

Hyperbaric oxygen suppresses stemness-associated properties and Nanog and oncostatin M expression, but upregulates β -catenin in orthotopic glioma models

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Abstract

Objective: This study aimed to explore whether initial hyperbaric oxygen treatment affects the stemness of glioma stem cells using an *in vivo* basal ganglia glioma model.

Methods: A basal ganglia glioma rat model was established. Rats were exposed to normal oxygen or hyperbaric oxygen on days 2, 4, 6, 8, 10, and 12. After 16 days of glioma cell inoculation, western blot, ELISA, and flow cytometry were performed to examine stemness-associated properties by examining the expression of CD133, A2B5, Nanog, oncostatin M, β -catenin, Oct-3/4, Sox2, and Nestin.

Results: Initial hyperbaric oxygen treatment began to affect glioma stemness-associated properties. The proportion of CD133⁺A2B5⁺ cells was significantly reduced after initial hyperbaric oxygen treatment. Additionally, the expression of stemness-related genes such as Nanog and oncostatin M was reduced, while TGF- β and β -catenin were increased.

Conclusions: Initial hyperbaric oxygen treatment not only alters the hypoxic microenvironment but also affects the stemness-associated properties of cancer stem cells.

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Introduction

Gliomas account for 30% of all brain and central nervous system (CNS) tumors, representing 80% of all malignant brain tumors.¹ In China, malignant glioma is the most common malignant brain tumor with a median survival time of only 12 to 14 months.² Currently, the standard treatment for malignant gliomas is surgical resection, followed by adjuvant radiotherapy and/or chemotherapy such as the second-generation alkylating agent temozolomide (TMZ). Retrospective analyses have shown that multimodality therapy with repeated surgical resection, radiotherapy and chemotherapy have better therapeutic efficacy than chemotherapy alone.³ Despite multimodality therapy having better effects, the prognosis of these glioma patients remains poor owing to high recurrence rates. The poor outcomes for these glioma patients are mainly due to recurrence and resistance to radiotherapy and chemotherapy.² In addition to the limitation of drug delivery across the blood–brain barrier, the poor diagnosis suggests the existence of multiple resistance mechanisms, such as resistance to DNA damage or apoptotic agents and the effect of a hypoxic microenvironment.

Tumor hypoxia is one characteristic of locally advanced malignant solid tumors including glioma. During tumor progression, tumor hypoxia is caused by an imbalance between the supply and consumption of oxygen. The oxygen-sensitive α -subunit of hypoxia-inducible factor-1 (HIF-1 α) regulates the sensitivity and resistance of glioma cells in different oxygen

concentrations.⁴ Glioma cells were identified as tumor propagators and classified as malignant, which suggests the presence of cancer stem cells (CSCs) with highly aggressive, self-renewal ability properties that can be phenotyped by cell markers. The cytoskeletal protein (Nestin) and pluripotency transcription factors (Sox2, Nanog, and Oct3/4) serve as neural stem biomarkers to identify glioma stem cells.⁵ Meanwhile, oncostatin M (OSM) is a cytokine that belongs to the interleukin-6 (IL-6) family and functions in cell migration and invasion.⁶ Treating cancer cells with OSM leads to increased stemness of CSCs (increased SOX2 expression) and promotes progression (phenotypic changes).⁶ CD133 (also known as prominin-1) is a cancer stem cell marker that illustrated the initiation function of brain cancer and is associated with the behaviors of cancer stem cells.⁷ A2B5⁺ is characterized as a glial progenitor marker and has been demonstrated to play an important role in the initiation and maintenance of cancer stem cells.⁸ Combining these CSCs markers, displays the level of stemness with regard to cell proliferation and regeneration.⁹ According to the epigenetic plasticity properties of tumor cells, we can distinguish CSCs from non-CSCs and then provide potential strategies for cancer therapy.¹⁰ Additionally, HIF-1 α plays crucial roles in tumor angiogenesis, migration, and chemoresistance¹¹ as well as in the transcriptional regulation of pluripotency transcription factors, such as Oct-3/4, Nanog, and Sox2.¹² Another substantial impact on glioma resistance is

the presence of glioma stem cells (GSCs), which exhibit the characteristics of self-renewal and resistance to radiotherapy and chemotherapy, including TMZ. GSCs are undifferentiated cells that prefer a hypoxic microenvironment and may represent a novel therapeutic target for chemo-radiotherapy-resistant malignant brain tumors. Finally, hypoxia was reported to promote the stemness of GSCs, resulting in high resistance to chemotherapy and radiotherapy.^{13,14}

Hyperbaric oxygen (HBO) therapy is the administration of 100% oxygen under elevated atmospheric pressure. Currently, HBO is widely used as an adjunct treatment for various tumors. A systemic review found that HBO treatment before irradiation therapy improved survival rates, progression-free survival, time to progression, and response rates.¹⁵ The combination of HBO and TMZ synergistically enhanced apoptosis in the glioma cell line U521 by altering the expression of VEGF and multi-drug resistance-associated protein-1.¹⁶ Additionally, the synergistic effect of this combination therapy was confirmed in a C6 rat glioma model.¹⁷ Furthermore, HBO slows glioma growth and induces apoptosis in a model of transplanted glioma in nude rats.¹⁸ Nevertheless, the results from multiple studies exploring these associations remain inconsistent and contradictory. In glioma cells, hypoxia was found to upregulate the expression of stem cell markers, such as CD133, A2B5, Oct-4, and Sox2^{19,20} but downregulate the expression of differentiation markers, such as glial fibrillary acidic protein (GFAP).¹¹ This implies that hypoxia may induce the conversion of glioma cells into cells consistent with tumorigenic GSCs. Moreover, HIF-1 α can upregulate the expression of Nanog,¹² a pluripotency transcription factor that contributes to reprogramming somatic cells into an embryonic stem cell-like state. HBO treatment also increases

osteogenic differentiation of bone marrow stromal cells by regulating Wnt3a secretion and signaling.²¹ Interestingly, several studies have shown that HBO treatment promotes neural stem cell (NSC) proliferation,^{21–25} which may lead to unintended outcomes in glioma patients with HBO treatment.

The inconsistent and contradictory studies may relate to the timing of HBO treatment. Additionally, the impact of HBO on GSCs remains unclear; therefore, it is important to determine whether initial HBO treatment affects the stemness-associated properties in glioma. Thus, this study aimed to explore the impact of initial HBO treatment on the stemness of GSCs using an *in vivo* basal ganglia glioma model. Here, we demonstrated for the first time that initial HBO treatment affects the stemness-associated properties of glioma. Importantly we found that initial HBO treatment downregulated Nanog and OSM expression but upregulated β -catenin.

Materials and methods

Establishment of an in vivo basal ganglia glioma model

Six 12-week-old male Sprague Dawley (SD) rats, weighing 250 to 280 g, were purchased from the Experimental Animal Center of Fudan University and housed in a pathogen-free environment. Rats were kept in standard animal facilities (22°C; 55% relative humidity) and maintained under a 12:12-hour light:dark cycle with water and food *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of Fudan University.

Rats were anesthetized using 10% chloral hydrate (3.6 mL/kg) via intraperitoneal injection. A midline incision was made into the head of each animal using a stereotactic instrument (AlcBio Co., Ltd., Shanghai, China) to expose the coronal and sagittal sutures. Next, a 1-mm hole was drilled

into the right coronal suture 3 mm from the midline. Rat C6 glioma cells (Cell Bank of Chinese Academy of Science, Shanghai, China) were prepared and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamax-1 and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Subsequently, rat C6 glioma cells ($1 \times 10^6/10 \mu\text{L}$) were injected using a microinjector (Gauge Industry and Trade Co., Ltd., Shanghai, China) at a depth of 6 mm along the bone edge. The microinjector was then withdrawn approximately 1 mm, and the cells were injected into the right caudate nucleus of each rat at a rate of $1 \mu\text{L}/\text{minute}$ using a micro-infusion pump (AlcBio Co., Ltd.). After the cells were injected, the microinjector was kept in the brain for 5 minutes and then slowly removed. Bone wax (Johnson & Johnson, New Brunswick, NJ, USA) was used to seal the hole in the skull, and the wounds were sutured. After surgery, all rats received 5% glucose in normal saline (2 mL) via intraperitoneal injection.

HBO treatment

After glioma cells were inoculated, three SD rats were exposed to 100% oxygen at 3 atm for 1 hour in an animal hyperbaric chamber (DWC150/300; Yangyuan Hyperbaric Chamber Co., Ltd., Shanghai, China) on days 2, 4, 6, 8, 10, and 12. After 1 hour of HBO treatment, the pressure was decompressed to 1 atm within 15 minutes. In contrast, tumor-bearing animals in the control group were exposed to normal atmospheric oxygen for 1 hour in an animal hyperbaric chamber. The animals were then sacrificed using CO_2 gas asphyxiation at 16 days after inoculation, and glioma tissues were harvested from the right caudate nucleus.

Examination of CD133 and A2B5 expression by flow cytometry

Surface expression of the CSC markers CD133 and A2B5 were examined and quantified by flow cytometry analysis. After glioma tissue samples were harvested, single cell suspensions were prepared by cutting the tissue into small pieces. The minced tissue was trypsinized in 3 to 5 mL of pre-warmed 0.05% trypsin-EDTA for 10 to 15 minutes in a 37°C water bath. An equal volume of soybean trypsin inhibitor in DMEM medium supplemented with 10% FBS was added at the end of the incubation period. After centrifugation at $220 \times g$ for 5 minutes, the isolated cells were resuspended in 1 mL of sterile medium. The cell surface markers CD133 and A2B5 were stained by fluorescein isothiocyanate (FITC)-conjugated anti-CD133 (Abcam, Cambridge, UK) antibodies and allophycocyanin (APC)-conjugated anti-A2B5 (Miltenyi Biotec, Bergisch Gladbach, Germany) antibodies. Subsequently, stained cells were subjected to flow cytometry analysis to determine and quantify the surface expression CD133 and A2B5. For negative control, isotype control IgG conjugated to Alexafluor 488 antibodies (Abcam, Cambridge, UK) were used.

Nuclear and cytosolic fractionation

Cytoplasmic and nuclear lysates were isolated from 40 mg of tissue using the NEPER Nuclear and Cytoplasmic Extraction Reagents (78833; Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After fractionation, the samples were kept on ice during the whole process, and extracted samples were stored at -80°C until further use.

Western blot analysis

Western blot analysis was used to determine protein levels of Oct-3/4, Nanog, Sox2, Oct-

3/4, Nestin, β -catenin, Nrf-2, β -actin, and TBP in glioma cells that were isolated from tumors of the control and HBO groups. Briefly, glioma tissues were snap-frozen in liquid nitrogen and stored at -80°C for later use or kept on ice for immediate homogenization. For approximately 5 mg tissue, 300 μL of ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) was added and homogenized using an electric homogenizer. This homogenization process was repeated twice with 300 μL of lysis buffer and then maintained under constant agitation on an orbital shaker for 2 hours at 4°C , followed by centrifugation for 20 minutes at $16,000 \times g$ at 4°C . Next, 30 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST) for 1 hour at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: Oct-3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Nanog (Abcam), Nestin (Abcam), Sox2 (Abcam), Nrf-2 (Abcam), TBP (Abcam), and β -actin (Abcam). After washing twice with TBST, the membranes were incubated with a peroxidase-conjugated secondary antibody for 1 hour at room temperature, then blots were developed using an enhanced chemiluminescence kit (Amersham, GE Healthcare, Little Chalfont, UK).

Determining the concentration of TGF- β and OSM by ELISA

Approximately 5 mg of fresh tumor tissue was rapidly homogenized using an electric homogenizer in 300 μL of ice-cold lysis buffer as previously described.²⁶ After centrifugation at $5000 \times g$ for 15 minutes at

4°C , the supernatant was aliquoted and stored at -20°C (analysis was performed within 1 month) or at 2 to 8°C (analysis was performed within 24 hours). The concentration of TGF- β and OSM were analyzed using the Rat TGF β 1 ELISA Kit (Abcam, ab119558) and Rat OSM ELISA Kit (LS-F5508; LifeSpan BioSciences, Seattle, WA, USA), respectively according to the manufacturers' instructions.

Statistical analysis

Statistical data are shown as mean \pm standard deviation (SD) for control and HBO groups and were compared using two-sample t-tests. All statistical assessments were two-tailed, and p -values < 0.05 were considered significantly different. Statistical analyses were performed using SPSS 17.0 statistics software (SPSS Inc, Chicago, IL, USA).

Results

HBO treatment attenuated glioma stemness-associated properties in vivo

To investigate whether HBO treatment affected the stemness of glioma, an *in vivo* basal ganglia glioma rat model was established. Glioma-bearing rats were then treated with HBO (HBO group) or normal atmospheric oxygen (control group) for 1 hour, and glioma tissues were harvested and analyzed. HBO treatment slightly reduced glioma tumor weights after 16 days of inoculation (Figure 1). To further understand whether initial HBO treatment affected the stemness of glioma, the surface expression of the CSC markers CD133 and A2B5 were determined and analyzed by flow cytometry. The percentage of A2B5⁺ or CD133⁺ cells were not significantly changed after HBO treatment (Figure 2a). It was worth noting that the percentage of A2B5⁺ CD133⁺ glioma cells were

significantly reduced (Figure 2b; $p = 0.015$), suggesting HBO treatment might attenuate the stemness properties of glioma.

HBO treatment downregulated Nanog and OSM expression and upregulated TGF- β

To further understand whether HBO affected glioma stemness by regulating stem

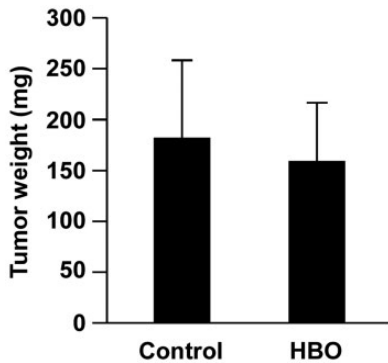


Figure 1. Effect of initial HBO treatment on glioma tumor weight. After glioma inoculation, rats were exposed to 100% oxygen at 3 atm for 1 hour on days 2, 4, 6, 8, 10, and 12. To examine the initial effect of HBO in glioma, animals were sacrificed on day 16, and tumor weights were measured. HBO, hyperbaric oxygen.

cell-related transcriptional factors, western blot analysis was performed to examine the expression of Nanog, Sox2, Oct-3/4, and Nestin (Figure 3a). Notably, HBO treatment significantly attenuated the expression of Nanog (Figure 3b; $p = 0.039$). However, levels of Sox2, Oct-3/4, and Nestin were not significantly changed between control and HBO groups. As TGF- β and OSM play crucial roles in the stemness of glioma cells, we next examined whether HBO treatment affected TGF- β and OSM expression. As shown in Figure 3c, HBO treatment significantly reduced OSM levels compared with control (111.81 ± 10.08 vs. 188.44 ± 6.72 ; $p < 0.001$). Additionally, TGF- β levels were increased in glioma tumor tissues after HBO treatment (Figure 3d; 13043 ± 1202.8 vs. 9820 ± 530.16 ; $p = 0.013$).

HBO increased β -catenin expression in a rat basal ganglia glioma model

Nuclear factor-erythroid 2-related factor 2 (Nrf-2) is involved in the quiescence, survival, and stress resistance of cancer stem cells.²⁷ Additionally, β -catenin signaling is involved in the stemness and early differentiation of murine stem cells.²⁸ Therefore,

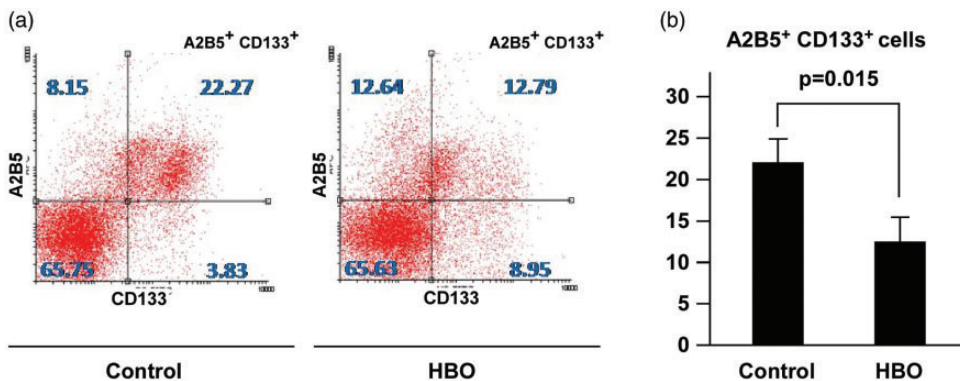


Figure 2. Initial HBO treatment attenuated the proportion of A2B5⁺ CD133⁺ glioma cells. (a) The surface cancer stem cell markers A2B5 and CD133 were determined by flow cytometry. (b) The proportions of A2B5⁺ CD133⁺ cells were quantified. Data represent mean \pm SD. Differences were found to be statistically significant at * $P < 0.05$. HBO, hyperbaric oxygen.

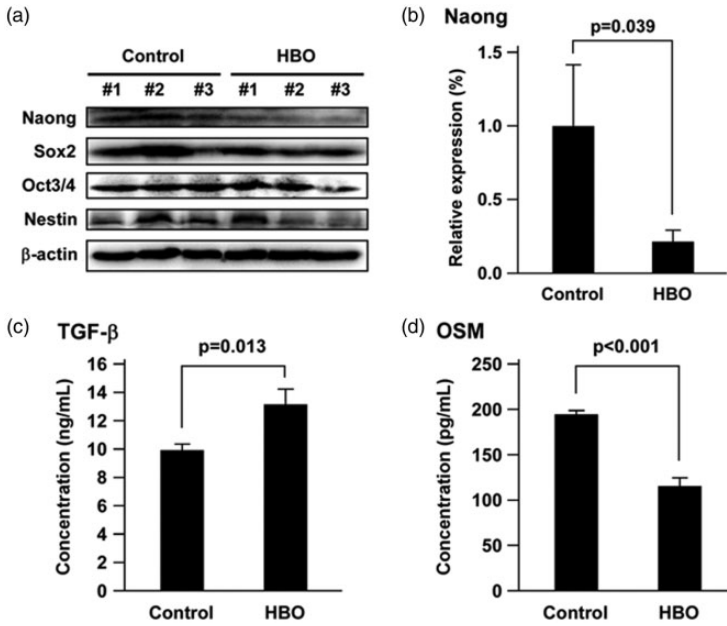


Figure 3. Initial HBO treatment upregulated Nanog expression. (a) Glioma tissues isolated from control or HBO-treated rats were homogenized, and the extracted protein were subjected to western blot analysis to determine the expression of Nanog, Sox2, Oct3/4, and Nestin. (b) Graph showing the quantification of relative signal intensities of Nanog. Data represent mean \pm SD. Differences were considered statistically significant at * $P < 0.05$. HBO, hyperbaric oxygen.

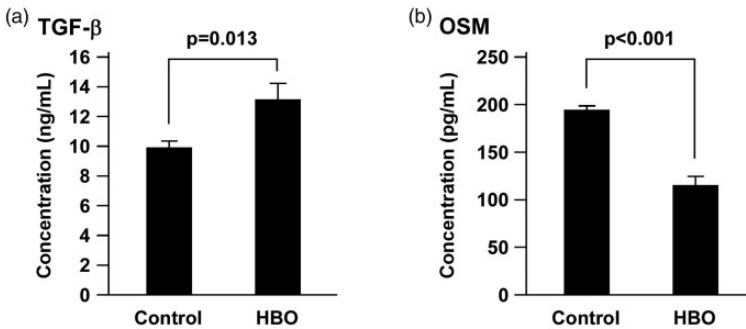


Figure 4. Initial HBO treatment of the basal ganglia glioma model upregulated TGF- β and downregulated OSM *in vivo*. Protein concentrations of (a) TGF- β and (b) OSM were analyzed by ELISA. Data represent mean \pm SD. Differences were considered statistically significant at * $P < 0.05$. HBO, hyperbaric oxygen; OSM, oncostatin M.

we investigated whether HBO affected β -catenin expression. The impact of HBO on β -catenin and Nrf-2 was evaluated by assessing cytosolic (Figure 4a) nuclear

(Figure 4b) protein levels by western blot analysis. As shown in Figure 5, HBO treatment upregulated β -catenin expression in both cytosolic and nuclear fractions.

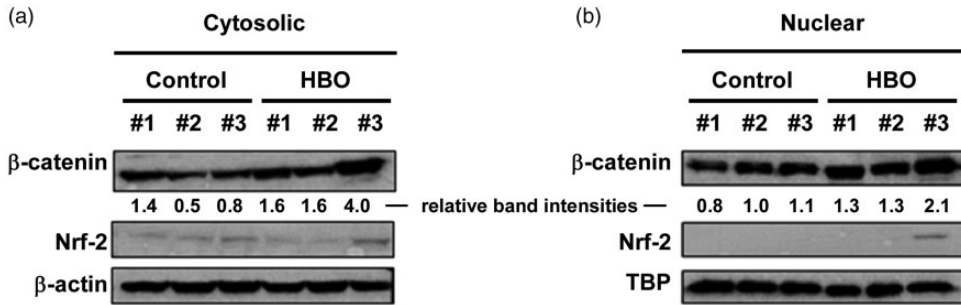


Figure 5. Initial HBO treatment increased β -catenin expression in both nuclear and cytosolic fractions in the rat basal ganglia glioma model. Glioma tissues isolated from control or HBO-treated rats were homogenized, and extracted proteins were subjected to nuclear and cytosolic fractionation. Western blotting was used to determine the expression of Nrf-2 and β -catenin. β -actin was used as an internal control for cytosolic proteins. TBP was used as an internal control for nuclear proteins. Relative band intensities of β -catenin are shown. HBO, hyperbaric oxygen.

However, there were no significant differences in Nrf-2 levels. Taken together, HBO treatment upregulated the expression of β -catenin in our rat basal ganglia glioma model.

Discussion

The current treatment for malignant glioma is surgical resection followed by adjuvant chemo-radiotherapy. During glioma progression or after therapeutic treatment, the necrotic area of glioblastoma develops into a hypoxia region, which promotes tumor development, angiogenesis, and recurrence. Recently, it has been reported that combining adjuvant HBO with therapeutic compounds or radiotherapy has synergistic effects on glioma treatment.^{16,17,29} However, several studies have demonstrated a different observation: that adjuvant HBO promotes glioma cell growth, neural stem cell proliferation, and osteogenic differentiation of bone marrow stromal cells.^{21,30,31} Thus, we investigated the impact of initial HBO on the stemness of glioma cells using an *in vivo* basal ganglia model. The results of our *in vivo* study demonstrated that HBO treatment attenuated glioma stemness-associated properties. HBO treatment

began to affect the glioma tumor weight after 16 days of inoculation. Importantly, the glioma stem cell markers A2B5 and CD133 were dramatically reduced in HBO-treated glioma tissues. Furthermore, HBO treatment downregulated Nanog and OSM expression, which are both involved in the self-renewal and pluripotency of stem cells. β -catenin expression, which is involved in the stemness and early differentiation of murine stem cells, was increased after HBO treatment. Taken together, these data suggest that *in vivo* HBO treatment can affect glioma stemness-associated properties and suppress glioblastoma stemness in an orthotopic glioma model.

It is interesting to observe that HBO treatment slightly reduced the glioma tumor weight in our basal ganglia rat model. The slight decrease of tumor weight may be due to the effect of HBO treatment being analyzed 16 days after tumor inoculation. This also indicated that short-term and repeated HBO treatment began to influence tumor progression. Thus, it will be worthwhile to perform a long-term investigation of the effect of HBO on glioma progression.

As glioblastoma multiforme are aggressive malignant brain tumors and GSCs are involved in neoplastic recurrence and

patient survival, the CSC markers CD133 and A2B5 were analyzed by flow cytometry to understand the characteristics of GSCs in response to HBO. In glioma patients, increased CD133 expression was associated with poor prognosis.³² Bao et al.³³ demonstrated that CD133-positive glioma tumor cells conferred resistance to radiotherapy and may be associated with tumor recurrence. Additionally, cancer stem cell properties were observed in A2B5⁺ human glioblastoma.^{7,34} The expression of A2B5 in human glioblastomas was associated with chemoresistance.³⁵ Thus, glioma cells with both A2B5 and CD133 expression are considered GSCs. In contrast, A2B5⁺, CD133⁺, and A2B5⁻CD133⁻ cells are considered glioma cells. Interestingly, our study found that the proportion of A2B5⁺CD133⁺ cells was dramatically reduced after HBO treatment (Figure 2). However, the proportion of A2B5⁺ or CD133⁺ cells were not reduced in the HBO-treated group. This implied that glioma stem cells (A2B5⁺CD133⁺ cells) were more sensitive to HBO treatment than glioma cells (A2B5⁺ or CD133⁺ cells).

We hypothesized that HBO treatment would force GSCs to escape from a quiescent state and differentiate into a glioma-associated phenotype. In other words, HBO treatment may decrease the stemness of GSCs or reduce the number of GSCs, thereby increasing the sensitivity of radiotherapy and chemotherapy.

It has been reported that hypoxia increases the self-renewal capacity of GSCs as well as non-stem cells.³⁶ Hypoxia upregulated the expression of Oct-4, Nanog, and c-myc; however, it is unclear whether short-term and repeated HBO treatment alters the stemness of GSCs. Thus, rats were analyzed after 4 days of HBO treatment. Interestingly, we observed that HBO treatment reduced Nanog expression, while levels of Sox2, Oct-3/4, and Nestin were not significantly changed.

This indicated that the expression of Nanog in glioma began to decrease after 4 days of HBO treatment, and the decreased expression of Nanog in HBO-treated glioma tissues suggested that HBO began to suppress glioblastoma stemness. Thus, long-term observations in future studies will be required to examine the expression of other stemness factors and their association with phenotypic changes, such as glioma cell proliferation and invasion.

In this study, HBO treatment increased TGF- β levels, similar to a previous study conducted in a rat model of traumatic brain injury.³⁷ TGF- β -responsive SMADs can bind to the proximal promoter of Nanog, thereby regulating its expression in human embryonic stem cells.³⁸ Inhibition of TGF- β signaling by small-molecule inhibitor induced Nanog expression.³⁹ In contrast, hypoxia-induced Nanog expression directly binds to the proximal promoter of TGF- β 1,⁴⁰ indicating that the regulatory mechanisms between TGF- β and Nanog are different under different conditions. Thus, further studies are necessary to fully elucidate the role of TGF- β in HBO-treated gliomas and the relationship with Nanog expression.

OSM regulates the proliferation and differentiation of glioma cells.⁴¹ In human proximal tubule cells, OSM attenuated TGF- β -induced expression of FoXC2, CTGF, TNC, TSP-1, and SPARC,^{41,42} implying that OSM negatively regulates TGF- β expression. In this study, initial HBO treatment significantly reduced OSM expression (Figure 4b, $p < 0.001$), inhibited stemness-associated properties (Figure 2 and 3) and began to reduce tumor weights (Figure 1). Consistently, we found that HBO treatment upregulated TGF- β (Figure 4a). Thus, it is likely that initial HBO treatment upregulated TGF- β expression by inhibiting OSM in our orthotopic glioma model, which warrants further investigation. Conversely, several

studies^{43–45} have reported a positive role for TGF- β in promoting or sustaining stemness in various types of malignant cancer tumor-initiating cells, including glioma.^{46,47} You et al.⁴³ demonstrated that TGF- β upregulated CD133 expression, and thus increased tumor initiation in Huh7 hepatocellular carcinoma cells. However, our results found that initial HBO treatment did upregulate TGF- β expression, but the proportion of CD133⁺ cells did not change significantly, suggesting that another unknown mechanism affected TGF- β -induced CD133 upregulation. It may be that initial HBO treatment had not begun to upregulate CD133 expression at the time of our analysis. CD133 expression may be increased after longer HBO treatment. It is worthwhile to investigate whether and how HBO-inhibited OSM expression upregulated TGF- β expression in the orthotopic glioma model. Additionally, future studies will be necessary to determine if longer HBO treatment increases CD133 expression and to elucidate the underlying regulatory mechanism.

In conclusion, this study emphasized that initial HBO treatment attenuated GSC stemness. Initial HBO treatment began to attenuate glioma stemness-associated properties, including a reduced proportion of A2B5⁺CD133⁺ cells and decreased Nanog and OSM expression. Thus, initial HBO treatment reduces glioma stemness and increases sensitivity to therapeutic compounds and/or radiotherapy.

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None.

Author contributions

All authors were involved in the experimental design, data collection and analysis, and manuscript preparation. Zhiyong Qin is the guarantor of the integrity of the entire study, and is responsible for the entire study concept, study design, and literature research. Kun Song, Junrui Chen and Jianbo Ding carried out experiments and

collected the data as well as data analysis and manuscript preparation. Hongzhi Xu and Hao Xu were responsible for manuscript editing and review.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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References

1. Goodenberger ML and Jenkins RB. Genetics of adult glioma. *Cancer Genet* 2012; 205: 613–621.
2. Chang L, Su J, Jia X, et al. Treating malignant glioma in Chinese patients: update on temozolomide. *Onco Targets Ther* 2014; 7: 235–244.
3. Scorsetti M, Navarria P, Pessina F, et al. Multimodality therapy approaches, local and systemic treatment, compared with chemotherapy alone in recurrent glioblastoma. *BMC Cancer* 2015; 15: 486.
4. Wang P, Wan W, Xiong S, et al. HIF1 α regulates glioma chemosensitivity through the transformation between differentiation and dedifferentiation in various oxygen levels. *Sci Rep* 2017; 7: 7965.
5. Jin X, Jin X, Jung JE, et al. Cell surface Nestin is a biomarker for glioma stem cells. *Biochem Biophys Res Commun* 2013; 433: 496–501.
6. West NR, Murray JI and Watson PH. Oncostatin-M promotes phenotypic changes associated with mesenchymal and stem cell-like differentiation in breast cancer. *Oncogene* 2014; 33: 1485–1494.
7. Tchoghandjian A, Baeza N, Colin C, et al. A2B5 cells from human glioblastoma have cancer stem cell properties. *Brain Pathol* 2010; 20: 211–221.

8. Ogden AT, Waziri AE, Lochhead RA, et al. Identification of A2B5⁺CD133⁻ tumor-initiating cells in adult human gliomas. *Neurosurgery* 2008; 62: 505–514; discussion 14–5.
9. Aponte PM and Caicedo A. Stemness in cancer: stem cells, cancer stem cells, and their microenvironment. *Stem Cells Int* 2017; 2017: 5619472.
10. Safa AR, Saadatzadeh MR, Cohen-Gadol AA, et al. Glioblastoma stem cells (GSCs) epigenetic plasticity and interconversion between differentiated non-GSCs and GSCs. *Genes Dis* 2015; 2: 152–163.
11. Soeda A, Park M, Lee D, et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. *Oncogene* 2009; 28: 3949–3959.
12. Mimeault M and Batra SK. Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells. *J Cell Mol Med* 2013; 17: 30–54.
13. Persano L, Pistollato F, Rampazzo E, et al. BMP2 sensitizes glioblastoma stem-like cells to Temozolomide by affecting HIF-1alpha stability and MGMT expression. *Cell Death Dis* 2012; 3: e412.
14. Semenza GL. Dynamic regulation of stem cell specification and maintenance by hypoxia-inducible factors. *Mol Aspects Med* 2016; 47-48: 15–23.
15. Chen JR, Xu HZ, Ding JB, et al. Radiotherapy after hyperbaric oxygenation in malignant gliomas. *Curr Med Res Opin* 2015; 31: 1977–1984.
16. Lu XY, Cao K, Li QY, et al. The synergistic therapeutic effect of temozolomide and hyperbaric oxygen on glioma U251 cell lines is accompanied by alterations in vascular endothelial growth factor and multidrug resistance-associated protein-1 levels. *J Int Med Res* 2012; 40: 995–1004.
17. Dagistan Y, Karaca I, Bozkurt ER, et al. Combination hyperbaric oxygen and temozolomide therapy in C6 rat glioma model. *Acta Cir Bras* 2012; 27: 383–387.
18. Stuhr LE, Raa A, Oyan AM, et al. Hyperoxia retards growth and induces apoptosis, changes in vascular density and gene expression in transplanted gliomas in nude rats. *J Neurooncol* 2007; 85: 191–202.
19. Li Z, Bao S, Wu Q, et al. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. *Cancer Cell* 2009; 15: 501–513.
20. Seidel S, Garvalov BK, Wirta V, et al. A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain* 2010; 133: 983–995.
21. Lin SS, Ueng SW, Niu CC, et al. Hyperbaric oxygen promotes osteogenic differentiation of bone marrow stromal cells by regulating Wnt3a/beta-catenin signaling—an in vitro and in vivo study. *Stem Cell Res* 2014; 12: 260–274.
22. Meng XE, Zhang Y, Li N, et al. Effects of hyperbaric oxygen on the Nrf2 signaling pathway in secondary injury following traumatic brain injury. *Genet Mol Res* 2016; 15: gmr.15016933.
23. Zhu J, Wang H, Sun Q, et al. Nrf2 is required to maintain the self-renewal of glioma stem cells. *BMC Cancer* 2013; 13: 380.
24. Yang YJ, Wang XL, Yu XH, et al. Hyperbaric oxygen induces endogenous neural stem cells to proliferate and differentiate in hypoxic-ischemic brain damage in neonatal rats. *Undersea Hyperb Med* 2008; 35: 113–129.
25. Zhang XY, Yang YJ, Xu PR, et al. The role of beta-catenin signaling pathway on proliferation of rats neural stem cells after hyperbaric oxygen therapy in vitro. *Cell Mol Neurobiol* 2011; 31: 101–109.
26. Rabuffetti M, Sciorati C, Tarozzo G, et al. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 2000; 20: 4398–4404.
27. Ryoo IG, Lee SH and Kwak MK. Redox modulating NRF2: a potential mediator of cancer stem cell resistance. *Oxid Med Cell Longev* 2016; 2016: 2428153.
28. Anton R, Kestler HA and Kuhl M. Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells. *FEBS Lett* 2007; 581: 5247–5254.

29. Kohshi K, Yamamoto H, Nakahara A, et al. Fractionated stereotactic radiotherapy using gamma unit after hyperbaric oxygenation on recurrent high-grade gliomas. *J Neurooncol* 2007; 82: 297–303.
30. Yang Y, Wei H, Zhou X, et al. Hyperbaric oxygen promotes neural stem cell proliferation by activating vascular endothelial growth factor/extracellular signal-regulated kinase signaling after traumatic brain injury. *Neuroreport* 2017; 28: 1232–1238.
31. Wang YG, Zhan YP, Pan SY, et al. Hyperbaric oxygen promotes malignant glioma cell growth and inhibits cell apoptosis. *Oncol Lett* 2015; 10: 189–195.
32. Rebetz J, Tian D, Persson A, et al. Glial progenitor-like phenotype in low-grade glioma and enhanced CD133-expression and neuronal lineage differentiation potential in high-grade glioma. *PLoS One* 2008; 3: e1936.
33. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; 444: 756–760.
34. Lathia JD, Mack SC, Mulkearns-Hubert EE, et al. Cancer stem cells in glioblastoma. *Genes Dev* 2015; 29: 1203–1217.
35. Balik V, Mirossay P, Bohus P, et al. Flow cytometry analysis of neural differentiation markers expression in human glioblastomas may predict their response to chemotherapy. *Cell Mol Neurobiol* 2009; 29: 845–858.
36. Heddleston JM, Li Z, McLendon RE, et al. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. *Cell Cycle* 2009; 8: 3274–3284.
37. Wee HY, Lim SW, Chio CC, et al. Hyperbaric oxygen effects on neuronal apoptosis associations in a traumatic brain injury rat model. *J Surg Res* 2015; 197: 382–389.
38. Xu RH, Sampsel-Barron TL, Gu F, et al. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell* 2008; 3: 196–206.
39. Ichida JK, Blanchard J, Lam K, et al. A small-molecule inhibitor of TGF-beta signaling replaces Sox2 in reprogramming by inducing Nanog. *Cell Stem Cell* 2009; 5: 491–503.
40. Hasmim M, Noman MZ, Messai Y, et al. Cutting edge: hypoxia-induced Nanog favors the intratumoral infiltration of regulatory T cells and macrophages via direct regulation of TGF-beta1. *J Immunol* 2013; 191: 5802–5806.
41. Halfter H, Lotfi R, Westermann R, et al. Inhibition of growth and induction of differentiation of glioma cell lines by oncostatin M (OSM). *Growth Factors* 1998; 15: 135–147.
42. Sarkozi R, Hauser C, Noppert SJ, et al. Oncostatin M is a novel inhibitor of TGF-beta1-induced matricellular protein expression. *Am J Physiol Renal Physiol* 2011; 301: F1014–F1025.
43. You H, Ding W and Rountree CB. Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. *Hepatology* 2010; 51: 1635–1644.
44. Bruna A, Greenwood W, Le Quesne J, et al. TGFbeta induces the formation of tumour-initiating cells in claudinlow breast cancer. *Nat Commun* 2012; 3: 1055.
45. Naka K, Hoshii T, Muraguchi T, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 2010; 463: 676–680.
46. Ikushima H, Todo T, Ino Y, et al. Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. *Cell Stem Cell* 2009; 5: 504–514.
47. Penuelas S, Anido J, Prieto-Sanchez RM, et al. TGF-beta increases glioma-initiating cell self-renewal through the induction of LIF in human glioblastoma. *Cancer Cell* 2009; 15: 315–327.