Glioblastoma (GBM) is the most prevalent and lethal primary intrinsic brain cancer. The disease is essentially incurable, with glioblastomas characterized by resistance to both chemotherapy and radiotherapy, as well as by rapid tumor progression, all of which are mainly ascribed to glioma stem-like cells (GSLCs). In the present study, an improved model that is more similar to clinical GBM was constructed. Twenty clinical glioma samples were collected to obtain primary low-grade tumor cells. The cells were either maintained in serum-free medium as primary glioma-based cells (PGBCs) or cultured in the same medium with CHIR99021 as GSLCs. Then, the molecular and ultrastructural differences between the two cell groups were determined. Furthermore, the proliferation and migration of the GSLCs were examined and the potential mechanisms were investigated. Finally, temozolomide resistance in vitro and in the mouse model was assessed to study the properties of the induced GSLCs. The primary low-grade tumor cells extracted from surgical samples were enriched with GSLC properties, with high expression levels of CD133 and Nestin in 100 nM CHIR99021. The GSLCs exhibited high proliferation and migration. Furthermore, the expression of the PI3K/AKT signaling pathway and that of related genes and proteins were significantly enhanced by CHIR99021. The animal study also revealed high levels of STAT3, mTOR, NF-κB, and VEGF in the GSLC-transplanted mice. CHIR99021 could stably enhance GSLC properties in patient-derived glioma samples. It may provide a useful model for further study, helping to understand the pathogenesis of therapeutic resistance and to screen drug candidates.

Introduction

Although multimodal treatment, including surgical resection, radiotherapy, and chemotherapy, prolongs survival, glioblastoma (GBM) is still the most fatal primary intrinsic brain cancer, with a median survival time of less than 2 years (1,2). GBMs contain self-renewing, tumorigenic cancer stem cells that contribute to tumor initiation and characteristic, malignant glioma stem-like cells (GSLCs) (3,4). In addition, these GSLCs have been reported to be closely associated with resistance to radiotherapy and chemotherapy, although the underlying mechanism needs to be further explored (5-7).

Conventional human glioma cell lines, such as U87, U251, and T98G, may not represent the real GBMs in patients, for several reasons. First, the serum-containing medium in which these cell lines are cultured could potentially change the genomes of the cells and their transcriptomes, giving rise to the depletion of stem-like cells (8). Second, tumor models constructed in mice by the injection of these cell lines into the brain fail to develop the typical morphological features of GBMs, such as diffuse infiltration into the surrounding tissue and microvascular proliferations (8-10). Therefore, it is unlikely that findings with these cell line models can be correlated with the parameters of patients (10).

It is well known that activation of the canonical Wnt/β-catenin signaling pathway inhibits the glycogen synthase kinase 3 (GSK-3)-mediated degradation of β-catenin, resulting in the accumulation of cytoplasmic β-catenin. Then, the β-catenin translocates into the nucleus and interacts with transcriptional complexes to regulate the downstream target genes, which can facilitate cell proliferation and inhibit cell apoptosis (11,12). CHIR99021 is a chemical compound that acts as an inhibitor of GSK-3. It has been demonstrated to be useful for applications in molecular biology for transforming the cell type into induced pluripotent stem cells (iPSCs) and neurons (13,14). However, there is no evidence that CHIR99021 can transform primary low-grade glioma cells into GSLCs. In...
the present study, for the first time to the best of our knowledge, the effect of CHIR99021 in enriching cells with GSLC properties was explored, which would enable the construction of a better model that is more similar to clinical GBM.

Materials and methods

Preparation of the tumor specimens. Glioma samples were obtained from the operation room of the Neurosurgical Department of the Army General Hospital from January 2017 to October 2017. All the patients, 8 females and 12 males aged from 18 to 65 years old, were informed and signed the informed consent. A total of 20 glioma samples with low grades (WHO I, 10; II, 10), as based on the WHO classification criteria, were collected and used in the experiment. The study was approved by the Ethics Committee of the Army General Hospital of Beijing (no. 2017-114).

Cell culture and experimental groups. Glioma specimens were placed in a saline solution after surgical extraction. Primary low-grade tumor cells were obtained after mechanical dissociation and enzymatic digestion, mainly based on a single method (15). Then, the cells were separated into two groups. In the first group, the primary glioma-based cell (PGBC) group, the cells were cultured in serum-free medium with penicillin-streptomycin (1:100), B-27 (1:50), and N2 supplement (1:100; all from Gibco; Thermo Fisher Scientific, Inc.), recombinant human fibroblast growth factor-2 (FGF-2; 20 ng/ml), epidermal growth factor (EGF; 20 ng/ml), and 5 µg/ml insulin (Sigma-Aldrich; Merck KGaA). In the second group, the GSLC group, the cells were cultured under the same conditions, except for the addition of 100 nM CHIR99021 (Stemgent).

Flow cytometric analysis. Flow cytometry was used in order to find a suitable concentration of CHIR99021 for cell survival and to detect the expression level of stem cell markers in the two groups. In brief, after being cultured in various concentrations of CHIR99021 (0, 10⁻¹ M, 10⁻² M, 10⁻³ M, 10⁻⁴ M, 10⁻⁵ M, 10⁻⁶ M) for 48 h, the cells were collected and washed with PBS. The apoptotic cells were detected using an Annexin V-FITC apoptosis detection kit.

After culturing the two groups of cells for 48 h, combinations of monoclonal antibodies against human CD133-FITC (1:200 dilution; cat. no. 566597; BD Biosciences) and Nestin-PE (1:400 dilution; cat. no. 561230; BD Biosciences) were added to the cell suspensions at concentrations recommended by the manufacturer. These cells were then analyzed by FACS (BD Accuri C6 flow cytometry) after being incubated at 4°C in a dark place for 60 min. This assay was performed in triplicate for the mixed cells derived from grade I and II gliomas, and the results were presented as average percentages.

Cell migration assay. Transwell chambers with an 8.0-µm pore (Costar; Corning Incorporated) were used to detect cell migration abilities after the cells were cultured at 37°C for 5 days. Briefly, the cells were seeded into the upper chamber of the Transwell plates (3.0x10⁵ cells/well in 200 µl serum-free medium), while the lower chamber was filled with 500 µl medium containing 10% FBS as a chemoattractant. After incubation for 24 h, the cells remaining on the upper surface of the filter were removed. The cells that had migrated into the lower compartment were fixed with methanol at room temperature (RT) for 20 min and stained with 0.5% crystal violet (0.5 g crystal violet in 100 ml of 20% methanol; cat. no. C0121; Beyotime Institute of Biotechnology) at RT for 15 min. The cells were counted visually in 5 random fields under a light microscope (20X objective lens). In addition, the migrated cells were dissociated, lysed, and quantified at 570 nm using a spectrophotometer.

Transmission electron microscopy. After being cultured in a suitable medium for 7 days, the cells were fixed in 2.5% glutaraldehyde in a 100-mM sodium cacodylate buffer (pH 7.4), dehydrated in a graded ethanol series after OS2 fixation, and embedded into Epon (catalyst). Sections of 60 nm were imaged with a Tecnai Spirit transmission electron microscope (FEI; Thermo Fisher Scientific, Inc.).

Quantitative real-time PCR. To determine the role of CHIR99021 in the PI3K/AKT signaling pathway in GSLCs, the gene expression levels of signal transducer and activator of transcription 3 (STAT3), protein kinase B (AKT), p85, p110, phosphoinositide 3-kinases (PI3K), vascular endothelial growth factor (VEGF), glycogen synthase kinase-3β (GSK-3β) and CD133 were analyzed. Total RNA was extracted from the two groups on day 5 using TRizol® Reagent. M-MLV reverse transcriptase was used for cDNA synthesis. In brief, a mixture containing 2 µg of total RNA, 0.75 µg oligo-dT primer (Tiangen Biotech Co., Ltd.), and nuclease-free water in a total volume of 13.5 µl was heated at 70°C for 5 min and then cooled on ice for another 5 min. The mixture was supplemented with 4 µl M-MLV buffer, 1.25 µl dNTP, 0.5 µl RNAsin, and 0.75 µl M-MLV-RT up to a final volume of 20 µl, followed by incubation at 42°C for 60 min.

The quantitative real-time PCR analysis was performed using the SYBR Green Master Mix Kit (Takara Biotechnology Co., Ltd.). Briefly, each PCR reaction mixture with a total volume of 20 µl, including 10 µl 2X SYBR-Green Master Mix, 1 µl sense and antisense primers (10 µmol/µl), and 1 µl cDNA, was run for 40 cycles, undergoing denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. For relative quantification, 2⁻ΔΔCq was calculated as an indication of the relative gene expression levels of STAT3, AKT, p85, p110, PI3K, VEGF, GSK-3β and CD133 (16). The primer sequences for the PCR amplification of the genes are presented in Table I.

Western blotting. The PGBCs and GSLCs were harvested by mechanical dissociation after being cultured for 5 days. The cells were lysed in ice-cold lysis buffer (RIPA) and phosphatase and protease inhibitors for 30 min. The supernatants were collected after centrifugation and quantified for protein content. BCA was used to test the concentration of each sample, then equal amounts of proteins (10 µg per lane) were electrophoretically fractionated in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes. After the transfer, the membrane was blocked with 5% skim milk and then incubated with the primary antibody overnight at 4°C. The specific antibodies were against...
Table I. Primer sequences for qPCR.

| Primer Name | Forward Sequence | Reverse Sequence |
|-------------|-----------------|-----------------|
| STAT3       | 5'-TCTCCACCAAGTGAAGTGACGC-3' | 5'-GGCAGATTCTCCTCACACCAAGAC-3' |
| AKT         | 5'-TGGTGTAACAGCCGCG-3'  | 5'-TTTGAGACGAGGACACC-3'  |
| p85         | 5'-CCCCAGGAACTCCACATA-3'  | 5'-TAACCATCCAGACCCAC-3'  |
| p110        | 5'-TCCTCAGCTCTGGTGTTG-3'  | 5'-TGCGTTTGAATCTTTGGC-3'  |
| PI3K        | 5'-AGGTAGGTGTGGGCCG-3'  | 5'-CTTGAGTCTCTGGGTG-3'  |
| VEGF        | 5'-CAGAAGTTGGACGAAATG-3'  | 5'-GCAGAAAGAGAAAAAGATG-3'  |
| GSK-3       | 5'-ACTTTCTTGAGGCACC-3'  | 5'-TTTCCTTCTTCTGTGGG-3'  |
| CD133       | 5'-TTACGGCCACCTTACCTC-3'  | 5'-TATTCCACAAAGCAACAA-3'  |
| β-actin     | 5'-AGCGAGCATCCCCCAAGTT-3'  | 5'-GGGCACAGGCTCATCATT-3'  |

AKT, protein kinase B; GSK-3β, glycogen synthase kinase-3β; PI3K, phosphoinositide 3-kinase; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

human STAT3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (1:1,000 dilution for both; product nos. 9139 and 8242, respectively; Cell Signaling Technology, Inc.), GSK-3β, mTOR and AKT (1:500 dilution for all of them; cat. nos. 615002, 610302 and 649002, respectively; BioLegend, Inc.), according to the protocol of the manufacturer. The GSLCs were transfected 24 h after being seeded into a 6-well plate at a density of 2x10^5 cells/well. STAT3 siRNAs (Santa Cruz Biotechnology, Inc.) were transfected with Lipofectamine RNAiMax transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Different concentrations of siRNA (200, 100 and 50 nM) were assessed. The knockdown effect was confirmed using western blotting. In addition, the migration capacity of the cells was detected as aforementioned. Temozolomide (TMZ) treatment. The cytotoxicity of the two groups was determined by culturing cells in various concentrations of TMZ (Sigma-Aldrich; Merck KGaA), ranging from 0 to 1,600 µM. In order to determine the half-maximal inhibitory concentration (IC_{50}), an MTS assay kit was performed according to the instructions after the cells were cultured in different densities of TMZ (75, 150, and 300 µM) for 3 days. Furthermore, the apoptotic cells were detected using an Annexin V-FITC apoptosis detection kit, in which vehicle solvent dimethyl sulfoxide (DMSO) was used as a negative control. Each assay was performed in triplicate, followed by an analysis of the statistical significances.

In vivo study. Thirty female C57BL/6 mice (6-8 weeks old, supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd.) were raised under sterile conditions. The use of animals was approved by the Institutional Animal Care and Use Committee of the Army General Hospital of Beijing (approval no. IACUC20170114-04). The mice were randomly divided into 3 groups: The saline group, the PGBC group, and the GSLC group. Then, the mice were sublethally irradiated with 2.5 Gy from a 137Cs source (2.115 Gy/min) before transplantation. The PGBCs and GSLCs, suspended in a total volume of 3 µl (approximately 1x10^5 cells), were intra-cerebrally infused into the frontal lobe (coordinates: 1.0 mm anterior, 2.5 mm ventral, and 1.8 mm lateral to the midline) using a Hamilton syringe after exposing the bregma. After the surgery, the animals were allowed to recover from the anesthesia and were placed in the cages. The mice were sacrificed with an overdose of pentobarbital (100 mg/kg) 14 days later. The weight of the mice and the survival ratio over that period were recorded. Then, brain tissue was cut into sections of 5 µm by using a cryostat (Leica CM 1850), and hematoxylin and
eosin (H&E) staining was conducted according to the instructions to reveal the cytomorphology of the cells. In addition, IF staining as well as western blotting were performed as in the in vitro procedure to reveal the expression of related proteins.

**Statistical analysis.** Results were expressed as the mean ± standard deviation, unless otherwise indicated. Statistically significant differences between the two groups were determined by a two-tailed Student's t-test or a one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All tests were performed using SPSS 20.0 (IBM Corp.).

**Results**

**CHIR99021 promotes the expression of GSLC markers.** The suitable concentration of CHIR99021 was revealed to be 100 nM after culturing the cells in different concentrations of CHIR99021. With this concentration, the survival rate of the primary low-grade glioma cells was ~90%. Moreover, the apoptotic rate increased by ~10% when the concentration was increased ten times (Fig. 1A). In the PGBCs, the average percentages of CD133 and Nestin were 15.13±4.40% and 61.17±6.26%, respectively. These percentages were 93.83±2.20% and 83.93±1.98%, respectively, in cells cultured under 100 nM CHIR99021, which were significantly higher percentages compared to those in the PGBCs (P<0.01, Fig. 1B).

**Proliferation and migration of the GSLCs.** Cellular cross-links and gliomashere formation were gradually formed by culturing in 100 nM CHIR99021 from day 3 to day 5 (Fig. 2A). In addition, the migration ratios were confirmed by colorimetric assay and revealed as the optical densities (OD) at 570 nm, which were ~15% and 38% in the PGBCs and GSLCs groups, respectively (Fig. 2B). Ultrastructural observation of cellular changes was carried out by using a transmission electron microscopy (TEM). The GSLCs had more filopodia and lamellipodia and a larger endoplasmic reticulum (ER), followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All tests were performed using SPSS 20.0 (IBM Corp.).

**Effect of CHIR99021 on the PI3K/AKT signaling pathway.** The analysis revealed that GSK-3β was expressed in the PGBCs but almost absent in the GSLCs. The PI3K gene was significantly enhanced in the GSLCs compared to the PGBCs (~288-fold). The heterodimers of PI3K, namely p85 and p110, were respectively ~150-fold and 11-fold higher in the GSLCs than in the PGBCs, which was in accordance with the high PI3K activity in the GSLCs.

The AKT and mTOR genes, which are well-characterized key downstream effectors of PI3K, were highly expressed in the GSLCs (Fig. 3A). The expression levels of the related proteins (PI3K, AKT and mTOR) were also enhanced in the GSLCs compared to the PGBCs (Fig. 3B). The expression levels of the STAT3, VEGF, and NF-κB genes, which are involved in cell migration and invasion, were increased ~152-fold, 25-fold, and 50-fold, respectively, in the GSLCs (Fig. 3A). Western blot analysis revealed that the expression of STAT3, p-STAT3, VEGF, and NF-κB was significantly increased in the GSLCs compared to the PGBCs (Fig. 3B).

**Activation of STAT3.** The IF staining of STAT3, AKT, VEGF and NF-κB was positive in the GSLCs (Fig. 4A). These results were in accordance with the changes in gene expression. In addition, the knockdown effect of STAT3 in the GSLCs was also detected by western blotting. In comparison to the control group (untreated GSLCs) and the negative group (without siRNA transfection), siRNA (200 and 100 nM) markedly inhibited the expression of the STAT3 protein in the GSLCs. However, only 200 nM siRNA could effectively decrease the p-STAT3 protein level (Fig. 4B). Further study revealed that 200 nM siRNA significantly decreased the expression of NF-κB and VEGF (Fig. 4C). In the Transwell experiment, 200 nM siRNA significantly inhibited the migration of the GSLCs (Fig. 4D).

**TMZ treatment.** The cytotoxicity of mixed PGBCs and induced GSLCs was determined by adding various concentrations of TMZ into the medium. A significant difference of cytotoxicity between the PGBCs and the GSLCs was observed when TMZ was added in a concentration of 400 µM (0.44±0.03 and 0.70±0.05, respectively, P<0.05; Fig. 5A). Further exploration revealed that the IC50 values of the PGBCs and GSLCs to TMZ were 430±40.0 and 3450±350 µM, respectively (Fig. 5B).

**Annexin V-FITC/PI analysis revealed that in the DMSO-treated group (vehicle control), the cell apoptosis percentages were ~2.00% and 1.80% for the PGBCs and GSLCs, respectively. When the concentration of TMZ increased, the proportion of apoptotic cells was increased in both groups; however, it was more significant in the PGBCs (Fig. 5C). Additionally, early apoptosis (Annexin+/PI-) only 200 nM siRNA could effectively decrease the p-STAT3 protein level (Fig. 4B). Further study revealed that 200 nM siRNA significantly decreased the expression of NF-κB and VEGF (Fig. 4C). In the Transwell experiment, 200 nM siRNA significantly inhibited the migration of the GSLCs (Fig. 4D).

**In vivo validations in mice.** The weight of the mice transplanted with the GSLCs was significantly decreased than the weight of the mice injected with the PGBCs and saline (Fig. 6A). In addition, no dead mice were found in the saline group, 1 death in the PGBC group, and 5 deaths in the GSLC group were identified after the transplantation (Fig. 6B). H&E staining demonstrated that there were numerous malignant cells with large nuclei and active mitosis in the brain sections of the GSLC-transplanted mice (Fig. 6C).

**Discussion**

**Use of CHIR99021.** Most glioma cell experiments are conducted using cell lines such as U87 and U251. With these cell lines, however, numerous clinical gliomas are not fully...
detected. Furthermore, these cell lines may not accurately represent GBM characteristics. The results of the present study revealed that CHIR99021, in a concentration of 100 nM, could enrich primary low-grade glioma cells with CD133-positive GSLC properties. Based on some of the following existing studies, the use of CHIR99021 was decided to establish a GSLC model of clinical glioma tissue. A recent study demonstrated that cordycepin, derived from cultured *Cordyceps militaris*, could induce a decrease in cell viability, a downregulation of...
β-catenin, an increase in apoptosis, and a reduction in TMZ resistance. However, all these effects could be reversed with CHIR99021 (17). Another study reported that CHIR99021 activated the Wnt/β-catenin signaling pathway and then
initiated the differentiation of human embryonic stem cells (hESCs) by inhibiting the degradation of β-catenin (18). Additionally, CHIR99021 was crucial for maintaining the metabolic activity of differentiated rat embryonic stem (ES) cells (19). Furthermore, a tumor cell line was established by culturing cells in a chemically defined N2B27 medium containing CHIR99021. The cell lines revealed some indications of malignancy, such as Oct4 expression and a long-term expansion ability (20).

**Proliferation and invasiveness of the GSLCs.** The present results revealed that the GSLCs had more filopodia and lamellipodia and a larger endoplasmic reticulum. These changes indicated that the proliferation and migration abilities of the GSLCs were enhanced by CHIR99021, which was consistent with the expression of related genes and proteins in the PI3K/AKT signaling pathway. Further exploration demonstrated that the GSLCs significantly expressed invasion-related genes and proteins, including STAT3, VEGF and NF-κB.

CHIR99021 specifically inhibits GSK-3, while AKT inactivates GSK-3 through its Ser9 phosphorylation (21). In the present study, qRT-PCR and western blot analysis were used to demonstrate that AKT was activated by CHIR99021 in the GSLCs. In addition, a similar genetic tendency in the classical PI3K/AKT signaling pathway was revealed in the GSLCs, including a heterodimer of PI3K, consisting of the regulatory subunit p85 and the catalytic subunit p110. Furthermore, the downstream key kinase mTOR in this pathway also exhibited the same trend. The mTOR gene plays an essential role in the activation of STAT3, which is followed by an upregulation of the expression of angiogenesis-promoting factors (VEGF). Both the activation and the upregulation contribute to cancer stem-like cell survival/proliferation (22,23). The NF-κB gene is another downstream transcription factor in the classical PI3K/AKT signaling pathway. It has been implicated in numerous hallmarks of cancer development, including growth-factor-independent proliferation, apoptosis prevention, unlimited replicative potential, and tissue invasion and metastasis (24,25).

**Figure 3.** Expression level of genes and proteins relating to cell proliferation, migration and invasion. (A) The gene expression levels detected by qRT-PCR. (B) The expression levels of corresponding proteins detected by western blotting. *P<0.05 and **P<0.01. AKT, protein kinase B; GSK-3β, glycogen synthase kinase-3β; GSLCs, glioma stem-like cells; mTOR, mammalian target of rapamycin; NF-κB, clear factor kappa-light-chain-enhancer of activated B cells; PGBCs, primary glioma-based cells; PI3K, phosphoinositide 3-kinase; P-STAT3, phospho-STAT3; STAT3, signal transducer and activator of transcription 3; Tp, total protein; VEGF, vascular endothelial growth factor.
Figure 4. Expression of STAT3 and related proteins and siRNA study. (A) IF staining of proteins in GSLCs (scale bar, 10 µm). (B) Knockdown effect of STAT3 in GSLCs by siRNA. (C) The effect of 200 nM siRNA on the expression of STAT3, p-STAT3, NF-κB and VEGF. (D) Transwell assay to detect the migration rates after the siRNA study (scale bar: 200 µm). *P<0.05 and **P<0.01. GSLCs, glioma stem-like cells; IF, immunofluorescence; NF-κB, clear factor kappa-light-chain-enhancer of activated B cells; p-STAT3, phospho-STAT3; siRNA, small interference RNA; STAT3, signal transducer and activator of transcription 3; Tp, total protein; VEGF, vascular endothelial growth factor.
As a signal transducer and activator, STAT3 regulates the expression of target genes involved in the cell cycle, apoptosis, cellular transformation, and tumor angiogenesis (23). In the present study, western blot analysis and IF staining revealed that CHIR99021 induced high expression of STAT3 and phosphorylated (p)-STAT3 proteins. Silencing STAT3 by siRNA also confirmed the effect of CHIR99021, by downregulating related proteins (p-STAT3, VEGF, and NF-κB). These findings indicated that STAT3 may function as an integrator of these cellular signals, controlling the migration and invasiveness of GSLCs, and thus could be a potential therapeutic target in glioma treatment.

Resistance to TMZ and an animal study. Existing publications have suggested that glioma cancer stem cells are closely associated with resistance to radiotherapy and chemotherapy.
Figure 6. *In vivo* experimental validations in mice. (A) Weights of the mice injected with saline, PGBCs and GSLCs. (B) Mortality rates of the mice transplanted with saline, PGBCs and GSLCs. (C) Malignant cells in the brains of mice revealed by H&E staining (scale bar, 200 µm). (D) Co-expression of STAT3 and AKT in the GSLC-transplanted mice (scale bar, 200 µm). (E and F) Expression levels of the STAT3, NF-κB, VEGF, and/or mTOR proteins detected by IF and western blotting (scale bar, 250 µm). *P<0.05 and **P<0.01. AKT, protein kinase B; GSLCs, glioma stem-like cells; IF, immunofluorescence; mTOR, mammalian target of rapamycin; NF-κB, clear factor kappa-light-chain-enhancer of activated B cells; PGBCs, primary glioma-based cells; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.
In addition, resistance to TMZ has been revealed to be closely related to MGMT-mediated DNA repair in glioma cancer stem cells (26-28), however, the underlying mechanism remains to be elucidated. The present research revealed that the high expression of STAT3 in the GSLCs rendered the cells significantly resistant to TMZ, which was not the case in the PGBCs. This may account for the recurrence of gliomas. Whether TMZ resistance is mainly related to the increased expression of STAT3 still requires further study. Irradiated mice instead of nude mice were used in our animal studies in order to mimic the clinical situation, in which the patient is often radiated after surgery but still has clinical relapses. This recurrence may be ascribed to the weak condition of the patient and the high malignant properties of the cancer stem cells. The present animal studies revealed that transplantation of the GSLCs had a tumor-initiating capacity in the irradiated cells. The present animal studies revealed that transplantation of the GSLCs had a tumor-initiating capacity in the irradiated cells, and the high expression of AKT, mTOR, STAT3, VEGF, and NF-κB in the brain sections two weeks later also indicated migration and invasion of the GSLCs in vivo.

Limitations. The present study focused only on the proliferation and invasiveness of GSLCs, without further exploration of other characteristics, such as tumor angiogenesis or inflammation. Furthermore, although the present study provided evidence that CHIR99021 could enhance the expression of malignancy-related biological features, only the well-known PI3K/AKT pathway and STAT3 were explored. Finally, the mechanism of TMZ resistance requires further investigation.

CHIR99021 has the potential to promote the migration and invasiveness of induced GSLCs both in vitro and in vivo, possibly by regulating the PI3K/AKT signaling pathway and activating STAT3. In addition, the drug resistance capability of the GSLCs was also enhanced. In conclusion, CHIR99021 may provide a useful GSLC model, generated from patient-derived low-grade glioma samples, for further study, helping to understand the pathogenesis of therapeutic resistance and to screen for potential drug candidates.

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Availability of data and materials
The datasets in the current study are available from the corresponding author on reasonable request.

Authors' contributions
YY, OB and SSW designed this study. QQW and SSW performed the cell experiments, and YY and YLS performed the animal study and data collection. OB and SSW helped to perform data analysis and interpretation. YY, QQW and YLS wrote the manuscript, and OB and RXC critically revised manuscript for important intellectual content. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
Written informed consent was obtained from the patients, and ethical approval was obtained from the Ethics Committee of the Army General Hospital of Beijing (No. 2017-114). The use of animals was approved by the Institutional Animal Care and Use Committee of the Army General Hospital of Beijing (approval no. IACUC20170114-04).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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