

## Research Article

# Hypoxia Promotes Glioma Stem Cell Proliferation by Enhancing the 14-3-3 $\beta$ Expression via the PI<sub>3</sub>K Pathway

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Glioma is a serious fatal type of cancer with the shorter median survival period and poor quality of living. The overall 5-year survival rate remains low due to high recurrence rates. Glioma stem cells (GSCs) play the important roles in the development of gliomas. Examination of the numerous biomarkers or cancer-associated genes involved in the development or prevention of glioma may therefore serve the discovery of novel strategies to treat patients with glioma. Hypoxia induced by using CoCl<sub>2</sub> application and 14-3-3 $\beta$  protein knockdown by specific small interfering RNA transfection were performed in GSCs both in vitro and in vivo to observe their role in glioma progression and metastasis occurrence by using western blot analysis and MTT assay. The results demonstrated that CoCl<sub>2</sub> application enhanced the 14-3-3 $\beta$  protein expression and mRNA levels via the PI<sub>3</sub>K pathway in GSCs. Furthermore, hypoxia promoted GSC cell proliferation and activated the expression of proliferating cell nuclear antigen, which was inhibited following 14-3-3 $\beta$  knockdown. In addition, tumor growth in mice was enhanced by CoCl<sub>2</sub> application but reversed following 14-3-3 $\beta$  knockdown, which also enhanced GSC cell apoptosis. In conclusion, the present study demonstrated that hypoxia promoted glioma growth both in vitro and in vivo by increasing the 14-3-3 $\beta$  expression via the PI<sub>3</sub>K signaling pathway. 14-3-3 $\beta$  and HIF-1 $\alpha$  may therefore be considered as the potential therapeutic target to treat patients with glioma.

## 1. Introduction

Glioma is a serious fatal type of tumor with a shorter median survival period and poor quality of living, and grade IV glioblastoma (GB) is the most malignant type of glioma [1–3]. Abnormal gene expression or mutation including tumor-suppressor inactivation or oncogene activation could be the certain genetic reasons for the development of gliomas. In addition, more recent evidence has been proved that glioma stem cells (GSCs), exhibiting stem cell-like self-renewal capabilities, involved in the process of initiation of tumors and played the important roles in the development of gliomas [4, 5]. Although progress has been obtained in the last 20 years in the development of numerous clinical treatments for glioma patients, the overall 5-year survival rate remains

low due to high recurrence rates [6, 7]. Thus, the novel therapeutic strategies for glioma patients are urgent and to also understand the underlying mechanism of recurrence to tailor specific treatment. Previous studies reported that numerous biomarkers or cancer-associated genes, which could be potential targets, serve crucial roles in the development or prevention of glioma, and that these novel targets will be developed as antitumor agents [8–10].

It has been demonstrated that hypoxia inducible factor (HIF), including HIF-1 $\alpha$  and HIF-1 $\beta$  subunits, is a heterodimeric transcription factor which may involve in the tumor angiogenesis and progression [11, 12]. HIF-1 $\alpha$  regulated numerous physiological functions and even tumorigenesis in the hypoxic tumor microenvironment (TME) [13, 14] and modulated the tumor development and survival by

involving in the tumor cell apoptosis, cell proliferation, differentiation, or migration, which serves a crucial role in the progression of breast, esophageal, lung, prostate, colon, and liver cancer [15–17]. Some studies reported that HIF-1 $\alpha$  inhibition could induce the changes in radio- or chemosensitivity and angiogenesis [18, 19]. Our previous studies also evidenced that the malignant progression of glioma was related to the HIF-1 $\alpha$  and vascular endothelial growth factor (VEGF) [20], and silencing of HIF-1 $\alpha$  by siRNA application could enhance the radiation sensitivity of human glioma growth in vitro and in vivo [21]. Generally, certain molecular pathways, including phosphoinositide dependent kinase 1, mTOR-RICTOR kinases and PI<sub>3</sub>K-AKT, could be affected or controlled by HIF-1 $\alpha$ , which is itself an attractive drug target for clinical tumor treatment [22, 23]. In addition, HIF-1 $\alpha$  was associated with the tumor angiogenesis and progression of glioma by regulating the apoptosis-related protein expression through the PTEN/Akt pathway [24, 25]. HIF-1 $\alpha$  combined with JAK1/2-STAT3 (Janus kinase 1/2-signal transducer and activator of transcription 3) axis could enhance the self-renewal of glioma stem-like cells [26]. Clinical study also demonstrated that high hypoxia signature was associated with poor prognosis for glioma patients [27]. And amplification of EGFR activated the PI3K pathway in approximately 45% of GBM cases [28], and the p85/PI3K regulatory effect has been obtained in approximately 15% of GBM patients [29]. Furthermore, multitherapy that would include HIF-1 $\alpha$  inhibition combined with chemotherapeutic regimens, radiotherapy, or other cancer gene-targeted reagents may be beneficial in clinical cancer therapy [30, 31].

Proteins 14-3-3 include seven distinct isoforms and constitute a family of acidic polypeptides found in mammals [32, 33]. There is strong evidence that the 14-3-3 overexpression promotes tumor growth and progression in glioma. 14-3-3 $\beta$  affects a wide range of biological processes, including signal transduction, DNA replication, DNA repair, cell cycle control, and vesicular transport, by binding to phosphoserine-containing sequence motifs in different pathways [34–36]. Previous studies demonstrated that 14-3-3 $\beta$ , which is considered as an oncogene and serves a crucial role in tumor angiogenesis and tumor formation, can modulate tumor cell proliferation, motility, cycle, and apoptosis via specific stimulator factors or downstream pathways, including mitogen-activated protein kinase- (MAPK-) dependent signal activation [37, 38].

Although several clinical studies reported that both 14-3-3 $\beta$  overexpression and HIF-1 $\alpha$  are associated with the pathological characteristics and progression of various types of tumor [21, 32, 33], few study has reported the association between HIF-1 $\alpha$  and 14-3-3 $\beta$  modulation in tumor progression and metastasis, notably in glioma. The present study is aimed therefore at clarifying the underlying mechanism of HIF-1 $\alpha$  promoted glioma growth in vivo and in vitro by enhancing the 14-3-3 $\beta$  expression, which activates the downstream PI<sub>3</sub>K pathway. Effective inhibition on HIF-1 $\alpha$  using novel inhibitors combined with 14-3-3 $\beta$  knockdown may be considered as a potential therapeutic strategy to treat patients with glioma.

## 2. Materials and Methods

**2.1. Cell Culture.** For GSC culture, clinical samples from patients were prepared as described in our previous reports [39, 40] with serum free Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Invitrogen) including epidermal growth factor (EGF, 20 ng/mL), basic fibroblast growth factor (bFGF, 20 ng/mL) and B27 (1:50), and placed at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

**2.2. Small Interfering (si) RNA Transfection.** GSCs were cultured and transfected with 100 nM 14-3-3 $\beta$  siRNA and control vector (Qiagen, Inc.) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The fresh medium was changed after 24 h following transfection, and the transfection efficiency was verified by western blotting after 48 h. The 14-3-3 $\beta$  targeting sequences were as follows: forward 5' -ATTGAGGAGGTACAGAGA AC-3' and reverse 5' -TTCATATCTGTTTTGGCCTG-3'.

**2.3. Western Blotting.** The expression of 14-3-3 $\beta$  in GSCs was first evaluated by western blotting after chloride hexahydrate (CoCl<sub>2</sub>) application (10, 50, and 150  $\mu$ M) or 14-3-3 $\beta$  siRNA transfection for 48 h. GSCs were lysed using lysis buffer (100 mg/mL PMSF, 1% NP-40, 0.01 M Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), and 1% (w/v) Triton X-100), and above chemicals were purchased from Sigma-Aldrich. Then, total proteins were obtained following cell lysate centrifugation at 12,000 x g for 30 min at 4°C. Protein concentration was measured by bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). After that, proteins were separated by SDS-PAGE (4% stacking gel and 12% resolving gel) and transferred onto polyvinylidene fluoride membranes. Membranes were blocked by 5% BSA (Sigma-Aldrich) for 1 h at 37°C and incubated with the primary antibodies against 14-3-3 $\beta$  (1:1,000), HIF-1 $\alpha$  (1:1,000), and  $\beta$ -actin (1:1,000; all Santa Cruz Biotechnology, Inc.) overnight at 4°C, respectively. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) was used to detect the signal on the membrane. Data were analyzed via densitometry using ImageJ (Version 1.8.0; National Institutes of Health, Bethesda, MD, USA). In addition, the protein expression of proliferating cell nuclear antigen (PCNA), which is a typical biomarker of cell proliferation [41, 42], was also detected by western blotting according to above protocol (anti-PCNA, 1:1,000; Santa Cruz Biotechnology, Inc.).

**2.4. Cell Proliferation Assay.** GSCs were harvested by trypsinization and cultured in triplicate in 96-well plates (2  $\times$  10<sup>3</sup> cells per well). MTT assay (Roche Diagnostics) was used to evaluate cell proliferation following CoCl<sub>2</sub> treatment (50  $\mu$ M for 24 h) or 14-3-3 $\beta$  siRNA transfection after incubation with 10  $\mu$ l MTT for 2 h. Absorbance was measured with a microplate spectrophotometer at 570 nm.

**2.5. Real-Time PCR Analysis.** Next, the mRNA levels were also detected by using real-time quantitative PCR (qPCR) analysis following the manufacturer's instructions. Total

RNA was isolated by using TRIzol Reagent (Invitrogen). The ABI Prism 7500 real time PCR system (Applied Biosystems) was used to evaluate mRNA changes following the above treatment and siRNA transfection.

**2.6. In Vivo Tumor Xenograft Model.** Mice were aged six to eight weeks old and half for male or female in each group. GSCs (control or 14-3-3 $\beta$  siRNA transfected cells) were injected into the left dorsal flank of nude mice to set up the animal model with subcutaneous tumor xenografts. Mice were then treated with 5 mM CoCl<sub>2</sub> by subcutaneous injections 4 weeks following GSC injection. Mice were anesthetized prior to injection and executed via an approved method of euthanasia after the treatment. The research protocol was approved by the Animal Ethics Committee. Tumor volumes were measured every 7 days according to the following equation:  $V = L \times W^2 \times \pi / 6$ , where  $V$  is the tumor volume,  $L$  is the tumor length, and  $W$  is the tumor width. Kaplan-Meier analysis was used to calculate the animal survival rates in order to evaluate the potential therapeutic effects of CoCl<sub>2</sub> and/or 14-3-3 $\beta$  siRNA transfection [43–45].

**2.7. Cell Apoptosis Analysis.** After CoCl<sub>2</sub> application or 14-3-3 $\beta$  siRNA transfection, GSC cell apoptosis rate was evaluated by annexin V-fluorescein isothiocyanate and propidium iodide staining (BD Biosciences) for 15 min at RT (25°C) in the dark and flow cytometry analysis (BD, FACSuite Research Assay Software). Bax/Bcl-2 assessment is a way of assessing cell apoptosis [46, 47]. Thus, Bax/Bcl-2 protein expression and mRNA levels were also detected by western blotting or qPCR following 14-3-3 $\beta$  siRNA transfection combined with CoCl<sub>2</sub> treatment (anti-Bax and anti-Bcl-2, 1:1,000; Santa Cruz Biotechnology, Inc.). In addition, caspase-3 activity was measured to determine GSC cell apoptosis after above treatments by using a kit (Promega, Inc.) according to the manufacturer's instructions [48].

**2.8. PI<sub>3</sub>K Effect.** To test the effect of PI<sub>3</sub>K on 14-3-3 $\beta$  siRNA transfection, the specific PI<sub>3</sub>K pan isoform inhibitor TG 100713 (Tocris Bioscience) was used at 10  $\mu$ M to treat GSCs for 48 h prior to CoCl<sub>2</sub> application [49]. Subsequently, the 14-3-3 $\beta$  protein expression was also detected using western blotting.

**2.9. Statistical Analysis.** Statistical analysis was performed using Student's  $t$ -test and one-way ANOVA followed by a Tukey post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference. Data were expressed as means  $\pm$  standard deviation.

### 3. Results

**3.1. Effect of Hypoxia on the 14-3-3 $\beta$  Expression in GSCs.** The expression of 14-3-3 $\beta$  and HIF-1 $\alpha$  in GSCs was determined by western blotting and qPCR. The high expression of 14-3-3 $\beta$  and HIF-1 $\alpha$  protein was observed in GSCs. In addition, 14-3-3 $\beta$  and HIF-1 $\alpha$  mRNA levels were significantly increased in GSCs (Figure 1).

To observe the effect of 14-3-3 $\beta$  knockdown, specific 14-3-3 $\beta$  siRNA was transfected into GSCs to reduce the 14-3-3 $\beta$  expression firstly. The transfection efficiency of 14-3-3 $\beta$  siRNA was evaluated by qPCR and western blotting. The result confirmed that the 14-3-3 $\beta$  expression in GSCs was inhibited after 14-3-3 $\beta$  siRNA transfection for 48 h. In addition, 14-3-3 $\beta$  mRNA level was also significantly reduced after 14-3-3 $\beta$  siRNA application (Figure 1). These results provided the direct evidence that using 14-3-3 $\beta$  siRNA could reduce the 14-3-3 $\beta$  expression in GSCs significantly.

It has been demonstrated that hypoxia enhanced the HIF-1 $\alpha$  expression [13, 16], and thus the effect of hypoxia induced by using CoCl<sub>2</sub> on the 14-3-3 $\beta$  expression was subsequently investigated in GSCs too. To do so, GSCs were incubated with 10, 50, and 150  $\mu$ M CoCl<sub>2</sub> for 8 h prior to mRNA level evaluation, respectively. Furthermore, cells were treated with different concentrations (10, 50, and 150  $\mu$ M) of CoCl<sub>2</sub> for 24 h to observe the changes on the 14-3-3 $\beta$  expression. The results from western blotting and qPCR demonstrated that GSC treatment with CoCl<sub>2</sub> could increase the 14-3-3 $\beta$  protein expression and mRNA level in a dose-dependent manner. The above results also confirmed that both 14-3-3 $\beta$  and HIF-1 $\alpha$  were overexpressed in GSCs and that hypoxia enhanced the 14-3-3 $\beta$  expression (Figure 1).

**3.2. Effects of 14-3-3 $\beta$  Knockdown on GSC Cell Proliferation.** Our previous study reported that inhibition on HIF-1 $\alpha$  could be important for the biological behavior changes of glioma cells [21]. In the current experiments, GSC cell proliferation was therefore evaluated by MTT assay following CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA transfection. The results demonstrated that, in the group treated with 14-3-3 $\beta$  siRNA alone, cell proliferation was significantly reduced compared with that of the untreated group, and that cell count was reduced by 38.2%; however, a noticeable enhancement in cell numbers was obtained using CoCl<sub>2</sub> alone. Notably, 14-3-3 $\beta$  knockdown by siRNA reversed the effect of CoCl<sub>2</sub> on cell proliferation, and GSC cell count decreased by 31.3% following CoCl<sub>2</sub> application combined with 14-3-3 $\beta$  siRNA transfection (Figure 2).

Furthermore, to observe the changes on cell proliferation after 14-3-3 $\beta$  knockdown, the expression of PCNA in GSCs was determined by western blotting and qPCR following CoCl<sub>2</sub> and/or 14-3-3 $\beta$  siRNA application. Hypoxia induced by using CoCl<sub>2</sub> enhanced PCNA protein expression and mRNA levels compared with the control group in GSCs (Figure 2). In addition, the PCNA protein expression in GSCs was reduced after 14-3-3 $\beta$  siRNA transfection, and PCNA mRNA levels were significantly inhibited by 14-3-3 $\beta$  siRNA application. And the reverse effect was also obtained in the group of CoCl<sub>2</sub> application, and the PCNA protein and mRNA levels were decreased by combining CoCl<sub>2</sub> treatment with 14-3-3 $\beta$  siRNA.

**3.3. Effects of 14-3-3 $\beta$  Knockdown on Subcutaneous Tumor Growth.** An in vivo tumor xenograft study was performed to evaluate the animal survival rate and tumor volume. The tumor weight in each group was also measured. The

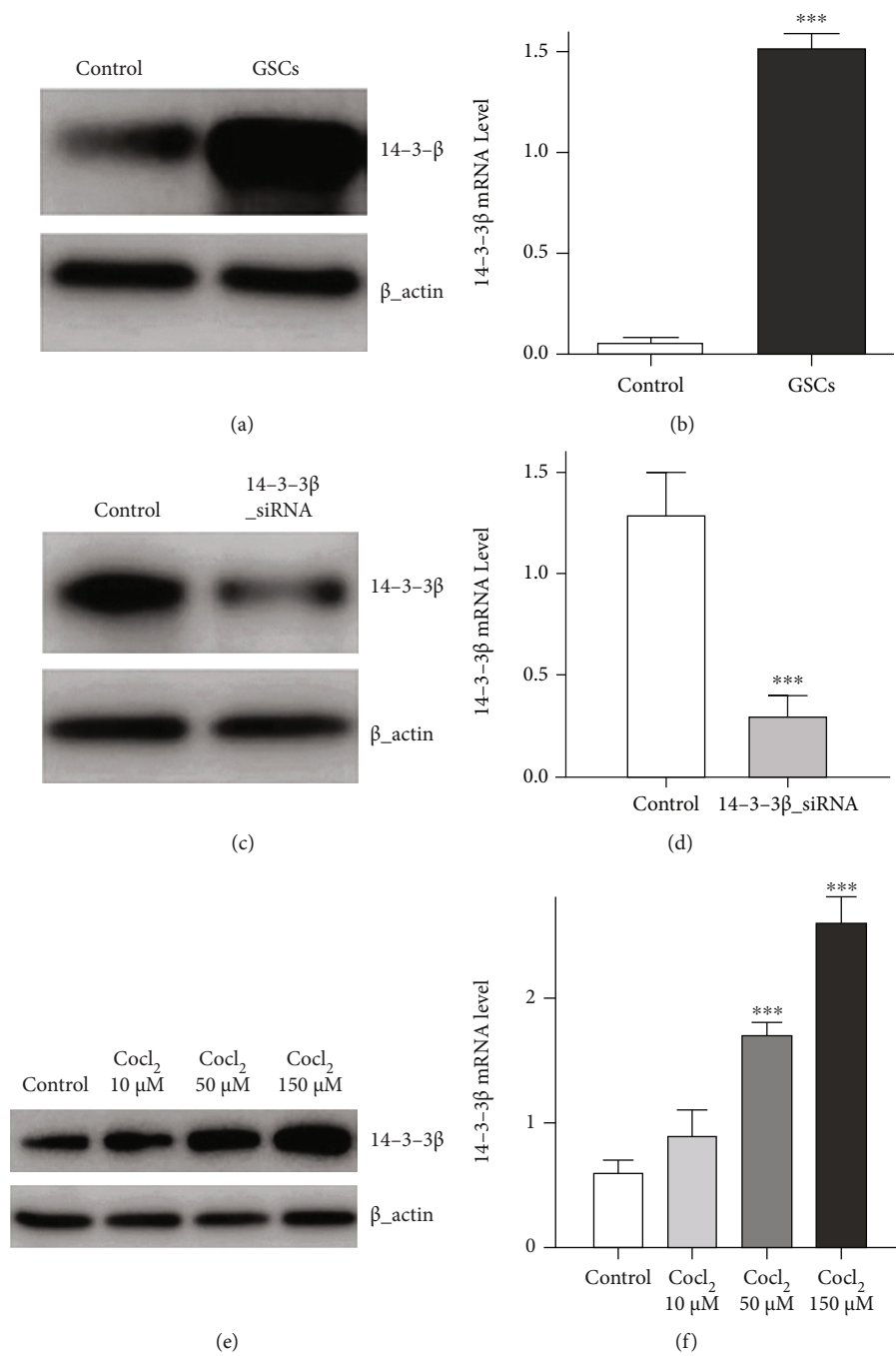


FIGURE 1: Continued.

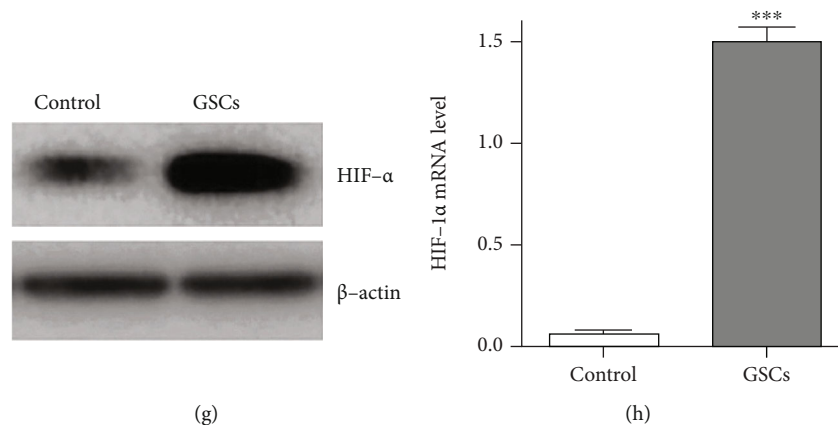


FIGURE 1: HIF-1 $\alpha$  and 14-3-3 $\beta$  expression in GSCs. (a) 14-3-3 $\beta$  protein expression in GSC cells. (b) 14-3-3 $\beta$  mRNA level in GSC cells (vs. control,  $P < 0.001$ ). (c) Effect of 14-3-3 $\beta$  siRNA transfection on the 14-3-3 $\beta$  expression in GSC cells. (d) Effect of 14-3-3 $\beta$  siRNA transfection on 14-3-3 $\beta$  mRNA level in GSCs cells (14-3-3 $\beta$  siRNA vs. control,  $P < 0.001$ ). (e) Effect of hypoxia induced by using CoCl<sub>2</sub> on the 14-3-3 $\beta$  protein expression in GSC cells. (f) Effect of hypoxia on 14-3-3 $\beta$  mRNA level in GSC cells (CoCl<sub>2</sub> 50  $\mu$ M vs. control,  $P < 0.001$ ; CoCl<sub>2</sub> 150  $\mu$ M vs. control,  $P < 0.001$ ). (g) HIF-1 $\alpha$  expression in GSC cells.  $\beta$ -Actin was used as a loading control. (h) HIF-1 $\alpha$  mRNA level in GSCs cells (vs. control,  $P < 0.001$ ).

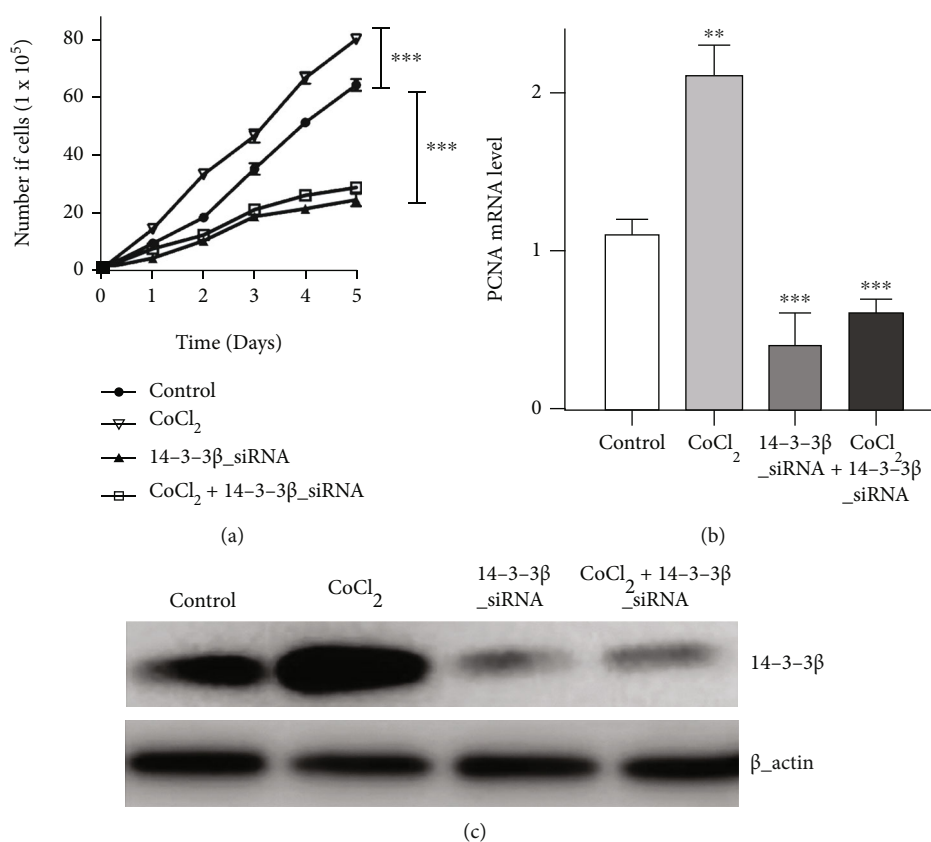


FIGURE 2: Effects on cell proliferation. (a) Effects of hypoxia and 14-3-3 $\beta$  siRNA transfection on GSC cell proliferation assessed by MTT assay (14-3-3 $\beta$  siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub> vs. control,  $P < 0.001$ ). (b) Effects of CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA transfection on PCNA mRNA level in GSC cells (CoCl<sub>2</sub> vs. control,  $P < 0.01$ ; 14-3-3 $\beta$  siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub>+14-3-3 $\beta$  siRNA vs. CoCl<sub>2</sub>,  $P < 0.001$ ). (c) Effects of CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA transfection on the PCNA protein expression in GSC cells.  $\beta$ -Actin was used as a loading control.

results demonstrated that CoCl<sub>2</sub> increased tumor weight, whereas 14-3-3 $\beta$  knockdown significantly reduced it. In the control group, the tumor volume was  $350 \pm 41$  mm<sup>3</sup> fol-

lowing injection for 28 days and increased to  $588 \pm 33$  mm<sup>3</sup> when treated with CoCl<sub>2</sub>. Conversely, tumor volume was reduced to  $100 \pm 12$  mm<sup>3</sup> after 14-3-3 $\beta$  siRNA application.

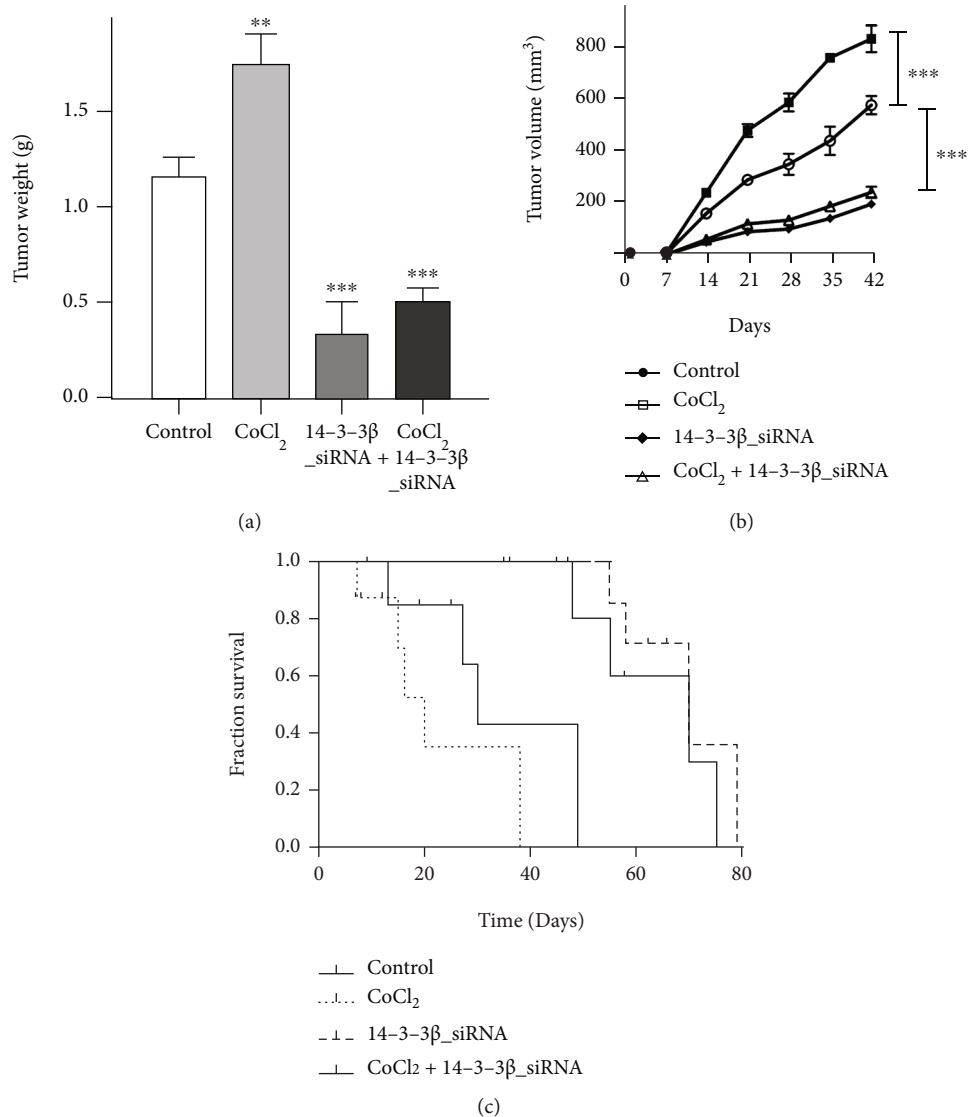


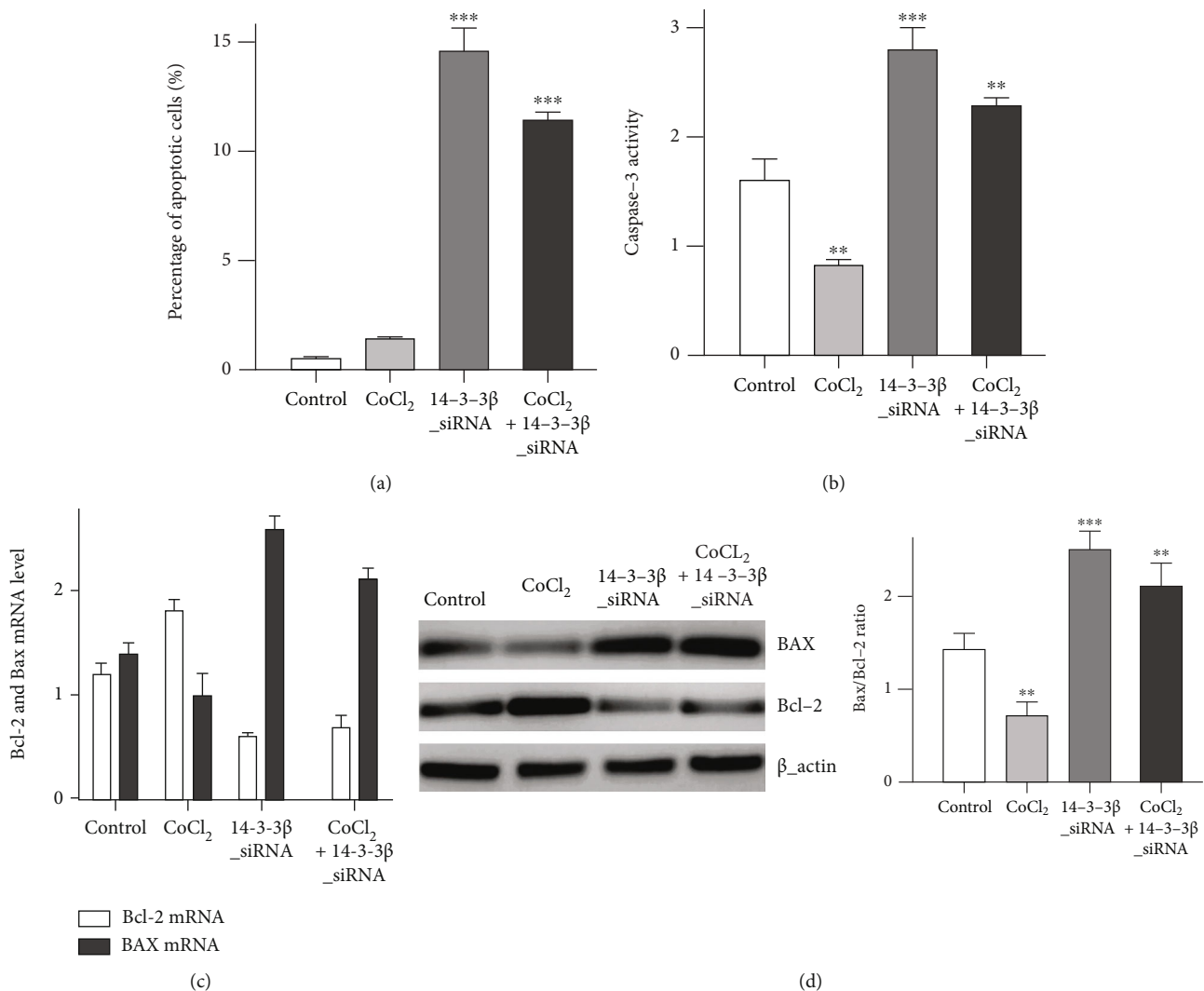
FIGURE 3: Effects of 14-3-3 $\beta$  knockdown on subcutaneous tumor growth. (a) Effect of CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA transfection on tumor weight in a tumor xenograft model (CoCl<sub>2</sub> vs. control,  $P < 0.01$ ; 14-3-3 $\beta$  siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub>+14-3-3 $\beta$  siRNA vs. CoCl<sub>2</sub>,  $P < 0.001$ ). (b) Effect of CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA transfection on tumor volume in a tumor xenograft model (14-3-3 $\beta$  siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub> vs. control,  $P < 0.001$ ). (c) Effect of CoCl<sub>2</sub> and 14-3-3 $\beta$  siRNA transfection on mice survival rate.

In addition, the enhancing effect of CoCl<sub>2</sub> application on tumor volume was reversed, and tumor volume reached  $135 \pm 8 \text{ mm}^3$  (Figure 3) in the combined group. These results indicated that 14-3-3 $\beta$  knockdown could inhibit tumor growth, and hypoxia induced by using CoCl<sub>2</sub> increases the tumor growth which could be also reversed by combining 14-3-3 $\beta$  siRNA transfection.

The survival rate of transplanted mice was also determined following CoCl<sub>2</sub> application and 14-3-3 $\beta$  knockdown. The results demonstrated that 14-3-3 $\beta$  siRNA application markedly prolonged mice survival rate; however, a lower survival rate was observed after CoCl<sub>2</sub> treatment which inducing the hypoxic tumor microenvironment (TME) (Figure 3). 14-3-3 $\beta$  knockdown reduced tumor growth and prolong mice survival rate, and such 14-3-3 $\beta$  inhibition could reverse the effect of hypoxia on tumor growth in vivo.

**3.4. Effects of 14-3-3 $\beta$  Knockdown on Cell Apoptosis.** The percentage of apoptotic cells was first evaluated by flow cytometry after 14-3-3 $\beta$  siRNA transfection or CoCl<sub>2</sub> application. The results evidenced that 14-3-3 $\beta$  knockdown increased cell apoptosis; and the combination of CoCl<sub>2</sub> application and 14-3-3 $\beta$  siRNA also induced a significant increase in cell apoptosis. Caspase-3 activity was investigated following CoCl<sub>2</sub> application or 14-3-3 $\beta$  knockdown. The results demonstrated that caspase-3 activity was significantly decreased following CoCl<sub>2</sub> application in GSCs; however, 14-3-3 $\beta$  knockdown increased caspase-3 activity (Figure 4). A rescue experiment also provided evidence that 14-3-3 $\beta$  siRNA application reversed the above effect of hypoxia induced by using CoCl<sub>2</sub>.

Furthermore, Bax and Bcl-2 mRNA levels were determined by qPCR. The results proved that CoCl<sub>2</sub> treatment



**FIGURE 4: Effects on GSC cell apoptosis.** (a) Effects of CoCl<sub>2</sub> application and 14-3-3β siRNA transfection on the apoptotic rate of GSCs (14-3-3β siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub>+14-3-3β siRNA vs. CoCl<sub>2</sub>,  $P < 0.001$ ). (b) Effects of CoCl<sub>2</sub> application and 14-3-3β siRNA transfection on caspase-3 activity in GSCs (CoCl<sub>2</sub> vs. control,  $P < 0.01$ ; 14-3-3β siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub>+14-3-3β siRNA vs. CoCl<sub>2</sub>,  $P < 0.01$ ). (c) Effects of CoCl<sub>2</sub> application and 14-3-3β siRNA transfection on Bcl-2 and Bax mRNA levels in GSCs. (d) Effects of CoCl<sub>2</sub> application and 14-3-3β siRNA transfection on Bcl-2 and Bax expression in GSCs, Bax/Bcl-2 ratio was also calculated. (CoCl<sub>2</sub> vs. control,  $P < 0.01$ ; 14-3-3β siRNA vs. control,  $P < 0.001$ ; CoCl<sub>2</sub>+14-3-3β siRNA vs. CoCl<sub>2</sub>,  $P < 0.01$ ). β-Actin was used as a loading control.

in GSCs decreased Bax mRNA level and increased Bcl-2 mRNA level, which was reversed when combined with 14-3-3β knockdown. In addition, after 14-3-3β knockdown, Bcl-2 mRNA level was reduced, whereas Bax mRNA level was increased (Figure 4). Western blotting results were used to observe Bax and Bcl-2 expression in GSCs, which are associated with the development of cell apoptosis. After CoCl<sub>2</sub> application, the Bax protein expression in GSCs was reduced compared with that in the untreated group, whereas the Bcl-2 expression was increased. Furthermore, in the 14-3-3β siRNA transfection group, the Bax expression was increased, whereas the Bcl-2 expression was decreased. The Bax/Bcl-2 ratio, which expresses the apoptotic process of a cell, was therefore calculated. The results demonstrated that the decrease in Bax/Bcl-2 ratio after CoCl<sub>2</sub> application was completely reversed following 14-3-3β knockdown.

**3.5. Effects of PI<sub>3</sub>K Inhibition.** In the following experiments, the pan isoform inhibitor of PI<sub>3</sub>K *TG 100713* was used to observe the association between hypoxia and 14-3-3β. The western blotting results confirmed that *TG 100713* application significantly reduced the 14-3-3β expression in GSCs. Furthermore, it was interesting that CoCl<sub>2</sub> application did not enhance 14-3-3β expression in the presence of *TG 100713* (Figure 5). Hypoxia induced by using CoCl<sub>2</sub> may promote therefore the 14-3-3β expression via the PI<sub>3</sub>K signaling pathway.

## 4. Discussion

Proteins of the 14-3-3 family are overexpressed in glioma, lung cancer, and astrocytoma and serve crucial roles in the biological behavior of various types of tumor [32, 33].

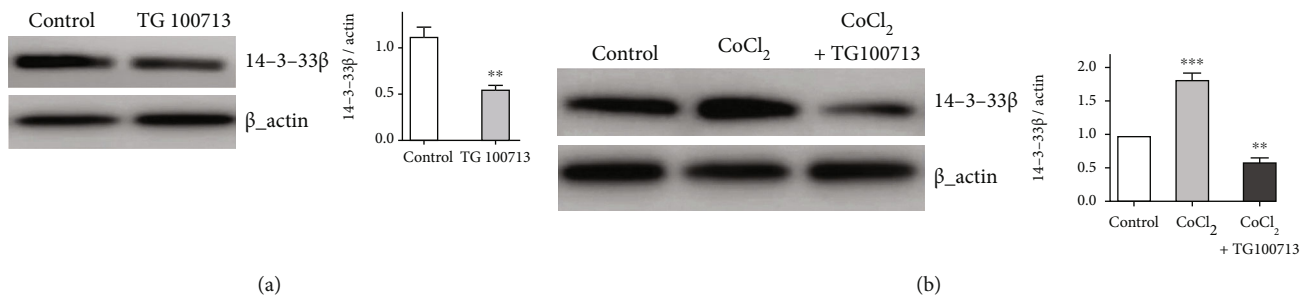


FIGURE 5: Effects of PI<sub>3</sub>K inhibition on the 14-3-3β expression. (a) Effects of TG 100713 on the 14-3-3β expression, as assessed by western blotting. Densitometry analysis of 14-3-3β/actin (TG100713 vs. control,  $P < 0.01$ ). (b) Effects of TG 100713 and CoCl<sub>2</sub> cotreatment on the 14-3-3β expression, as assessed by western blotting. Densitometry analysis of 14-3-3β/actin (CoCl<sub>2</sub> vs. control,  $P < 0.001$ ; CoCl<sub>2</sub> + TG100713 vs. CoCl<sub>2</sub>,  $P < 0.01$ ). β-Actin was used as a loading control.

Furthermore, 14-3-3 regulates glioma cell proliferation through the glycogen synthase kinase 3/β-catenin signaling pathway [50, 51]. It has been reported that inhibition of HIF-1α by siRNA could reduce tumor growth, and hypoxic tumor microenvironment (TME) enhances tumor angiogenesis and progression [14, 15].

The results from the present study were as follows: (1) HIF-1α and 14-3-3β were highly expressed in GSCs and in tumor xenografts; (2) hypoxia induced by using CoCl<sub>2</sub> enhanced 14-3-3β protein expression and mRNA levels in GSCs; (3) hypoxia promoted cell proliferation and associated PCNA expression, which was rescued by 14-3-3β knockdown; (4) tumor growth was significantly promoted by CoCl<sub>2</sub> application, which was reversed following 14-3-3β knockdown; (5) cell apoptosis was increased after 14-3-3β siRNA application, which altered Bax/Bcl-2 ratio and caspase-3 activity in the hypoxic tumor microenvironment; and (6) hypoxia activated 14-3-3β via the PI<sub>3</sub>K signaling pathway. The present study therefore provided evidence that hypoxia may promote GSC growth via the PI<sub>3</sub>K signaling pathway both in vitro and in vivo by increasing 14-3-3β expression, which could be considered as a potential therapeutic target for patients undergoing clinical siRNA treatment.

Since glioma was characterized by high recurrence and low survival rate [3, 52], it is crucial to develop the novel strategies for the treatment of patients with glioma. The high HIF-1α expression was observed in the hypoxic tumor microenvironment (TME); thus, specific inhibition of HIF-1α may provide certain benefits in clinical cancer therapy when combined with a multitherapeutic approach. Previous studies also reported that 14-3-3β serves a key role in tumor angiogenesis and tumor formation, and that it could stimulate cell proliferation, cell cycle, and cell apoptosis to promote tumor growth and progression [32–34]; however, few study has described the association between hypoxia and 14-3-3β modulation in glioma progression and metastasis and the biological behaviors of GSCs in vitro.

In the current experiments, the results demonstrated that both 14-3-3β and HIF-1α proteins were overexpressed in GSCs cells. Subsequently, the effect of hypoxia induced by using CoCl<sub>2</sub>, which can specifically enhance HIF-1α level [11, 12], on the 14-3-3β expression, was investigated in GSCs cells. The results provided the evidence that 14-3-3β protein expression and mRNA level were increased follow-

ing hypoxia induced by using CoCl<sub>2</sub> in a dose-dependent manner. Thus, it is hypothesized that 14-3-3β-induced GSC cell proliferation may be a potential downstream effect of hypoxia. Multitherapeutic approaches could therefore increase glioma therapeutic efficiency by combining 14-3-3β and HIF-1α inhibitors or siRNA application.

The results from cell proliferation experiments demonstrated that hypoxia promoted GSC cell proliferation by enhancing 14-3-3β expression level. These data indicated that HIF-1α and 14-3-3β may be associated in the tumor growth process. Additional evidence was obtained in the animal experiments by analyzing the survival rate and tumor volume. After 14-3-3β siRNA application, survival rate of the transplanted mice was enhanced, and tumor volume and weight were decreased, which indicated that the effect of hypoxia on tumor growth was reversed by 14-3-3β knockdown. In addition, a previous study also reported that 14-3-3β downregulation inhibits osteosarcoma cell proliferation and migration [53]. The present study therefore hypothesized that 14-3-3β may serve a crucial role in the hypoxia-induced tumor growth in glioma.

14-3-3β is highly expressed in various types of tumor and is associated with numerous human malignancies, including glioma, lung cancer, astrocytoma, breast cancer, squamous cell carcinoma, ovarian cancer, and liver cancer [32–35]. Tumor cell apoptosis is an important biological process associated with tumor growth. To clarify the underlying mechanism of hypoxia-induced tumor growth, the effect of CoCl<sub>2</sub> application and 14-3-3β knockdown on GSC cell apoptosis was investigated. By observing the biomarker or apoptotic rate, the effect of hypoxia and 14-3-3β knockdown on cell apoptosis were evaluated both in vitro and in vivo. To do so, Bax/Bcl-2 ratio and caspase-3 activity were selected to reflect the changes in tumor apoptosis following the mentioned treatments by using 14-3-3β siRNA and CoCl<sub>2</sub> application. Bax/Bcl-2 ratio was significantly decreased following CoCl<sub>2</sub> treatment, which reduced tumor cell apoptosis. Furthermore, 14-3-3β knockdown enhanced GSC cell apoptosis via Bax/Bcl-2 ratio increase. The results from caspase-3 activity revealed similar tendencies. Above results suggested that hypoxia may enhance tumor growth or reduce tumor cell apoptosis via 14-3-3β modulation in GSCs. The underlying mechanism or downstream targets should be clarified in the further experiments.



The PI<sub>3</sub>K signaling pathway is closely associated with cell proliferation and cell apoptosis [23]. Hypoxia activates PI<sub>3</sub>K, which serves critical roles in cell survival and apoptosis [54, 55]. Furthermore, HIF-1 $\alpha$  is known to relate to the Raf/MAPK/ERK axis, and hypoxia is a key event to leads to PI<sub>3</sub>K and Ras/MAPK signaling pathway activation [56]; however, whether hypoxia enhances 14-3-3 $\beta$  expression in GSCs via PI<sub>3</sub>K activation has not been investigated to date. In the present study, a specific inhibitor of PI<sub>3</sub>K reduced the 14-3-3 $\beta$  expression, even after CoCl<sub>2</sub> application, which suggested that hypoxia may upregulate 14-3-3 $\beta$  via the PI<sub>3</sub>K signaling pathway. Hypoxia may therefore activate PI<sub>3</sub>K, which may promote 14-3-3 $\beta$  expression and influence the biological behavior of GSCs, including cell proliferation, survival, and apoptosis. PI<sub>3</sub>Ks, including the  $\alpha$ ,  $\beta$ ,  $\gamma$ , or  $\delta$  isoforms, are lipid kinases that serve crucial roles in the regulation of cell proliferation, cell cycle, and cell apoptosis [23]. The role of these different isoforms on cell proliferation, cell cycle, and cell apoptosis may be further investigated to clarify the underlying mechanism of the association between PI<sub>3</sub>Ks and 14-3-3 $\beta$ . Furthermore, it has been demonstrated that hypoxia induced HIF-1 $\alpha$  modulates the communication between p53 and AKT-mTOR pathways in cancer cells [57–59]. The results from the present study also indicated that hypoxia may promote GSCs or glioma growth by activating the 14-3-3 $\beta$ . Subsequently, targeted inhibition of HIF-1 $\alpha$  or 14-3-3 $\beta$  may provide novel clinical therapeutic strategies to treat patients with glioma.

14-3-3 $\beta$  and HIF-1 $\alpha$  overexpression in tumors have been previously demonstrated [13, 32]. The present study therefore investigated whether 14-3-3 $\beta$  knockdown and PI<sub>3</sub>K inhibition could increase the therapeutic efficacy of glioma treatment in vitro and in vivo. In addition, since HIF-1 $\alpha$  and the Raf/MAPK/ERK axis are associated, future works will investigate the changes between 14-3-3 $\beta$  and the Raf/MAPK signaling pathway. Through in vivo and in vitro experiments, the present study demonstrated that hypoxic tumor microenvironment (TME) may promote glioma progression via PI<sub>3</sub>K signaling pathway activation following the 14-3-3 $\beta$  overexpression. In conclusion, HIF-1 $\alpha$  inhibition by using novel PI<sub>3</sub>K inhibitors combined with 14-3-3 $\beta$  knockdown by specific siRNA may represent an efficient therapeutic strategy to treat patients with glioma.

### Data Availability

The data used to support the findings of this study are included within the article.

### Conflicts of Interest

The authors declare that they have no competing interests.

### Authors' Contributions

WC and JZ designed the experiments and wrote the manuscript. WC, QZ, HW, WR, SG, and GC and FY performed all experiments. WC and QZ analyzed the data. All authors read and approved the final manuscript. Wei-

dong Cao, Qiang Zhou, and Hongwei Wang contributed equally to this work.

### References

- [1] M. Lim, Y. Xia, C. Bettgowda, and M. Weller, "Current state of immunotherapy for glioblastoma," *Nature Reviews. Clinical Oncology*, vol. 15, no. 7, pp. 422–442, 2018.
- [2] E. G. Van Meir, C. G. Hadjipanayis, A. D. Norden, H. K. Shu, P. Y. Wen, and J. J. Olson, "Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma," *CA: a Cancer Journal for Clinicians*, vol. 60, no. 3, pp. 166–193, 2010.
- [3] D. Hsieh, "Collateral damage control in cancer therapy: defining the stem identity in gliomas," *Current Pharmaceutical Design*, vol. 17, no. 23, pp. 2370–2385, 2011.
- [4] A. Dirkse, A. Golebiewska, T. Buder et al., "Stem cell-associated heterogeneity in glioblastoma results from intrinsic tumor plasticity shaped by the microenvironment," *Nature Communications*, vol. 10, no. 1, p. 1787, 2019.
- [5] S. K. Singh, C. Hawkin, and I. D. Clarke, "Identification of human brain tumour initiating cells," *Nature*, vol. 432, no. 7015, pp. 396–401, 2004.
- [6] S. DeCordova, A. Shastri, A. G. Tzolaki et al., "Molecular heterogeneity and immunosuppressive microenvironment in glioblastoma," *Frontiers in Immunology*, vol. 11, p. 1402, 2020.
- [7] B. Campos, L. R. Olsen, T. Urup, and H. S. Poulsen, "A comprehensive profile of recurrent glioblastoma," *Oncogene*, vol. 35, no. 45, pp. 5819–5825, 2016.
- [8] J. R. Molina, N. K. Agarwal, F. C. Morales et al., "PTEN, NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma," *Oncogene*, vol. 31, no. 10, pp. 1264–1274, 2012.
- [9] D. Krex, B. Klink, C. Hartmann et al., "Long-term survival with glioblastoma multiforme," *Brain*, vol. 130, no. 10, pp. 2596–2606, 2007.
- [10] Y. Liu, X. Li, Y. Zhang et al., "An miR-340-5p-macrophage feedback loop modulates the progression and tumor microenvironment of glioblastoma multiforme," *Oncogene*, vol. 38, no. 49, pp. 7399–7415, 2019.
- [11] J. W. Shih, W. F. Chiang, A. T. H. Wu et al., "Long noncoding RNA *LncHIFCAR/MIR31HG* is a HIF-1 $\alpha$  co-activator driving oral cancer progression," *Nature Communications*, vol. 8, no. 1, p. 15874, 2017.
- [12] T. T. Wei, Y. T. Lin, S. P. Tang et al., "Metabolic targeting of HIF-1 $\alpha$  potentiates the therapeutic efficacy of oxaliplatin in colorectal cancer," *Oncogene*, vol. 39, no. 2, pp. 414–427, 2020.
- [13] A. Palazon, P. A. Tyraklis, D. Macias et al., "An HIF-1 $\alpha$ /VEGF-A axis in cytotoxic T cells regulates tumor progression," *Cancer Cell*, vol. 32, no. 5, pp. 669–683.e5, 2017.
- [14] M. C. Lin, J. J. Lin, C. L. Hsu, H. F. Juan, P. J. Lou, and M. C. Huang, "GATA3 interacts with and stabilizes HIF-1 $\alpha$  to enhance cancer cell invasiveness," *Oncogene*, vol. 36, no. 30, pp. 4243–4252, 2017.
- [15] V. Mukund, M. S. Saddala, B. Farran, M. Mannavarapu, A. Alam, and G. P. Nagaraju, "Molecular docking studies of angiogenesis target protein HIF-1 $\alpha$  and genistein in breast cancer," *Gene*, vol. 701, pp. 169–172, 2019.
- [16] M. B. Gariboldi, E. Taiana, M. C. Bonzi et al., "The BH3-mimetic obatoclax reduces HIF-1 $\alpha$  levels and HIF-1 transcriptional activity and sensitizes hypoxic colon adenocarcinoma

- cells to 5-fluorouracil," *Cancer Letters*, vol. 364, no. 2, pp. 156–164, 2015.
- [17] K. Wei, S. M. Pieciewicz, L. M. McGinnis et al., "A liver Hif-2 $\alpha$ -Irs2 pathway sensitizes hepatic insulin signaling and is modulated by Vegf inhibition," *Nature Medicine*, vol. 19, no. 10, pp. 1331–1337, 2013.
- [18] Y. Liu, X. Wang, W. Li et al., "Oroxlylin A reverses hypoxia-induced cisplatin resistance through inhibiting HIF-1 $\alpha$  mediated XPC transcription," *Oncogene*, vol. 39, no. 45, pp. 6893–6905, 2020.
- [19] H. J. Lee, C. Yoon, D. J. Park et al., "Inhibition of Vascular endothelial growth factor A and hypoxia-inducible factor 1 $\alpha$  maximizes the effects of radiation in sarcoma mouse models through destruction of tumor vasculature," *International Journal of Radiation Oncology • Biology • Physics*, vol. 91, no. 3, pp. 621–630, 2015.
- [20] W. D. Cao, N. Kawai, K. Miyake, X. Zhang, Z. Fei, and T. Tamiya, "Relationship of 14-3-3zeta ( $\zeta$ ), HIF-1 $\alpha$ , and VEGF expression in human brain gliomas," *Brain Tumor Pathology*, vol. 31, no. 1, pp. 1–10, 2014.
- [21] Z. Luo, M. Bai, X. Xiao et al., "Silencing of HIF-1 $\alpha$  enhances the radiation sensitivity of human glioma growth *in vitro* and *in vivo*," *Neuropharmacology*, vol. 89, pp. 168–174, 2015.
- [22] M. Aoki and T. Fujishita, "Oncogenic roles of the PI3K/AKT/mTOR Axis," *Current Topics in Microbiology and Immunology*, vol. 407, pp. 153–189, 2017.
- [23] S. H. Park, B. R. Kim, J. H. Lee et al., "GABARBP downregulates HIF-1 $\alpha$  expression through the VEGFR-2 and PI3K/mTOR/4E-BP1 pathways," *Cellular Signalling*, vol. 26, no. 7, pp. 1506–1513, 2014.
- [24] J. Karar, G. J. Cerniglia, T. Lindsten, C. Koumenis, and A. Maity, "Dual PI3K/mTOR inhibitor NVP-BEZ235 suppresses hypoxia-inducible factor (HIF)-1 $\alpha$  expression by blocking protein translation and increases cell death under hypoxia," *Cancer Biology & Therapy*, vol. 13, no. 11, pp. 1102–1111, 2012.
- [25] J. H. Park, J. Y. Lee, D. H. Shin, K. S. Jang, H. J. Kim, and G. Kong, "Loss of Mel-18 induces tumor angiogenesis through enhancing the activity and expression of HIF-1 $\alpha$  mediated by the PTEN/PI3K/Akt pathway," *Oncogene*, vol. 30, no. 45, pp. 4578–4589, 2011.
- [26] D. A. Almiron Bonnin, M. C. Havrda, M. C. Lee et al., "Secretion-mediated STAT3 activation promotes self-renewal of glioma stem-like cells during hypoxia," *Oncogene*, vol. 37, no. 8, pp. 1107–1118, 2018.
- [27] W. Lin, S. Wu, X. Chen et al., "Characterization of hypoxia signature to evaluate the tumor immune microenvironment and predict prognosis in glioma groups," *Frontiers in Oncology*, vol. 10, p. 796, 2020.
- [28] D. Kita, Y. Yonekawa, M. Weller, and H. Ohgaki, "PIK3CA alterations in primary (de novo) and secondary glioblastomas," *Acta Neuropathologica*, vol. 113, no. 3, pp. 295–302, 2007.
- [29] I. K. Mellingshoff, M. Y. Wang, I. Vivanco et al., "Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors," *The New England Journal of Medicine*, vol. 353, no. 19, pp. 2012–2024, 2005.
- [30] R. Guo, Y. Li, Z. Wang et al., "Hypoxia-inducible factor-1 $\alpha$  and nuclear factor- $\kappa$ B play important roles in regulating programmed cell death ligand 1 expression by epidermal growth factor receptor mutants in non-small-cell lung cancer cells," *Cancer Science*, vol. 110, no. 5, pp. 1665–1675, 2019.
- [31] A. Lo Dico, S. Valtorta, L. Ottobrini, and R. M. Moresco, "Role of metformin and AKT Axis modulation in the reversion of hypoxia induced TMZ-resistance in glioma cells," *Frontiers in Oncology*, vol. 9, p. 463, 2019.
- [32] W. Cao, X. Yang, J. Zhou et al., "Targeting 14-3-3 protein, difopein induces apoptosis of human glioma cells and suppresses tumor growth in mice," *Apoptosis*, vol. 15, no. 2, pp. 230–241, 2010.
- [33] S. Jasinski-Bergner, F. Stehle, E. Gonschorek et al., "Identification of 14-3-3 $\beta$  gene as a novel miR-152 target using a proteome-based approach," *The Journal of Biological Chemistry*, vol. 289, no. 45, pp. 31121–31135, 2014.
- [34] S. B. Seo, J. J. Lee, H. H. Yun et al., "14-3-3 $\beta$  depletion drives a senescence program in glioblastoma cells through the ERK/SKP2/p27 pathway," *Molecular Neurobiology*, vol. 55, no. 2, pp. 1259–1270, 2018.
- [35] H. Wang, C. Z. Zhang, S. X. Lu et al., "A coiled-coil domain containing 50 splice variant is modulated by serine/arginine-rich splicing factor 3 and promotes hepatocellular carcinoma in mice by the Ras signaling pathway," *Hepatology*, vol. 69, no. 1, pp. 179–195, 2019.
- [36] Y. J. Wu, Y. J. Jan, B. S. Ko, S. M. Liang, and J. Y. Liou, "Involvement of 14-3-3 proteins in regulating tumor progression of hepatocellular carcinoma," *Cancers*, vol. 7, no. 2, pp. 1022–1036, 2015.
- [37] S. Colin, B. Darné, A. Kadi et al., "The antiangiogenic insulin receptor substrate-1 antisense oligonucleotide aganirsen impairs AU-rich mRNA stability by reducing 14-3-3 $\beta$ -tristetraprolin protein complex, reducing inflammation and psoriatic lesion size in patients," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 349, no. 1, pp. 107–117, 2014.
- [38] L. Liu, Z. Liu, H. Wang et al., "14-3-3 $\beta$  exerts glioma-promoting effects and is associated with malignant progression and poor prognosis in patients with glioma," *Experimental and Therapeutic Medicine*, vol. 15, no. 3, pp. 2381–2387, 2018.
- [39] X. Zhang, W. Zhang, X. G. Mao, H. N. Zhen, W. D. Cao, and S. J. Hu, "Targeting role of glioma stem cells for glioblastoma multiforme," *Current Medicinal Chemistry*, vol. 20, no. 15, pp. 1974–1984, 2013.
- [40] X. G. Mao, X. Zhang, X. Y. Xue et al., "Brain tumor stem-like cells identified by neural stem cell marker CD15," *Translational Oncology*, vol. 2, no. 4, pp. 247–257, 2009.
- [41] A. Renzi, S. DeMorrow, P. Onori et al., "Modulation of the biliary expression of arylalkylamine N-acetyltransferase alters the autocrine proliferative responses of cholangiocytes in rats," *Hepatology*, vol. 57, no. 3, pp. 1130–1141, 2013.
- [42] M. Cardano, C. Tribioli, and E. Prosperi, "Targeting proliferating cell nuclear antigen (PCNA) as an effective strategy to inhibit tumor cell proliferation," *Current Cancer Drug Targets*, vol. 20, no. 4, pp. 240–252, 2020.
- [43] K. Beyer, L. Normann, M. Sendler et al., "TRAIL promotes tumor growth in a syngeneic murine orthotopic pancreatic cancer model and affects the host immune response," *Pancreas*, vol. 45, no. 3, pp. 401–408, 2016.
- [44] H. Cho, T. C. Lai, and G. S. Kwon, "Poly(ethylene glycol)-block-poly( $\epsilon$ -caprolactone) micelles for combination drug delivery: evaluation of paclitaxel, cyclophosphamide and gossypol in intraperitoneal xenograft models of ovarian cancer," *Journal of Controlled Release*, vol. 166, no. 1, pp. 1–9, 2013.
- [45] T. G. Lam, Y. S. Jeong, S. A. Kim, and S. G. Ahn, "New metformin derivative HL156A prevents oral cancer progression by

- inhibiting the insulin-like growth factor/AKT/mammalian target of rapamycin pathways,” *Cancer Science*, vol. 109, no. 3, pp. 699–709, 2018.
- [46] J. L. Major, M. Salih, and B. S. Tuana, “E2F6 protein levels modulate drug induced apoptosis in cardiomyocytes,” *Cellular Signalling*, vol. 40, pp. 230–238, 2017.
- [47] V. R. Pidugu, N. S. Yarla, A. Bishayee, A. M. Kalle, and A. K. Satya, “Novel histone deacetylase 8-selective inhibitor 1,3,4-oxadiazole-alanine hybrid induces apoptosis in breast cancer cells,” *Apoptosis*, vol. 22, no. 11, pp. 1394–1403, 2017.
- [48] S. B. Kntayya, M. D. Ibrahim, N. Mohd Ain, R. Iori, C. Ioannides, and A. Abdull Razis, “Induction of apoptosis and cytotoxicity by isothiocyanate sulforaphene in human hepatocarcinoma HepG2 cells,” *Nutrients*, vol. 10, no. 6, p. 718, 2018.
- [49] J. Doukas, W. Wrasidlo, G. Noronha et al., “Phosphoinositide 3-kinase gamma/delta inhibition limits infarct size after myocardial ischemia/reperfusion injury,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 52, pp. 19866–19871, 2006.
- [50] F. Gong, G. Wang, J. Ye, T. Li, H. Bai, and W. Wang, “14-3-3 $\beta$  regulates the proliferation of glioma cells through the GSK3 $\beta$ / $\beta$ -catenin signaling pathway,” *Oncology Reports*, vol. 30, no. 6, pp. 2976–2982, 2013.
- [51] A. A. Dar, A. Belkhir, and W. El-Rifai, “The aurora kinase A regulates GSK-3 $\beta$  in gastric cancer cells,” *Oncogene*, vol. 28, no. 6, pp. 866–875, 2009.
- [52] J. R. Molina, F. C. Morales, Y. Hayashi, K. D. Aldape, and M. M. Georgescu, “Loss of PTEN binding adapter protein NHERF1 from plasma membrane in glioblastoma contributes to PTEN inactivation,” *Cancer Research*, vol. 70, no. 17, pp. 6697–6703, 2010.
- [53] Q. Wu, J. Zhu, F. Liu, J. Liu, and M. Li, “Downregulation of 14-3-3 $\beta$  inhibits proliferation and migration in osteosarcoma cells,” *Molecular Medicine Reports*, vol. 17, no. 2, pp. 2493–2500, 2018.
- [54] J. Karar and A. Maity, “PI3K/AKT/mTOR pathway in angiogenesis,” *Frontiers in Molecular Neuroscience*, vol. 4, p. 51, 2011.
- [55] S. Nicolas, S. Abdellatef, M. A. Haddad, I. Fakhoury, and M. el-Sibai, “Hypoxia and EGF stimulation regulate VEGF expression in human glioblastoma Multiforme (GBM) cells by differential regulation of the PI3K/rho-GTPase and MAPK pathways,” *Cell*, vol. 8, no. 11, p. 1397, 2019.
- [56] A. Mori, C. Moser, S. A. Lang et al., “Up-regulation of Krüppel-like factor 5 in pancreatic cancer is promoted by interleukin-1 $\beta$  signaling and hypoxia-inducible factor-1 $\alpha$ ,” *Molecular Cancer Research*, vol. 7, no. 8, pp. 1390–1398, 2009.
- [57] D. Shrimali, M. K. Shanmugam, A. P. Kumar et al., “Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer,” *Cancer Letters*, vol. 341, no. 2, pp. 139–149, 2013.
- [58] A. L. Chédeville and P. A. Madureira, “The role of hypoxia in glioblastoma radiotherapy resistance,” *Cancers*, vol. 13, no. 3, p. 542, 2021.
- [59] P. Ataie-Kachoie, M. H. Pourgholami, F. Bahrami-B, S. Badar, and D. L. Morris, “Minocycline attenuates hypoxia-inducible factor-1 $\alpha$  expression correlated with modulation of p53 and AKT/mTOR/p70S6K/4E-BP1 pathway in ovarian cancer: in vitro and in vivo studies,” *American Journal of Cancer Research*, vol. 5, no. 2, pp. 575–588, 2015.