far has primarily focused on GSK3β. GSK3β is subject to multiple levels of regulation mediated by its phosphorylation, subcellular localization, and protein–protein interactions. GSK3β protein itself undergoes multiple phosphorylation events, which impact its activity depending on the amino acid being modified (Doble & Woodgett, 2003). Tyrosine phosphorylation of the GSK3β kinase domain at Tyr216 leads to its activation (Dajani et al., 2001), whereas phosphorylation of the N-terminal Ser9 results in inhibition of its activity and plays an important role in the regulation of GSK3β function (Stambolic & Woodgett, 1994) (Figure 1).

![Phosphorylation and activation of GSK3β](image)

Fig. 1. Phosphorylation and activation of GSK3β. Akt and MAPKAP-K1 phosphorylate Ser9 of GSK3β, resulting in inhibition of activity. The inhibition of GSK3β decreases the phosphorylation of glycogen synthase, leading to an increase in the active form, since phosphorylated glycogen synthase is less active. As a result, glycogen synthesis is promoted. MAPK: mitogen-activated protein kinase, MAPKAP-K1: MAPK-activated protein kinase-1 (also called RSK), Circled P: phosphorylation.
One of the main regulators of GSK3β activity is the phosphoinositide 3-kinase (PI3-kinase)/Akt pathway. PI3-kinase-induced activation of Akt mediates Ser9 phosphorylation of GSK3β (Cross et al., 1995; Pap & Cooper, 1998), resulting in the inhibition of GSK3β activity. GSK3β can also be phosphorylated at Ser9 by the most downstream kinase of the classical mitogen-activated protein kinase (MAPK) cascade, called MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK) (Frame & Cohen, 2001). Apart from this, GSK3β functions as a suppressor protein in the Wnt signaling pathway, which is the protein network associated with embryo development and cancer progression (Cook et al., 1996; Manoukian & Woodgett, 2002; Fuchs et al., 2005). The Wnt pathway was found to have essential roles in promoting the survival, proliferation, differentiation, and migration of cells in many different tissues, including nervous tissue, as well as in synapse formation in the nervous system. Briefly, the Wnt pathway involves the inhibition of an inhibitor, leading to activation of a transcription factor. When Wnt signal is absent, GSK3β associates with other proteins (e.g., axin, adenomatous polyposis coli [APC]) and functions as a critical mediator of the pathway. In this situation, the proto-oncoprotein β-catenin is constitutively phosphorylated, rapidly removed by degradation, and thus, will not build up in the cell to a significant level (Hagen & Vidal-Puig, 2002). In contrast, when Wnt binds to frizzled (Frz), its receptor, dishevelled (Dsh) is recruited to the cell membrane. GSK3β is inhibited by the activation of Dsh. Consequently, β-catenin, having escaped ubiquitination-dependent proteasomal degradation mediated by GSK3β phosphorylation, accumulates in the cytoplasm. It is subsequently shifted to the nucleus, where it assembles with other proteins (e.g., T-cell factor [Tcf]/lymphoid enhancer factor [Lef]) to switch on transcription of specific target genes, leading to its function as an oncoprotein. In this mechanism, GSK3β is thought of as a tumor suppressor protein (Figure 2).

GSK3β acts as a downstream regulatory switch that determines the output of numerous signaling pathways initiated by diverse stimuli (Frame & Cohen, 2001). Phosphorylation of GSK3β (Ser9) leads to the dephosphorylation of substrates, including glycogen synthase and translation factor eukaryotic protein synthesis initiation factor-2B (eIF-2B) (Welsh et al., 1998), resulting in their functional activation and consequent increase in glycogen synthesis, release of a number of transcription factors from tonic inhibition, and protein synthesis (Cohen & Frame, 2001). Thus, GSK3β affects both key components of the response to stimuli, reprogramming of gene expression, and activation of protein synthesis. Additionally, GSK3β phosphorylates a broad range of substrates: c-myc (Gregory et al., 2003), c-Jun (Boyle et al., 1991), c-Myb (Kitagawa et al., 2010), cyclin D1 (Diehl et al., 1998), Cdc25A (Kang et al., 2008), nuclear factor of activated T-cells (Beals et al., 1997), heat shock factor-1 (He et al., 1998; Xavier et al., 2000), Mcl-1 (Ding et al., 2007), cAMP response element-binding protein (Bulloch & Habener, 1998), and so on. GSK3β can target these substrates for degradation or inactivation, resulting in inhibition of cell proliferation and self-renewal. GSK3β also regulates nuclear factor (NF)-xB stability and activity (Demarchi et al., 2003; Hoeflich et al., 2000). These findings identified GSK3β as a key determinant in both physiological and pathological conditions, such as glycogen metabolism, insulin signaling, cell fate, neuronal function, and oncogenesis.

2.2 Physiological function

GSK3β participates in a number of different cellular pathways in a context-dependent manner and is implicated in the regulation of a wide range of cellular processes, including apoptosis, cell proliferation, and migration. Multiple lines of evidence indicate that the
function of GSK3β is opposing in different cell types. This functional dichotomy suggests that the function of GSK3β seems to be dependent on cell-types and/or cell conditions, which are physiological and pathological.

Fig. 2. Role of GSK3β in the Wnt pathway. When Wnt signal is absent, GSK3β promotes the proteasomal degradation of β-catenin. In contrast, when Wnt binds to frizzled (Frz), its receptor, dishevelled (Dsh) is recruited to the cell membrane. GSK3β is inhibited by the activation of Dsh. Consequently, β-catenin accumulates in the cytoplasm and is subsequently translocated into the nucleus to switch on Tcf/Lef-dependent transcription of specific target genes, leading it to act as an oncoprotein. Note that there are other regulatory components of each pathway; this schema was simplified for clarity.

2.2.1 Apoptosis
GSK3β is a component of signaling cascades involved in the process of apoptosis (Iqbal & Grundke-Iqbal, 2008) and is a critical downstream element of the PI3-kinase/Akt cell survival pathway (Pap & Cooper, 1998). Transient overexpression of GSK3β was found to induce spontaneous apoptosis in PC12 cells, used as a model system for neuronal differentiation, in a caspase-3-dependent manner (Pap & Cooper, 1998). Inhibition of GSK3β activity prevented cell death by blocking mitochondrial membrane potential changes and subsequent caspase-9 and caspase-3 activation in murine TSM1 neuronal cells (Petit-Paitel et al., 2009).
2.2.2 Proliferation

GSK3β is involved in cell proliferation through the canonical Wnt/β-catenin signaling pathway. GSK3β inhibition promotes translocation of dephosphorylated and stabilized β-catenin to the nucleus and its interaction with transcription factors, resulting in the induction of genes responsible for cell proliferation. Lithium chloride (LiCl), a chemical GSK3β inhibitor, significantly increased the proliferative potency of thyrocytes that appeared to be mediated by β-catenin (Rao et al., 2005). Similarly, the small molecule 6-bromoindirubin-3'-oxime (BIO), a specific inhibitor for GSK3, promotes proliferation in mammalian cardiomyocytes by elevated β-catenin activity (Tseng et al., 2006). In addition, it is known that inhibition of GSK3β promotes vascular cell proliferation, suggesting that active GSK3β inhibits angiogenesis (Hou et al., 2010). GSK3β signaling also plays an essential role in regulating the differentiation and proliferation of adult neural stem cells. Inhibition of GSK3β results in transcriptional activation of distinct target genes via β-catenin, leading to an increase in the number of neurons that differentiated from neurospheres (Maurer et al., 2007).

2.2.3 Migration

It has also been shown that, during cell migration, GSK3β plays a positive role in activating Rac, a Rho family member, and ADP-ribosylation factor 6 (Arf6), a related small GTPase, in adherent cells. Rac is responsible for forming lamellipodia during cell migration. Arf6 is also involved in vesicle trafficking, membrane ruffling, as well as lamellipodia formation (Turner & Brown, 2001). It has been shown that GSK3β activity is required for keratinocytes to form lamellipodia and migrate directionally in response to wound signaling (Koivisto et al., 2003). Similarly, GSK3β activates Rac in response to wound signaling in intestinal epithelial cells. When GSK3β is inhibited, and thus Rac activation prevented, these cells stop moving (Vaidya et al., 2006). Although the mechanisms by which guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP) regulate Rac or Arf6 have been extensively studied, no direct evidence has demonstrated that GSK3β modifies these GEFs and GAPs.

Focal adhesion kinase (FAK) is another candidate that contributes to the regulation of cell migration by GSK3β. FAK tyrosine phosphorylation and Rac activation were suppressed in GSK3β knockdown HeLa S3 (human cervical carcinoma cell line) cells, suggesting that GSK3β mediates the disassembly of focal adhesions to promote cell migration (Kobayashi et al., 2006). In contrast, Bianchi et al. showed that GSK3β reduces FAK kinase activity and cell motility in rat fibroblasts and HEK-293 (human embryonic kidney 293) cells. The influence of GSK3β on migration of various types of cells (e.g., neoplastic and non-neoplastic) appears to be complex. Further identification of additional GSK3β targets and more detailed studies of the pathways affecting cell migration will be necessary to clarify the function of GSK3β in cell migration (Sun et al., 2009).

2.3 Pathological function

GSK3β is ubiquitously expressed at high levels in the brain. Numerous studies have indicated that GSK3β is involved in key functions of the brain and is associated with a variety of neurological disorders like Alzheimer, Parkinson, and Huntington diseases, as well as affective disorders and other neurodegenerative disorders (Grimes & Jope, 2001; Jope & Roh, 2006). Additionally, it is not surprising that GSK3β has been implicated in...
glucose intolerance, considering its primary role is as a negative regulator of insulin-mediated glycogen synthesis and glucose homeostasis. Indeed, the activity of GSK3β has been reported in type II diabetes mellitus and obese animal models (Eldar-Finkelman et al., 1999; Nikouлина et al., 2000). GSK3β is also involved in inflammation that accompanies various kinds of diseases (reviewed in Jope et al., 2007). GSK3β inhibition attenuates activation of the pro-inflammatory transcription factor NF-κB and activates the immunomodulatory transcription factor β-catenin (Gong et al., 2008). GSK3β inhibition also induces secretion of the anti-inflammatory cytokine IL-10 (Hu et al., 2006).

More recent studies indicate a role for GSK3β in the control of neoplastic transformation and tumor development (reviewed in Miyashita et al., 2009b). Overexpression and activation of GSK3β was confirmed in various kinds of cancers such as colorectal, stomach, pancreatic, and liver cancers, as well as leukemia and GBM (Shakoori et al., 2005; Shakoori et al., 2007; Wang et al., 2008; Miyashita et al., 2009a; Mai et al., 2009). Previous studies have shown that inhibition of GSK3β suppresses cancer cell proliferation and induces apoptosis (Ougolkov et al., 2005; Ougolkov et al., 2007). In these cancers, the function of GSK3β is critical for malignant phenotype with respect to proliferation and invasion. Accumulated evidence supports the role of GSK3β in the regulation of apoptosis and proliferation appears to be diverse between physiological and pathological conditions.

However, the exact role of GSK3β in malignancies remains highly controversial due to the conflicting results from different tumor models. It has been shown that GSK3β is a tumor suppressor protein that controls cellular fate determination and stem cell maintenance through inhibition of the Wnt, Hedgehog, and Notch pathways. These pathways are aberrantly activated in several cancers (Saldanha, 2001; Waaler et al., 2011). This suggests that GSK3β inhibitors could exert a therapeutically negative, pro-survival effect on tumor cells. In addition, some studies found that GSK3β is part of a tumor suppressor complex consisting of axin and APC that phosphorylates the oncoprotein β-catenin and that, when GSK3β is inactivated, could possibly lead to tumor promotion (Hinoi et al., 2000; Rask et al., 2003). Available evidence indicates that GSK3β may function as a “tumor suppressor” for certain types of tumors such as skin and mammary tumors (Farago et al., 2005; Ma et al., 2007). These findings suggest that the mechanisms underlying the function of GSK3β as a tumor promoter or suppressor might depend on cell type and tissue context.

3. GSK3β biology in glioma

Recently, 3 independent research groups, including our group, simultaneously reported that GSK3β is a key promoter of malignant GBM phenotypes and is thus a promising candidate for molecular-targeted therapy (Kotliarova et al., 2008; Nowicki et al., 2008; Miyashita et al., 2009a).

3.1 Expression and activation

GSK3β is consistently expressed in primary GBM (Korur et al., 2009; Li et al., 2010). High expression levels of GSK3β and phosphorylated GSK3β (Tyr216) were detected in GBM compared with non-neoplastic brain tissues (Miyashita et al., 2009a). This finding identified GSK3β as an important regulator of malignant phenotype in GBM cells. GSK3β is constitutively active in GBM cells, despite the fact that PI3K/Akt, which can inhibit GSK3β activity, is a major signaling pathway in GBM. It is possible that an undetermined pathway...
3.2 Localization in tumor cells
Overexpression of GSK3β was observed in the cytoplasm of neoplastic cells in GBM, whereas only weak expression was observed in the cytoplasm of neurons from non-neoplastic tissue (Miyashita et al., 2009a).

3.3 Function
GSK3β function in glioma has been investigated by inhibiting GSK3β using small interfering RNA (siRNA), the small-molecule inhibitors LiCl or thiazolidinediones (TZD), and a small heterocyclic compound first described as a non-ATP competitive inhibitor of GSK3β. Inhibition of GSK3β activity attenuated proliferation, inhibited cell survival, enhanced tumor cell apoptosis, induced tumor cell differentiation, impaired formation of neurospheres, and reduced clonogenicity of GBM cells in a dose-dependent manner (Aguilar-Morante et al., 2010; Korur et al., 2009; Kotliarova et al., 2008; Miyashita et al., 2009a) (Table 1). The cytotoxic effects are directly correlated with decreased enzyme-activating phosphorylation of GSK3β (Tyr216) (Kotliarova et al., 2008). Furthermore, specific pharmacologic GSK3β inhibitors and siRNA knockdown of GSK3β reduced glioma cell motility (Nowicki et al., 2008). Importantly, administration of a highly specific GSK3β inhibitor, AR-A014418 (Bhat et al., 2003), at a low dose sensitized GBM cells to chemotherapeutic agents such as temozolomide and ionizing radiation, resulting in reduced cell viability (Miyashita et al., 2009a).

Table 1. Disadvantageous effects of GSK3β inhibition for glioma

<table>
<thead>
<tr>
<th>Inhibitory effect of GSK3β</th>
<th>Target molecules</th>
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<td>Apoptosis promotion</td>
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<td>Stemness reduction of the number and volume of neurospheres</td>
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<tr>
<td>Induction of differentiation</td>
<td>interruption of cyclin D1 proteolysis</td>
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<tr>
<td>Metabolism increase of intracellular glycogen</td>
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<td>Chemosensitivity enhancement</td>
<td>methylation of MGMT promoter?</td>
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<td>Radioresistance enhancement</td>
<td>unknown</td>
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<td>Neuroprotection protection of hippocampal neurons from apoptosis</td>
<td>acceleration of double strand-break repair efficiency</td>
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4. GSK3β-mediated signaling in glioma

4.1 Apoptosis
The molecules associated with GSK3β were also assessed by GSK3β inhibition. Several signaling pathways are associated with the decreased cell survival related to GSK3β inhibition. Inhibition of GSK3β activates the oncogenic transcription factor c-myc, leading to the induction of apoptosis promoting factors such as Bax, Bim, DR4/DR5, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), as well as subsequent cytotoxicity (Kotliarova et al., 2008). In addition, inhibition of GSK3β was associated with increased expression of p53 and p21 in GBM cells with wild-type p53. Simultaneously, the phosphorylated fraction of retinoblastoma protein (Rb) (inactive form) decreased, which is associated with down-regulation of cyclin-dependent kinase 6 (CDK6) (Miyashita et al., 2009a). It has been reported that decreased phosphorylation of Rb (activation of Rb) could be attributed to the down-regulation of CDK6 (Classon & Harlow, 2002). These signaling pathways probably induce apoptosis by inhibition of GSK3β in GBM cells. In contrast to the GSK3β phosphorlyates the Thr366 residue of PTEN that negatively regulates PI3K and then reduces Akt activity. Blocking phosphorylation of PTEN by either mutating or inhibiting GSK3β in GBM cell lines leads to stabilization of the PTEN protein (Maccario et al., 2007). In this situation, PTEN can work stably as a suppressor for PI3K/Akt, leading to apoptosis (Figure 3).

4.2 Proliferation
One of the major targets of GSK3β is NF-κB, which is an intracellular protein complex that controls DNA transcription and is a pro-survival factor in glioma (Kasuga et al., 2004; Robe et al., 2004). Inhibition of GSK3β activity by GSK3β-specific inhibitors such as LiCl and by GSK3β siRNA caused a dramatic decrease in intracellular NF-κB activity in U251, T98, and U87 GBM cell lines (Kotliarova et al., 2008). NF-κB inhibition then resulted in decreased glioma cell survival in vitro and inhibition of tumor growth in vivo (Kotliarova et al., 2008) (Figure 3). TZD-8 can inhibit GSK3β activity not only by directly interacting with this enzyme, but also by phosphorylating the Ser9 residue of GSK3β via MAPK pathway activation. TZD-8 suppresses the growth of glioma cells in vivo and exerts anti-proliferative and pro-apoptotic activities in glioma cells in vitro (Aguilar-Morante et al., 2010). These effects were accompanied by an activation of the MAPK signaling pathway, concomitant phosphorylation of Ser9, inactivation of GSK3β, and an inhibition of NF-κB activity. These results are consistent with previously published data, showing that an activation of MAPK is associated with a reduction in cell survival in different tumor cell lines, including GBM cell lines (Tewari et al., 2008). In contrast, GSK3β inhibition promotes entrance of β-catenin to the nucleus and the interaction of β-catenin with transcription factors. While this could promote cell proliferation, it does not take place, presumably, because of the simultaneous action of other pathways, which inhibit glioma cell proliferation (Kotliarova, 2008).

4.3 Migration/invasion
Specific inhibitors and siRNA knockdown of GSK3β both reduced glioma cell motility. The effects are dose dependent and reversible (Nowicki et al., 2008). However, the mechanisms underlying the effect of GSK3β on glioma cell migration and invasion required further study. Migration of GBM cells requires the formation of lamellipodia at the cell front and stress fibers consisting of actomyosin at the rear; contraction of these stress fibers causes the cell to reacquire front-rear symmetry. The migrating morphology of GBM cells is dependent
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Fig. 3. GSK3β-mediated signaling in glioma. The signaling pathways associated with apoptosis, proliferation, angiogenesis, cell stemness, and glycogen synthesis are depicted.

**Apoptosis:** GSK3β activity results in c-myc inhibition that consequently decreases expression of Bax, Bim, DR4/DR5, and TRAIL, preventing subsequent cytotoxicity. GSK3β increases the levels of Rb phosphorylation, resulting in Rb inactivation. Decreased Rb phosphorylation is associated with down-regulation of CDK6. GSK3β destabilizes PTEN, which negatively regulates PI3K and reduces Akt activity.

**Proliferation:** siRNA targeting GSK3β causes a dramatic decrease in intracellular NF-κB activity. NF-κB inhibition results in decreased glioma cell survival.

**Angiogenesis:** Hypoxia activates RhoB. HIF-1α may be regulated by RhoB through the Akt/GSK3β pathway under hypoxic conditions, although there is no data proving direct inhibition of HIF-1α by GSK3β.

**Stemness:** Expression of GSK3β is reduced by down-regulation of polycomb group protein Bmi1. Inhibition of Bmi1 induces a differentiation phenotype and reduces expression of Nestin and Sox2 in glioma cells.

on the balance between Rac1, whose activity is largely responsible for lamellipodia formation, and RhoA, which is related to stress fiber formation. Interference with lamellipodia by the inhibition of Rac1 reduces migration. This also causes GBM cells to acquire a relatively round shape without extending cell processes (Chuang et al., 2004). In contrast, collapse of actin stress fiber formation by the inhibition of RhoA promotes migration (Salhia et al., 2005). As LiCl treatment is associated with a marked change in GBM cell morphology, with cells retracting their long extensions at their leading edge and losing lamellipodia formation (Nowicki et al., 2008), GSK3β signaling may involve small GTPases such as Rac1 and RhoA.

4.4 Angiogenesis (signal induced by hypoxia)
A hypoxic microenvironment is a striking characteristic of GBM that is the collective consequence of morphologically and functionally immature neovascularization, irregular blood flow, anemia, and high oxygen consumption due to rapidly proliferating malignant cells (Jensen, 2009). The hypoxic microenvironment is a powerful stimulus for the expression of genes involved in tumor cell proliferation and angiogenesis (Carmeliet et al., 1998). Hypoxia markedly increases the inactive GSK3β fraction and decreases the active GSK3β fraction in U87 GBM cells. In U87 cells under hypoxia, depletion of Ras homolog gene family member B (RhoB) by siRNA decreases the inactive form of GSK3β and increases active GSK3β. At the same time, RhoB inhibition induces degradation of hypoxia-inducible factor 1α (HIF-1α) in the proteasome (Skuli et al., 2006). These experimental data suggest that GSK3β controls RhoB-dependent HIF-1α stabilization under hypoxic conditions (Figure 3). The transcription factor, HIF-1, is an essential regulator of oxygen homeostasis by controlling a battery of target genes involved in angiogenesis, glycolysis, proliferation, and pH regulation (Semenza, 2009). According to this mechanism, it was shown that inhibition of RhoB in GBM xenografts leads to a decrease in vessel density (Ader et al., 2003). Considering the role of RhoB in the stimulation of angiogenesis, as well as the presumed connection between RhoB expression and GSK3β activity, it is reasonable to speculate that GSK3β activity in hypoxic conditions can regulate angiogenesis in GBM. However, the precise effect of GSK3β inhibition on angiogenesis has yet to be identified.

4.5 Cell stemness
GSK3β activity appears to regulate glioma stem cell populations. GSK3β protein, as well as stem cell markers Nestin and Notch2, are highly expressed in CD133+ populations, which was identified as a surface marker of cancer stem cells in brain tumors (Singh et al., 2004). Down-regulating GSK3β specifically decreased the subpopulation of cancer cells, with a 50–60% depletion of CD133+ cells that possessed a cancer stem cell-like signature. This depletion was attributed to the differentiation of the cell subtype. Additionally, reduction of Nestin in GBM stem cell cultures treated with GSK3β inhibitor at the molecular level indicated loss of cell stemness (Aguilar-Morante, 2010). Indeed, inhibition of GSK3β reduces the GBM stem cell pool and induces phenotypic switch towards differentiation in GBM cell cultures. This increases the expression of differentiation markers such as neuronal marker β-tubulin III, oligodendrocyte-specific marker CNPase, and astrocytic marker GFAP (Korur et al., 2009). One explanation for this mechanism is as follows. shRNA-mediated depletion of Bmi1, a polycomb group protein that is required for neural stem cell self-renewal, reduced expression of GSK3β in glioma. This decreased expression of Sox2 and Nestin in glioma cells
and induced cell differentiation, suggesting a putative functional link between GSK3β and Bmi1 in glioma cell stemness (Figure 3).

Neurosphere formation is a representative feature of glioma stem cells. GSK3β inhibition significantly reduced the number and volume of neurospheres in glioma cells (Korur et al., 2009). Primary neurosphere cultures treated with TZD-8 failed to give rise to secondary neurospheres, indicating that self-renewing stem cells are lost under TZD-8 treatment (Aguilar-Morante et al., 2010). TZD-8 inhibits the proliferation and expansion of these neurospheres and hampered their capacity for self-renewal, suggesting that TZD-8 could reduce the tumor-initiating cells (Aguilar-Morante et al., 2010). Furthermore, reduction in the levels of Nestin protein in GBM stem cell cultures treated with TZD-8 indicated a loss of cell stemness induced by GSK3β inhibition (Aguilar-Morante et al., 2010). Taken together, GSK3β activation is identified as a key element in maintaining stem cell-like characteristics in a subset of glioma cells, providing these cells with a higher self-renewal capacity.

In contrast, there is a recent contradictory report showing that forced expression of GSK3β induces cellular differentiation of malignant glioma cells to normal astrocytes. Conversely, GSK3β suppression inhibits differentiation and is accompanied by the interruption of cyclin D1 proteolysis, which is necessary for the astrocytic differentiation of malignant glioma cells (Li et al., 2010). The exact mechanisms underlying the effect of GSK3β on glioma cell differentiation require more detailed study.

### 4.6 Metabolism

Increased glycolysis is characteristic of malignancy. Glioma cell growth is closely associated with glucose metabolism. Down-regulation of GSK3β activity results in changes of intracellular glucose metabolism (Kotliarova et al., 2008). The activity of mitochondrial hexokinase, an enzyme that localizes at the outer mitochondrial membrane and metabolizes glucose in rapidly-growing glioma cells, is about 3 times higher than that in slow-growing cells. Consistently, the intracellular glucose concentration is undetectable in rapidly-growing glioma cells, suggesting that glucose catabolism is activated in these cells (Nagamatsu et al., 1996). The dissociation of hexokinase from the outer mitochondrial membrane by GSK3β inhibition is partially responsible for the reduction in intracellular glucose concentration (Kotliarova et al., 2008). However, the reduction of intracellular glucose after GSK3β inhibition is mainly due to decreased GSK3β-dependent glycogen synthase phosphorylation. This leads to glycogen synthase activation and consequently to the increased intracellular glycogen (Figure 1). Further studies are necessary to examine whether the forced glucose consumption and subsequent accumulation of glycogen by GSK3β inhibition affects the glioma phenotype. However, it may be possible that GBM cells cannot use glucose effectively for cell proliferation and survival due to the lack of glucose under the condition induced by GSK3β inhibition.

### 4.7 Chemosensitivity

One of the potential molecules involved in the chemosensitization associated with GSK3β inhibition in GBM cells is O6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme and major determinant of temozolomide cytotoxicity. Recently, clinical research has revealed that the methylation status of the MGMT promoter is associated with the prognostic outcome of GBM patients treated with temozolomide (Hegi et al., 2005). Reduction of MGMT expression induced by p53 in GBM cells renders them sensitive to
temozolomide (Natsume et al., 2005). It is reasonable to speculate that increased expression of p53 by GSK3β inhibition enhances temozolomide chemosensitivity through the reduction of MGMT (Miyashita et al., 2009a).

4.8 Neuroprotection
Radiation is a standard post-operative therapy for patients with GBM. Intellectual impairment, reduction in performance IQ, memory loss, and dementia have been reported after exposure of the brain to radiation. However, the exact mechanisms of radiation-induced brain injury remain unknown and prevention of cranial radiation-induced morbidity remains challenging. Recent studies indicate that inhibition of GSK3β protects hippocampal neurons from radiation-induced apoptosis and attenuation of neurocognitive dysfunction resulting from cranial radiation (Thotala et al., 2008; Yang et al., 2011). Inhibition of GSK3β accelerated double strand-break repair efficiency in irradiated mouse hippocampal neurons, whereas, none of these effects were observed in GBM cells, suggesting potential clinical application of neuroprotection with GSK3β inhibitors during cranial radiation.

5. GSK3β as a therapeutic target
In the field of medicinal chemistry, GSK3β has recently emerged as one of the most attractive therapeutic targets for the development of selective inhibitors as promising new drugs for diabetes. Several potent GSK3 inhibitors have been developed by pharmaceutical companies in preclinical models for diabetes treatment. Apart from this, inhibitors of GSK3β have enormous potential as therapeutics for a number of serious pathologies, including Alzheimer's disease, bipolar disorders, chronic inflammatory processes, and cancer. GSK3β inhibitors are being actively developed as drugs for the treatment of these various disorders. The therapeutic effect of GSK3β inhibition has been confirmed to inhibit inflammation in several studies (Jope et al., 2007). Concerns for the therapeutic use of GSK3β inhibitors remain because they may activate oncogenic (e.g., Wnt) signaling, thus promoting cell proliferation. Certainly, however, this concern has not deterred preclinical studies of GSK3β inhibitors in the treatment of many types of cancers, as discussed above, or Phase II clinical trials for the treatment of neurological diseases (Chico et al., 2009).

5.1 GSK3β-targeted therapy for cancers in clinic
The need for accurate determination of GSK3β status is illustrated by the excellent results of therapies targeting GSK3β in clinic. These strategies have been shown to benefit only tumors overexpressing the GSK3β protein. In other words, tumors that do not express GSK3β do not benefit from GSK3β-targeted therapies. To date, there are no clinical trial reports describing the use of specific GSK3β inhibitors for cancers, although many basic research results identified GSK3β as a tumor promoter and suitable candidate for targeted treatment. Chemical drugs already prescribed for other diseases were shown to have an inhibitory effect on GSK3β.

5.1.1 Lithium chloride (LiCl)
LiCl is highly effective in the treatment of bipolar disorder (Bowden et al., 2005). Results from an epidemiological study indicated that cancer prevalence in psychiatric patients on
long-term LiCl medication was lower than in the general population (Cohen et al., 1998), suggesting that administration of LiCl induces cell differentiation and inhibits proliferation and, therefore, might effectively inhibit tumor formation and progression. GSK3β has emerged as a key target that is central to the effects of LiCl treatment. There are 2 mechanisms by which LiCl inhibits GSK3β (Figure 4). Firstly, LiCl promotes Ser9 phosphorylation in GSK3β, resulting in a less active form of GSK3β (Jope, 2003). Secondly, LiCl blocks the function of activated GSK3β by competing with magnesium ions (Mg2+) (Jope, 2003). Mg2+ is required for activated GSK3β to phosphorylate its substrates, which are involved in the propagation of chemical signals required for cell survival, proliferation, and differentiation. As a consequence of these inhibitory effects, GSK3β can no longer regulate many important biological processes. It is notable that LiCl protects hippocampal neurons from radiation-induced apoptosis by promoting the DNA repair pathway, probably a result of the effect of GSK3β inhibition (Yang et al., 2009; Yang et al., 2011).

Fig. 4. Therapeutic mechanism of GSK3β inhibition in clinic. Lithium chloride (LiCl) inhibits phosphorylation of GSK3β substrates by competing with Mg2+, which is required for GSK3β-dependent substrate phosphorylation. Simultaneously, LiCl and olanzapine increase the phosphorylation of the Ser9 residue. Valproic acid (VPA) inhibits GSK3β by direct interaction. LiCl and VPA show additive effects on GSK3β inhibition. Like a pharmacological GSK3β inhibitor AR-A014418, olanzapine can be successfully docked within the adenosine triphosphate (ATP)-binding pocket of GSK3β. Cimetidine, gemifloxacin, and hydroxychloroquine are potent GSK3β inhibitors with at least 2 distinct binding modes accessible to ligands within the GSK3β binding pocket. Circled P, phosphorylation.
5.1.2 Valproic acid (VPA)
VPA is now an established drug for the treatment of epileptic seizures (absence, tonic-clonic, and complex partial seizures) and mania in bipolar disorder (Kostrouchova & Kostrouch, 2007). VPA affects multiple cell regulatory pathways. The best substantiated molecular mechanism of VPA action is its inhibitory effect on histone deacetylase (HDAC) activity, a key regulator in the dynamics of chromatin structure and function. It was proposed that VPA activates Wnt-dependent gene expression through inhibition of HDAC, which generated interest for its use in cancer therapy (Phiel et al., 2001). Apart from this, VPA, like LiCl, exerts significant inhibitory effects on the activity of GSK3β both directly in vitro and also on endogenous GSK3β in intact human neuroblastoma SY5Y cells (Chen et al., 1999) (Figure 4). The dual inhibition of HDAC and GSK3β by VPA may provide a basis for its anticancer activity. As expected, clinical trials using VPA for cancer showed some effects (Chateauvieux et al., 2010).

Significant inhibitory effects for GSK3β are clearly observed at VPA concentrations approximating those attained clinically during treatment. Furthermore, addition of LiCl at therapeutic concentrations results in additive inhibitory effects to that of VPA. These additive effects of LiCl and VPA on GSK3β suggest that the 2 drugs may exert their effects at different sites, but additional studies will be necessary to establish this definitively (Chen et al., 1999).

5.1.3 Olanzapine
Olanzapine is broadly used for patients with schizophrenia. Recently, olanzapine was identified as a GSK3β inhibitor by a docking simulation experiment, which validates the interaction between the drug and its target molecule. Olanzapine, as well as the well-known GSK3β inhibitor AR-A014418, were found to readily fit within the adenosine triphosphate (ATP)-binding pocket of GSK3β and to inhibit its activity (Mohammad et al., 2008). Additionally, the administration of olanzapine, similar to LiCl, increased phospho-Ser9-GSK3β in brain (Li et al., 2007) (Figure 4). The inhibition of GSK3β by olanzapine was accompanied by a decrease in the blood glucose level and accumulation of glycogen in the liver in a dose-dependent manner. This is consistent with the effect of GSK3β inhibition (Mohammad et al., 2008). Olanzapine-induced low blood glucose level is also consistent with clinical reports (Budman & Gayer, 2001). This result contrasts that of a previous report, where olanzapine induced hyperglycemia as a major side effect (Fertig et al., 1998). The molecular mechanism of these contradictory findings is currently unknown.

On the basis of the reported reduced cancer risk in schizophrenic patients (Catts et al., 2008), a recent study demonstrated the anti-tumor effect of a number of antipsychotic drugs, including olanzapine, except for risperidone (Wiklund et al., 2010). The effect of these drugs against cancer cells was associated with changes in the expression of genes acting on cholesterol homeostasis and the biophysical properties of the cellular membrane. Inhibition of GSK3β activity might be an alternate mechanism by which olanzapine acts against cancer.

5.1.4 Cimetidine
Cimetidine was the first registered histamine H2 receptor antagonist, and its frequent prescription was based on its clinical effectiveness in healing gastrointestinal ulcers by inhibiting gastric acid secretion (Somogyi & Gugler, 1983). Cimetidine has been demonstrated to possess anti-tumor activity against colon, gastric, and kidney cancers and...
melanomas. This activity involves a number of different mechanisms of action, including blocking the cell growth-promoting activity of histamine (Lefranc et al., 2006). With respect to GBM, cimetidine combined with temozolomide was superior to temozolomide alone in extending the survival of nude mice with human GBM cells orthotopically xenografted into their brain (Lefranc et al., 2005).

In silico screening is a powerful method to analyze large chemical databases in order to identify possible new drug candidates. Recently, in silico screening revealed that cimetidine, as well as hydroxychloroquine (an antimalarial and anti-lupus erythematosus agent) and gemifloxacin (a new quinolone antibiotic), have an inhibitory effect on GSK3β (Taha et al., 2008) (Figure 4).

5.1.5 Enzastaurin
Enzastaurin, a selective serine/threonine protein kinase inhibitor already under clinical evaluation to treat recurrent GBM, potently inhibits GSK3β in addition to its primary target, protein kinase C (PKC)β. In phase I/II clinical trials, enzastaurin showed potentially encouraging efficacy in a subset of patients with recurrent malignant glioma, but does not appear to have enough single-agent activity to be useful as a monotherapy (Kreisl et al., 2009; Kreisl et al., 2010). In this trial, phosphorylation of GSK3β in peripheral blood mononuclear cells was identified as a potential biomarker of drug activity.

6. Perspective
GSK3β has been recognized as a key component in a wide range of cellular functions and is involved in the vast number of signaling pathways that converge on this enzyme, and subsequently, an even greater number of biological targets. GSK3β is undoubtedly a promising target not only for diabetes, bipolar disorder, Alzheimer’s, and several other neurological disorders, but also for human cancers, including GBM, on the basis of the accumulated evidence. Inhibitors of GSK3β have enormous therapeutic potential. Of great importance is understanding the precise molecular mechanisms of GSK3β-mediated signal transduction, including signaling elements involved in proliferation, apoptosis, invasion, differentiation, chemosensitivity, radiosensitivity, and neuroprotection, as well as the precise functions of GSK3β proteins in cellular responses induced in human normal and malignant cell types. The emerging understanding of GSK3β function would also give rise to new insights in tumor biology. In the future, it is hoped that increasing knowledge of GSK3β will be translated into molecularly targeted therapies against intractable cancers represented by glioblastoma multiforme.

7. References


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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.

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