

Original Article

Glycogen synthase kinase-3 β regulates astrocytic differentiation of U87-MG human glioblastoma cells

Yan LI^{1,2, #}, Hui-min LU^{2, #}, Gang LI^{1, *}, Guang-mei YAN^{2, *}

¹Department of Infectious Diseases, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China; ²Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China

Aim: To evaluate the role of glycogen synthase kinase-3 β (GSK-3 β) in the induced differentiation of human glioblastoma cells.

Methods: Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation assay. The protein level of p-GSK-3 β , GSK-3 β , glial fibrillary acidic protein (GFAP) and proliferating cell nuclear antigen (PCNA) were determined using Western blots. The overexpression of mutant GSK-3 β was analyzed by immunocytochemistry.

Results: The biotoxin cholera toxin is capable of inducing differentiation of U87-MG human glioblastoma cells, which is characterized by morphological changes to astrocytic phenotype, increase in differentiation marker protein GFAP and decrease in proliferation. GSK-3 β activation is induced during this differentiation. Small interfering RNA against GSK-3 β suppresses the induced-differentiation in U87-MG cells. Conversely, overexpression of a constitutively active form of human GSK-3 β (pcDNA3-GSK-3 β -S9A) mutant leads to differentiation of U87-MG cells.

Conclusion: Our findings suggest that GSK-3 β plays an important role in astrocytic differentiation of human glioblastoma cells and may be a novel therapeutic target in the malignant tumor.

Keywords: glycogen synthase kinase-3 β ; differentiation; glial fibrillary acidic protein; glioblastoma; astrocytes

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Introduction

Glioblastoma are the most common primary tumor arising within the central nervous system (CNS) and the prognosis is poor, in particular with high grade tumors such as glioblastoma multiforme^[1]. Despite modern treatments with neurosurgical resection, radiotherapy and chemotherapy, the median life expectancy for patients with malignant gliomas is approximately 12 months with less than 5% of patients alive at 5 years after diagnosis^[2,3].

Differentiation-inducing therapy, which modifies cancer cell differentiation and is the most successful treatment for acute promyelocytic leukemia^[4], has been proposed to be a novel potential approach to treat malignant tumors^[5]. The biotoxin cholera toxin is reported to be capable of inducing differentiation of rat C6 malignant glioma cells^[6]. However, little has been known about its possible role in human glioma cell lines. And importantly, the key factors governing glioblastoma dif-

ferentiation, and therefore tumor malignancy, are still largely unknown.

The cytoplasmic serine/threonine protein kinase glycogen synthase kinase-3 (GSK-3) was first described as a component of the metabolic pathway for glycogen synthase regulation^[7]. There are two homologous mammalian isoforms encoded by different genes, GSK-3 α and GSK-3 β ^[8]. It has been implicated in fundamental processes including cell fate determination, metabolism, transcriptional control^[9]. More recent studies indicate a role for GSK-3 β in the control of neoplastic transformation and tumor development, suggesting that GSK-3 β is a potential therapeutic target in human cancers^[7,10-12]. However, the vast majority of research has been focused on the aspects of proliferation and apoptosis, and little is known about its possible role involved in the process of cancer cell differentiation.

Here we report that the traditional biotoxin cholera toxin is capable of inducing differentiation in U87-MG human glioblastoma cell line and further determine the underlying mechanism. Utilizing molecular biological approaches, we showed that GSK-3 β had a hitherto unrecognized pathologic role in cellular differentiation in human malignant glioblastoma. These findings may open new avenues for identifying novel therapeutic targets in the differentiation therapy of cancers.

These two authors contributed equally to the manuscript.

* To whom correspondence should be addressed.

E-mail ligang131@hotmail.com (Gang LI); ygm@mail.sysu.edu.cn (Guangmei YAN)

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Materials and methods

Cell culture and drug treatment

U87-MG cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen) in a humidified atmosphere of 5% CO₂ at 37 °C. Cell differentiation was induced with cholera toxin (Sigma, St Louis, MO) in DMEM containing 10% FBS. Control was treated with an equivalent volume of DMEM.

Morphological evaluation

The morphologies of living cells were studied using an Olympus (Melville, NY) IX71 inverted microscope along with an Olympus DP Controller software.

Proliferation assay

For cell proliferation assay, a 5-bromo-2-deoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. Cells seeded in 96-well plates at 2×10³ cells/well with triplicate wells for each condition were labeled with BrdU for 4 h, then anti-BrdU-POD Fab fragments and substrate were added in sequence. The OD was determined at 405 nm using an EXL800 microimmunoanalyzer (Bio-Tek Instruments, Burlington, VT). The results were obtained from three independent experiments.

Western blot analysis

After lysis of cells and measurement of protein concentration, the cells were dissolved in SDS sample buffer [62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mmol/L DTT, and 0.1% bromophenol blue]. Equal amount of proteins were analyzed by SDS-PAGE on 12% poly-acrylamide gels. Proteins were electroblotted on a nitrocellulose membrane. Membranes were incubated in 5% nonfat dry milk in TBST (Tris-buffered saline, 0.05% Tween-20) and then overnight at 4 °C with antibodies against GFAP, p-GSK-3β^{Ser9}, GSK-3β (1:1000, Cell Signaling Technology, Beverly, MA), p-GSK-3β^{Y216} (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), PCNA (1:10000, Cell Signaling Technology) and GAPDH (1:2000, Cell Signaling Technology), respectively. After incubation with horseradish peroxidase-labelled secondary antibody (1:1000, Cell Signaling Technology), visualization was achieved with enhanced chemiluminescence (Pierce, Rockford, USA) using a GeneGnome chemiluminescence imaging and analysis system (Syngene Bio Imaging, Cambridge, UK).

RNA silencing of GSK-3β

Human GSK-3β siRNA and negative siRNA (control) were purchased from Invitrogen. U87-MG cells with 30%–50% confluence were transfected via Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. GSK-3β protein expression was assessed by immunoblot analysis 48 h after transfection. At 36 h posttransfection, cells were incubated with cholera toxin for additional 48 h for further analysis.

Transient transfection

pcDNA3-GSK-3β S9A mutant is a kind gift of Prof Z MAO, Emory University. Asynchronously growing cells were plated in 6-well dishes 24 h before transfection. Transient transfections were performed with 4 μg plasmid DNA per well using 12 μL FuGENE HD transfection reagent (Roche Diagnostics Corp). Cells were replaced with fresh medium 12 h after the transfection. To monitor transfection efficiency, the constructs were co-transfected with pEGFP vector and fluorescent cells were counted 48 h posttransfection. Such analyses indicated that 60%–70% of the cell population expressed the foreign protein (data not shown).

Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS and then penetrated with PBS containing 0.2% Triton X-100 for 30 min and blocked with 5% bovine serum albumin for 1 h. The blocking buffer was removed and cells were incubated with antibody against GFAP (1:200, Cell Signaling Technology) overnight at 4 °C. Cells were then washed and incubated with cy3 conjugated goat anti-mouse IgG (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. DNA was stained with Hoechst 33258 (5 μg/mL, Sigma) for 5 min at room temperature. Images were observed by a fluorescence microscope (Olympus).

Statistical analysis

Data are presented as mean±standard deviation (SD) of three separate experiments. Statistical significance was determined by Student's *t*-test. A result with a *P*-value of less than 0.05 is considered statistically significant.

Results

Cholera toxin induces differentiation of U87-MG glioblastoma cells

Microscopic observation of U87-MG glioblastoma cells treated with cholera toxin in our study revealed that the shape of cholera toxin-treated cells was similar to that of mature astrocytes, being smaller cell bodies and much longer, fine, tapering processes. In contrast, most cells in the control were flattened and spindle-shaped and had no thin process (Figure 1A). We further examined whether morphologic changes were accompanied by expression of glial fibrillary acidic protein (GFAP), a well-established marker of mature astrocytes^[13]. Indeed, Western-blotting analysis confirmed significant up-regulation of GFAP protein expression in cholera toxin treated cells compared to controls in a dose-dependent manner (Figure 1B). At the same time, the level of proliferating cell nuclear antigen (PCNA), a well-accepted marker of proliferation that facilitates fast processing of DNA^[14] was markedly reduced (Figure 1B). Furthermore, BrdU incorporation analysis showed that cholera toxin led to a time-dependent decrease in proliferation of the U87-MG cells (Figure 1C). This indicates that cholera toxin induces differentiation of glioblastoma cells into the maturation process of astrocytic lineage and provides a reliable model

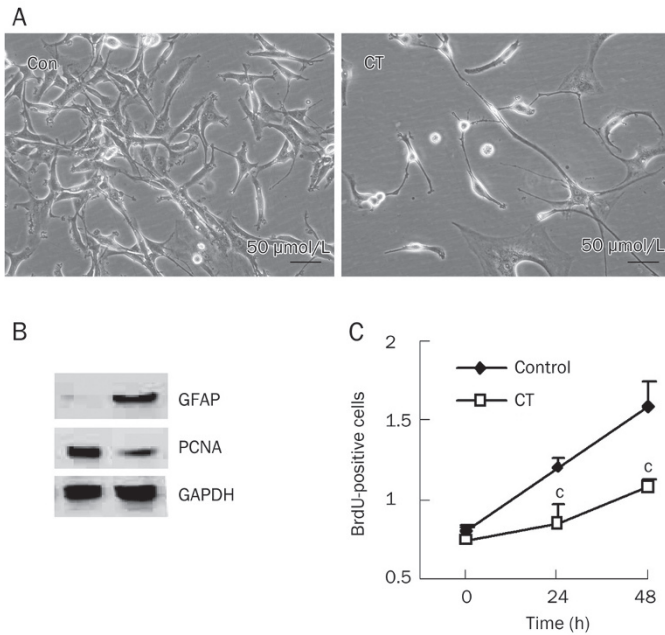


Figure 1. Cholera toxin induces differentiation of U87-MG glioblastoma cells. (A) Morphological transformation induced by 10 ng/mL cholera toxin (CT) for 48 h (original magnification, $\times 320$). (B) Effect of CT on GFAP and PCNA expression. Cells were treated with 10 ng/mL CT for 48 h. (C) Proliferating cells determined by measuring the amount of BrdU incorporation in cells. Cells were treated with 10 ng/mL CT for the time indicated. Results are means \pm SD. $n=3$. ^b $P<0.05$, ^c $P<0.01$ compared with the controls.

of differentiation.

Activation of GSK-3 β in the induced-differentiation of glioblastoma cells

We also found activation of GSK-3 β during the course of cholera toxin-induced differentiation. p-GSK-3 β^{Y216} which represents the active kinase form of GSK-3 β was detected by western blot analysis to assess the activation state of the protein. Figure 2A revealed significant up-regulated GSK-3 β^{Y216} protein level in U87-MG cells after incubation with cholera toxin for 9 h, which was maintained at a high level. GSK-3 β phosphorylation at the Ser-9 residue, which represents the inactive form of GSK-3 β kinase, was also determined. As shown in Figure 2B, 12 h after cholera toxin stimulation in U87-MG cells, there is a decrease in the intensity of the phosphorylated form of GSK-3 β , while total GSK-3 β remains stable throughout. Together, these data suggest the activation of GSK-3 β during the differentiation process of glioblastoma cells.

GSK-3 β gene knockdown abrogates differentiation ability of U87-MG glioblastoma cells

To further confirm the role of GSK-3 β in U87-MG glioblastoma cell differentiation, we examined the effects of GSK-3 β siRNA on cholera toxin induced-differentiation of U87-MG cell. To selectively down-regulate expression of GSK-3 β protein, we first evaluated the effect of GSK-3 β gene silencing mediated by

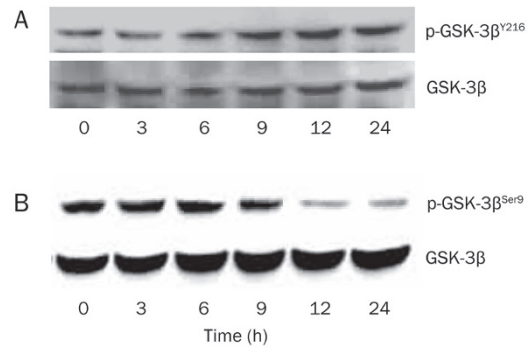


Figure 2. GSK-3 β is activated by cholera toxin in U87-MG cells. Immunoblot of p-GSK-3 β^{Y216} (A) and p-GSK-3 β^{Ser9} (B) levels in U87-MG cells treated with 10 ng/mL cholera toxin (CT) for the time indicated.

siRNA. As shown in Figure 3A, compared with those transfected with negative siRNA, cells transfected with GSK-3 β siRNA resulted in the most dramatically knockdown of the protein levels. In addition, morphological transformation and elevation of GFAP during U87-MG cell differentiation were

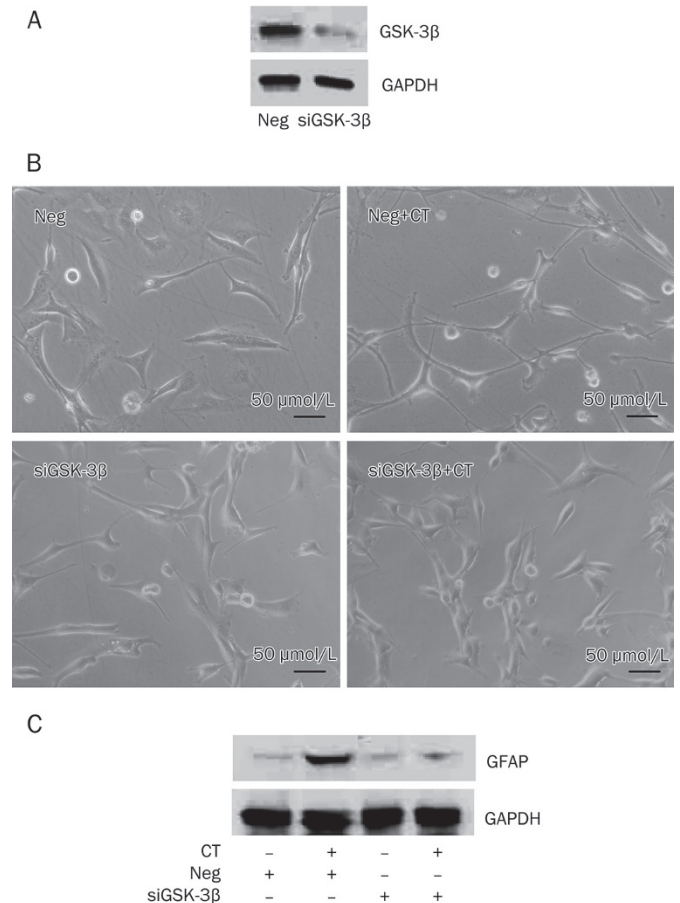


Figure 3. Silencing GSK-3 β blocks differentiation of U87-MG cells. (A) Immunoblot of the GSK-3 β protein levels after transfection with negative control (Neg) or 10 nmol/L siGSK-3 β for 36 h. (B-C) Morphology (original magnification, $\times 320$) (B) and immunoblot of the GFAP levels (C) in GSK-3 β knockdown cells subsequently stimulated with CT for 48 h.

blocked by GSK-3 β specific siRNA (Figure 3B and 3C). These results indicate the requisite role of GSK-3 β in the differentiation of glioblastoma cells and suggest that GSK-3 β positively regulates the differentiation.

Overexpression of active GSK-3 β leads to differentiation of U87-MG glioblastoma cells

We next examined whether forced expression of GSK-3 β is sufficient to induce differentiation in U87-MG cells. Cells were transfected with a constitutively active pcDNA3-GSK-3 β S9A mutant (GSK-3 β S9A) in which the N-terminal serine-9 residue was substituted with an alanine residue and therefore cannot undergo inhibitory phosphorylation. Western-blotting analysis confirmed marked enhancement of GSK-3 β protein level in GSK-3 β S9A gene transferred cells compared to those in the empty vector controls (Figure 4A). As seen in Figure 4B, transfection of GSK-3 β S9A initiates morphological transformation from polygon appearance in the empty vector (pcDNA3) group to smaller cell bodies with much longer, fine, tapering processes, similar to that of mature astrocytes. Immunohistochemical analysis further confirmed significant up-regulation of GFAP protein expression in GSK-3 β S9A gene transferred cells compared to those in the pcDNA3 controls (Figure 4C). That is, U87-MG cells transfected with GSK-3 β S9A exhibited hallmarks of differentiation. These data indicate that overexpression of active GSK-3 β is necessary and sufficient to promote differentiation in U87-MG human glioblastoma cells.

Discussion

The present study was undertaken to clarify the exact role of cholera toxin and GSK-3 β in cancer differentiation. Our results demonstrate that the traditional biotoxin cholera toxin is able to induce differentiation of human glioblastoma cells. GSK-3 β suppression via siRNA-triggered gene silencing inhibits cholera toxin-induced differentiation. Conversely, overexpression of GSK-3 β enables glioblastoma cells to acquire differentiation ability. These data lead to the identification of GSK-3 β as a positive regulator of astrocytic differentiation in human malignant glioblastoma cells.

Deviation from the tissue/lineage-specific differentiation program is one of the fundamental aspects of tumorigenesis^[15]. The aberrantly differentiated cells show abnormal growth characteristics and distinct invasive and metastatic properties^[16]. Upon appropriate stimulus, malignant cells result in reprogramming, loss in proliferative capacity as well as induction of differentiation^[5, 16]. Cholera toxin and elevated cAMP appeared to differentiate rat C6 glioma cells to express astrocytic phenotype^[6, 17]. Here we showed that exposure of U87-MG malignant glioblastoma cells to cholera toxin could also result in their morphological changes to astrocytic phenotype, increase in astrocytic differentiation marker protein GFAP and decrease in proliferation. This might serve as a faithful model to study molecular mechanisms underlying differentiation defects in human cancer cell lines.

GSK-3 β is a multifunctional serine/threonine kinase that

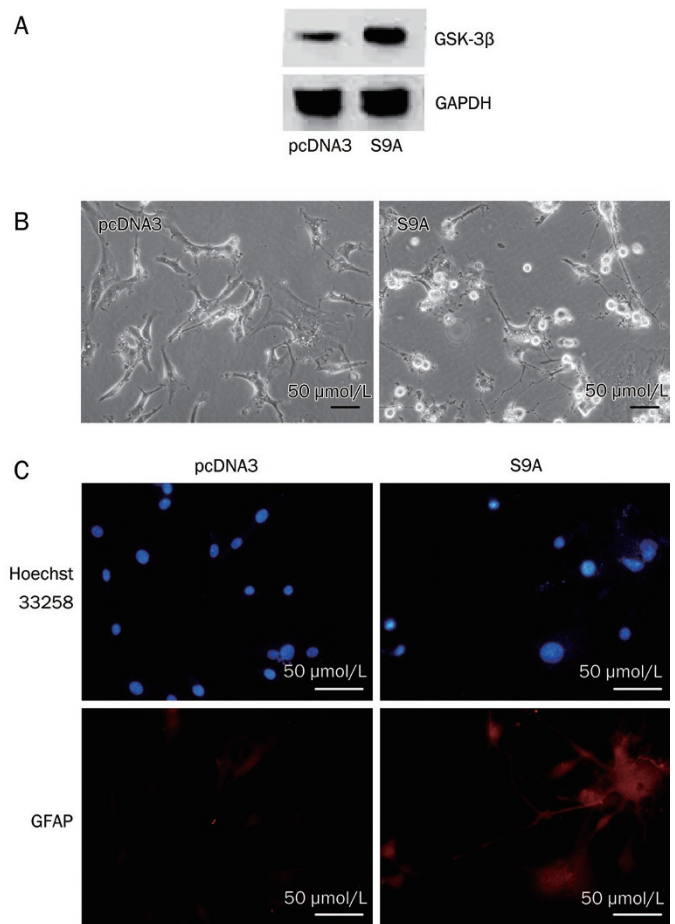


Figure 4. Overexpression of active GSK-3 β promotes differentiation of U87-MG glioblastoma cells. Cells transfected with GSK-3 β S9A mutants (S9A) or empty vector (pcDNA3) for 48 h followed by Western-blotting for GSK-3 β (A), morphological evaluation (original magnification, $\times 320$) (B) or immunohistochemistry for GFAP expression (C).

regulates various cellular pathways, depending on its substrates for phosphorylation^[9, 18]. Since oncogenic transcription factors (eg, c-Jun, c-Myc) and proto-oncoproteins (ie, β -catenin, Gli proteins) are putative GSK-3 β substrates for phosphorylation-dependent inactivation^[19], it is hypothesized that GSK-3 β interferes with cellular neoplastic transformation and tumor development, as exemplified by its activity in Wnt/ β -catenin signaling^[20]. However, only a few studies have addressed its role(s) in human cancer, and these studies have reported differing effects of GSK-3 β on cancer cells^[21, 22]. Using GSK-3 β deficient mouse embryonic fibroblasts, it was shown that GSK-3 β plays a crucial role in cell survival mediated by the nuclear factor- κ B (NF- κ B) pathway^[23, 24]. Thus, these observations bring forward apparently opposing notions regarding the functions of GSK-3 β in neoplastic cells on the one hand, removing a neoplastic trigger by phosphorylation-dependent degradation of β -catenin oncoprotein, and on the other, contributing to a cell proliferation and survival pathways by regulating NF- κ B.

In all, our study demonstrates a novel pathological role of

GSK-3 β in human malignant glioblastoma, both by genetically modulating the activity and expression of this kinase and by substantiating its activity in established glioblastoma cells. We demonstrate that GSK-3 β initiates U87-MG glioblastoma cells susceptible to differentiation and that loss of GSK-3 β activity was necessary for the blockage of astrocytic differentiation of malignant glioblastoma cells. Our data provide strong evidence that GSK-3 β contributes to cellular differentiation. GSK-3 β may be a novel therapeutic target and a regulator of cellular differentiation in malignant tumors.

Furthermore, investigating broader mechanisms underlying the potential pro-differentiation role of GSK-3 β may provide insights into molecular pathways leading to glioblastoma tumorigenesis, and support development of novel strategies for treatment targeting this kinase and the molecular epidemiology of glioblastoma.

One thing we would mention here is that the morphology of GSK-3 β S9A mutant transfected cells was not as obvious as that of cholera toxin treated cells. This may be attributed to the multiple-targets of cholera toxin besides GSK-3 β . We have previously reported that PKA/CREB pathway mediated the differentiation-inducing activity of cholera toxin in rat C6 and primary human malignant glioma cells^[6]. Recent reports provide evidence that cholera toxin inhibits dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function^[25]. It was also documented that ganglioside GM1 reaction with B subunit of cholera toxin induces neuron-like differentiation of PC12 and neuroblastoma cells^[26, 27]. Therefore, it seems reasonable to postulate that GSK-3 β might not be the unique molecular targets of cholera toxin that might contribute to the differentiation of human glioblastoma cells. And thus it was not surprising that GSK-3 β overexpression-initiated morphological alteration was not as obvious as that induced by cholera toxin. However, further studies are warranted to conclusively clarify this important issue.

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Author contribution

Yan LI and Guang-mei YAN designed the research; Yan LI and Hui-min LU performed the research; Yan LI, Hui-min LU, Gang LI and Guang-mei YAN analyzed the data; Yan LI wrote the paper.

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