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GLI1 is involved in HIF-1α-induced migration, invasion, and epithelial-mesenchymal transition in glioma cells

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Abstract

Introduction. Glioma is characterized by hypoxia that activates the hypoxia inducible factor (HIF) pathway and controls a myriad of genes that drive cancer progression. HIF-1α promotes GLI1 transferring to the nucleus by activating the hedgehog pathway under hypoxic conditions. However, their mechanisms in glioma cells under hypoxia remain unknown.

Material and methods. Human glioma cell lines (LN229 and LN18) were transfected with HIF-1α or GLI1-specific short hairpin RNAs (shRNAs) and cultured under normoxic or hypoxic conditions. The protein levels of HIF-1α, GLI1, and epithelial-mesenchymal transition (EMT) markers including E-cadherin and vimentin were measured by Western blot analysis. RT-qPCR analysis was performed for the detection of HIF-1α and GLI1 mRNA expression. Cell migratory and invasive capacities were evaluated by wound healing and Transwell assays, respectively.

Results. Hypoxia blocked the breakdown of the HIF-1α protein and upregulated GLI1 expression in glioma cells. Downregulation of HIF-1α expression inhibited hypoxia-induced cell migration and invasion, as well as reversed the effects of hypoxia on GLI1, E-cadherin, and vimentin expression in LN229 and LN18 cells. Depletion of GLI1 inhibited glioma cell migration and invasion induced by hypoxia. Silenced GLI1 did not affect HIF-1α expression but completely offset hypoxia-regulated expression of E-cadherin and vimentin in glioma cells.

Conclusions. GLI1 is involved in HIF-1α-induced migration, invasion, and EMT in glioma cells, thus revealing a novel molecular mechanism for glioma research. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 2, 156–166)*

Keywords: GLI1; glioma; LN229 and LN18 cells; HIF-1α; epithelial-mesenchymal transition; hypoxia

Introduction

Glioma is one of the most common primary intracranial tumors, which represents 81% of malignant brain tumors in humans [1]. The most important feature of glioma is its extensive aggressiveness represented by invasion and migration, which are also the main cause of therapeutic failure and recurrence [2]. Glioma cells with widespread invasiveness always respond poorly to treatment since they destroy the function of normal brain parenchyma [3]. Patients afflicted with glioma have unfavorable prognoses although great advances have been made in the treatment of this disease with chemotherapy and radiation following surgical resection [4–6]. Identifying the underlying mechanisms regarding the invasiveness of glioma is significant to designing effective therapeutic interventions.

Ever-increasing evidence has indicated that the tumor microenvironment (TME) equal importance to the intrinsic properties of tumor cells in determining tumor invasiveness. As one of the basic features of TME, hypoxia is well-known as an important stimulator of tumor invasion, metastasis, and angiogenesis [7–9]. In recent years, hypoxia has been validated to stimulate cell migration and invasion in tumors including glioma, but the mechanism is still not well studied [10]. Hypoxia inducible factor 1 (HIF-1) consists of

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HIF-1α and HIF-1β subunits, which can be activated by hypoxic conditions. Under normoxic conditions, HIF-1α subunit is degraded rapidly, whereas under hypoxic conditions, HIF-1α tends to be stabilized [11]. It has been shown that hypoxia-activated HIF-1α can elevate the expression of related genes such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) in glioblastoma cells, thereby regulating cell migration, invasion, and angiogenesis [12]. Epithelial-mesenchymal transition (EMT) is crucial for cancer metastasis, which can be promoted by the tumor microenvironment. During the EMT process, E-cadherin expression is downregulated, and vimentin and fibronectin expression is upregulated [13–16]. Previous studies revealed that the EMT, invasion, and proliferation of cancer cells can be directly modulated by HIF-1α [17–19]. Moreover, they can be promoted by HIF-1α through crosstalking with some signal pathways, such as Wnt/β-catenin, Hedgehog (Hh), and NF-κB [20–22]. However, the molecular mechanisms of HIF-1α-induced glioma cell migration, invasion, and EMT under hypoxia have not been fully elucidated.

Glioma-associated oncogene homolog 1 (GLI1) is a transcriptional factor of the Hh pathway, facilitating EMT and related to proliferation and invasion of multiple tumor cells. Several reports show that GLI1 promotes the proliferative and invasive abilities of glioma cells and exerts effects on glioma cell apoptosis by the regulation of Cyclin D1 and Bcl-2 expression [23, 24]. GLI1 expression has been found to be upregulated in breast cancers and is related to breast cancer aggressiveness [25]. Of note, it has been found that EMT can be induced by hypoxia that promotes the invasiveness of pancreatic cancer cells *via* elevation of GLI1 expression [26]. Nonetheless, the functions of GLI1 in hypoxia-induced migration, invasion, and EMT of glioma cells as well as its relationship with HIF-1α have not been elucidated.

In our current study, an *in vitro* hypoxia culture model was established to make a hypoxic microenvironment of human glioma LN229 and LN18 cells. We hypothesized that GLI1 is related to HIF-1α-induced migration, invasion, and EMT in glioma cells and we focused on the biological functions and the mechanisms of HIF-1α and GLI1 in hypoxia-stimulated glioma cells, which might provide a novel therapeutic target for the treatment of glioma patients.

Material and methods

Cell culture. Human glioma cell lines (LN229 and LN18) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Catalog No. 30-2002, ATCC) containing 5% fetal bovine serum (FBS; Catalog No. 30–2020, ATCC). Normoxic condition for cell incubation was 37°C with 5% CO_2. To assess the influence of hypoxia, LN229 and LN18 cells were cultured under normoxic conditions until 65–70% confluence and were then cultured under hypoxic conditions with consistent 3% O_2 for 2 days.

Cell transfection. The shRNA vector pGPU6/GFP/Neo-shRNA-HIF-1α (sh-HIF-1α; GenePharma, Shanghai, China) or pGPU6/GFP/Neo-shRNA-GLI1 (sh-GLI1; GenePharma) was used to transfect cells to silence HIF-1α or GLI1. Plasmids (0.6 μg) in the shRNA and control groups were diluted in 250 μL Opti-Mem medium (Thermo Fisher Scientific, Inc.) in one tube. Similarly, 5 μL Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted in 250 μL Opti-MEM in another tube. The contents of the two tubes were mixed and incubated for 5 min at room temperature (RT). The mixture was subsequently added to the cells in 6-well plates and the plates were cultured at 37°C. After 24 h, the medium was replaced with DMEM containing 10% FBS. The cells were collected for subsequent experimentation following 48 h of further culture. Transfection efficiency was determined by counting the number of GFP-positive cells under a fluorescent microscope. Empty vector pGPU6/GFP/Neo-shRNA (sh-NC) acted as negative control. The sequences for shRNA are presented as follows: HIF-1α: 5′-UUUAAUUCAUCAGUGGUGGTT-3′, 5′-CCACCACUGAUGAAUUAAATT-3′; GLI1 5′-AUUACACACAAGCUGAGCCTT-3′, 5′-GGCUCAGCUUGUGUGUAAUTT-3′; sh-NC 5′-ACGUGACACGUUCGGAGAATT-3′, 5′-UUCUCCGAACGUGUCACGUTT-3′.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol Reagent (B0201, HaiGene, Ha'erbin, China) was used to extract total RNA from LN229 and LN18 cells when cells reached 90% confluence. Reverse transcription of mRNA into cDNA was performed using a PrimeScript RT reagent Kit (DRR037A, TaKaRa, Dalian, China). According to the product manuals, RT-qPCR was conducted to determine the relative levels of mRNA transcripts using SYBR Green Master Mix (Q131-02/03, Vazyme, Nanjing, China) on a 7500 Realtime PCR System (4351151, Thermo Fisher, USA). The sequences of primers are shown as below: GLI1 forward 5′-GGGATGATCCCACATCCTCAGTC-3′, reverse 5′-CTGGAGCAGCCCCCCCAGT-3′; HIF-1α forward 5′-CGCAAGTCCTCAAAGCACAGTTAC-3′, reverse 5′-GCAGTGGTAGTGGTGGCATTAGC-3′; 18S RNA forward 5′-CAGATCAAAACCAACCCG-3′, reverse 5′-GCCCTATCAACTTTCGATGG-3′. The $2^{-\Delta\Delta Ct}$ method was used for the calculation of the relative expression of

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target gene mRNA transcripts with 18S RNA as a housekeeping gene [27].

Western blot analysis. LN229 and LN18 cells (1×10^6) under hypoxic or normoxic conditions were lysed in RIPA lysis buffer (CST Biological Reagents Co., Ltd.) containing phenylmethylsulphonyl fluoride (Thermo Fisher Scientific, Inc.), protease inhibitor cocktail (TransGen Biotech Co., Ltd.) and phosphatase inhibitor cocktail (TransGen Biotech Co., Ltd.). The protein lysates were centrifuged at 15,000 g for 15 min at 4°C. The protein concentration was measured by a Pierce BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (50 μg/lane) were separated by 10% SDS-PAGE and were transferred onto PVDF membranes (FFP24, Beyotime, Shanghai, China). The membranes were blocked with 5% skimmed milk powder, followed by incubation with primary antibodies including rabbit anti-HIF-1α (ab179483; 1:1000), anti-GLI1 (ab134906; 1:1000), anti-E-cadherin (ab40772; 1:10000), anti-Vimentin (ab92547; 1:1000), and anti-GAPDH (ab181602; 1:10000) at 4°C overnight. After washing with phosphate-buffered saline (PBS), the membranes were incubated with HRP-conjugated anti-rabbit secondary (ab205718; 1:5000) for 1.5 h at RT. Protein bands were visualized using an enhanced chemiluminescence detection system (EMD Millipore). Densitometric analysis was performed using ImageJ 1.48 software. GAPDH acted as a reference protein.

Wound healing assay. The migratory ability of cells was evaluated by wound healing assay as previously described [28]. LN229 and LN18 cells under hypoxic or normoxic conditions were seeded in 6-well plates and cultured in DMEM with 10% FBS to 80% confluence. A 200-μL pipette tip was applied to make a scratch on the cell monolayer. After washing with PBS, cells ere cultured in serum-free DMEM at 37°C and then imaged at 0 h and 24 h using a light microscope (magnification 100×) in five randomly selected fields of view. The cell migratory distance into the wound area was calculated using ImageJ 1.48 software.

Transwell assay. The invasive ability of glioma cells under hypoxic or normoxic conditions was assessed using invasion assays in a Millicell invasion chamber (MCHT06H48, Merck, USA). Briefly, 3×10^4 glioma cells in serum-free DMEM were plated on the membrane pre-coated with Matrigel (wlb1062, BD, Shanghai, China) in the upper chamber. DMEM supplemented with 5% FBS was added to the lower chamber as a chemoattractant. After incubation for 24 h at 37°C, non-invaded cells on the upper face of the membrane were removed with a cotton swab. Then, 0.1% crystal violet was used to stain the cells invaded to the lower surface. The images in five randomly selected fields were captured by a microscope at a magnification 100× and the number of invaded cells was calculated using ImageJ 1.48 software.

Statistical analysis. The data were analyzed using SPSS18.0 software (SPSS, Chicago, IL, USA) and are expressed as means ± standard errors. Each experiment was repeated at least three times. For comparison of the differences between the two groups, student's *t*-test was adopted. One-way ANOVA followed by Tukey's multiple comparison test was utilized for the analysis of statistical differences among multiple groups. P value less than 0.05 was considered statistically significant.

Results

Effects of hypoxia on HIF-1α and GLI1 expression in glioma cells

HIF-1α, which can be induced by hypoxia, crosstalks with other signaling pathways and activates the NF-κB pathway to modulate the metastasis, apoptosis, and proliferation of cancer cells [29]. It has been revealed by a previous report that the HIF-1α and GLI1 expression can be elevated by hypoxia in pancreatic cancer cells and breast cancer cells [26, 30]. To probe into the function of hypoxia in modulating the metastasis of glioma cells, LN229 and LN18 cells were cultured under hypoxic or normoxic conditions for 48 h and the expression levels of HIF-1α and GLI1 were determined using Western blot and RT-qPCR analysis. As a result, the protein levels of HIF-1α and GLI1 were higher in hypoxia-cultured cells than in normoxia-cultured cells (Fig. 1A). Meanwhile, HIF-1α mRNA expression in LN229 and LN18 cells under hypoxic and normoxic conditions showed no significant change (Fig. 1B). Compared with the cells cultured under normoxic conditions, LN229 and LN18 cells under hypoxic conditions exhibited high levels of GLI1 mRNA expression (Fig. 1C). Thus, hypoxia blocked the breakdown of the HIF-1α protein inside the cell and promoted GLI1 expression in glioma cells.

Silencing of HIF-1α inhibits hypoxia-induced glioma cell migration and invasion

To explore the biological function of HIF-1α in glioma cells, LN229 and LN18 cells were transfected with HIF-1α-specific shRNA (sh-HIF-1α) or control shRNA (sh-NC). RT-qPCR analysis showed significantly reduced HIF-1α expression in LN229 and LN18 cells transfected with sh-HIF-1α compared with sh-NC (Fig. 2A). Successful knockdown efficiency of HIF-1α was also demonstrated by Western blot analysis showing that the protein level of HIF-1α was downregulated by sh-HIF-1α in LN229 and LN18 cells (Fig. 2B). Wound healing and Transwell assays showed that glioma cell migration and invasion in the hypoxia group were significantly increased compared with the normoxia group. Downregulation of HIF-1α

had no effects on glioma cell migration and invasion under normoxic conditions compared with control. However, under hypoxic conditions, the migration and invasion of glioma cells were significantly decreased in the HIF-1α knockdown group compared with the control group (Fig. 2C, D). These results suggested that HIF-1α knockdown inhibited hypoxia-induced glioma cell migration and invasion.

HIF-1α knockdown reverses hypoxia-promoted GLI1 protein level and EMT

To investigate the impact of HIF-1α on GLI1 progression and EMT process in normoxia- and hypoxia-cultured glioma cells, Western blot analysis was carried out. In LN229 and LN18 cells transfected with sh-NC, the protein levels of GLI1 and vimentin were increased whereas the protein levels of E-cadherin were reduced by hypoxia compared with normoxia induction. No significant change was observed in sh-HIF-1α-transfected cells under normoxic conditions. However, silencing of HIF-1α reversed hypoxia-induced promotion of the protein levels of GLI1 and Vimentin as well as the suppression of the protein levels of E-cadherin (Fig. 3A, B). Overall, HIF-1α knockdown reversed the GLI1 levels and EMT induced by hypoxia.

Silenced GLI1 suppresses hypoxia-induced glioma cell migration and invasion

The influence of GLI1 on hypoxia-induced migration and invasion was further examined. GLI1 knockdown efficiency was determined by RT-qPCR and Western blot analysis, and the results showed that GLI1 expression levels were successfully downregulated by sh-GLI1 transfection in glioma cells (Fig. 4A, B). The migratory and invasive capacities of glioma cells were then measured by wound healing and Transwell assays. The results showed that, under either hypoxic or normoxic conditions, GLI1 downregulation significantly inhibited the migration and invasion of glioma cells compared with the control (Fig. 4C, D), showing that silencing of GLI1 inhibited hypoxia-evoked migration and invasion of glioma cells.

Silenced GLI1 reverses hypoxia-promoted EMT

We further explored the effects of GLI1 on the hypoxia-induced HIF-1α expression and EMT process. After transfection of LN229 and LN18 cells with sh-GLI1 or sh-NC under hypoxic or normoxic conditions, Western blot analysis was conducted for the examination of the protein levels of GLI1, E-cadherin, and vimentin. There was an increase in the protein levels of HIF-1α and vimentin and a decrease in the protein levels of E-cadherin in sh-NC- or sh-GLI1-transfected cells cultured under hypoxic conditions relative to normoxic conditions. Compared with normoxia- or hypoxia-cultured cells with the transfection of sh-NC, GLI1 knockdown significantly increased E-cadherin protein expression and decreased vimentin protein expression but had no effects on HIF-1α protein expression (Fig. 5A, B). Therefore, GLI1 knockdown mitigated hypoxia-induced EMT process in glioma cells.

Discussion

EMT exerts a crucial role in the migration, invasion, and metastasis of cancers [31]. Previous literature manifested that hypoxia contributes to the EMT process *via* upregulation of HIF-1α expression and upregulation of other transcription factors associated with EMT, such as SNAIL [32, 33]. In addition, it has been

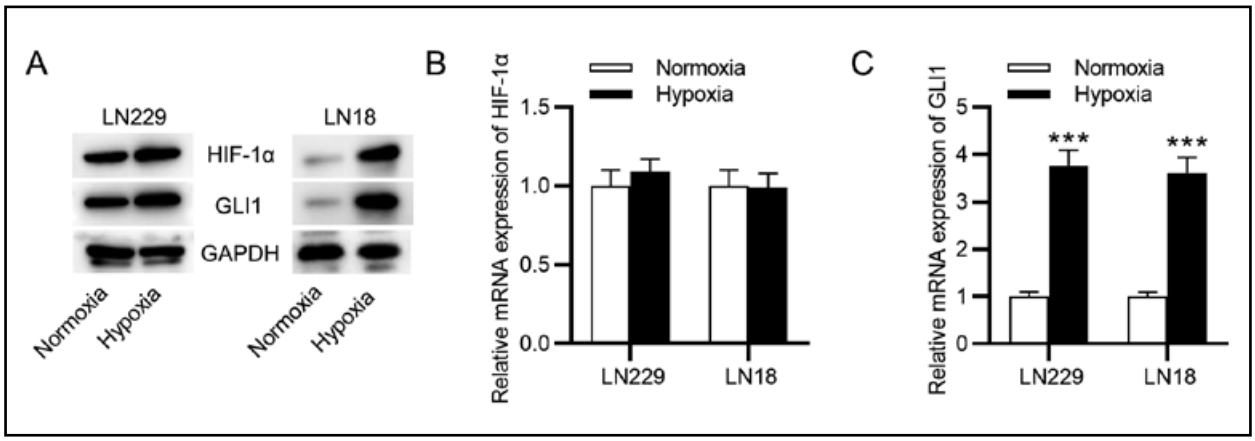

Figure 1. Effects of hypoxia on HIF-1α and GLI1 expression in glioma cells. LN229 and LN18 cells were cultured in hypoxic or normoxic conditions for 48 h and subjected to Western blot (A) and RT-qPCR analysis (B, C) for the measurements of the protein and mRNA levels of HIF-1α and GLI1. All bars were normalized to normoxia control (= 1). ***P < 0.001.

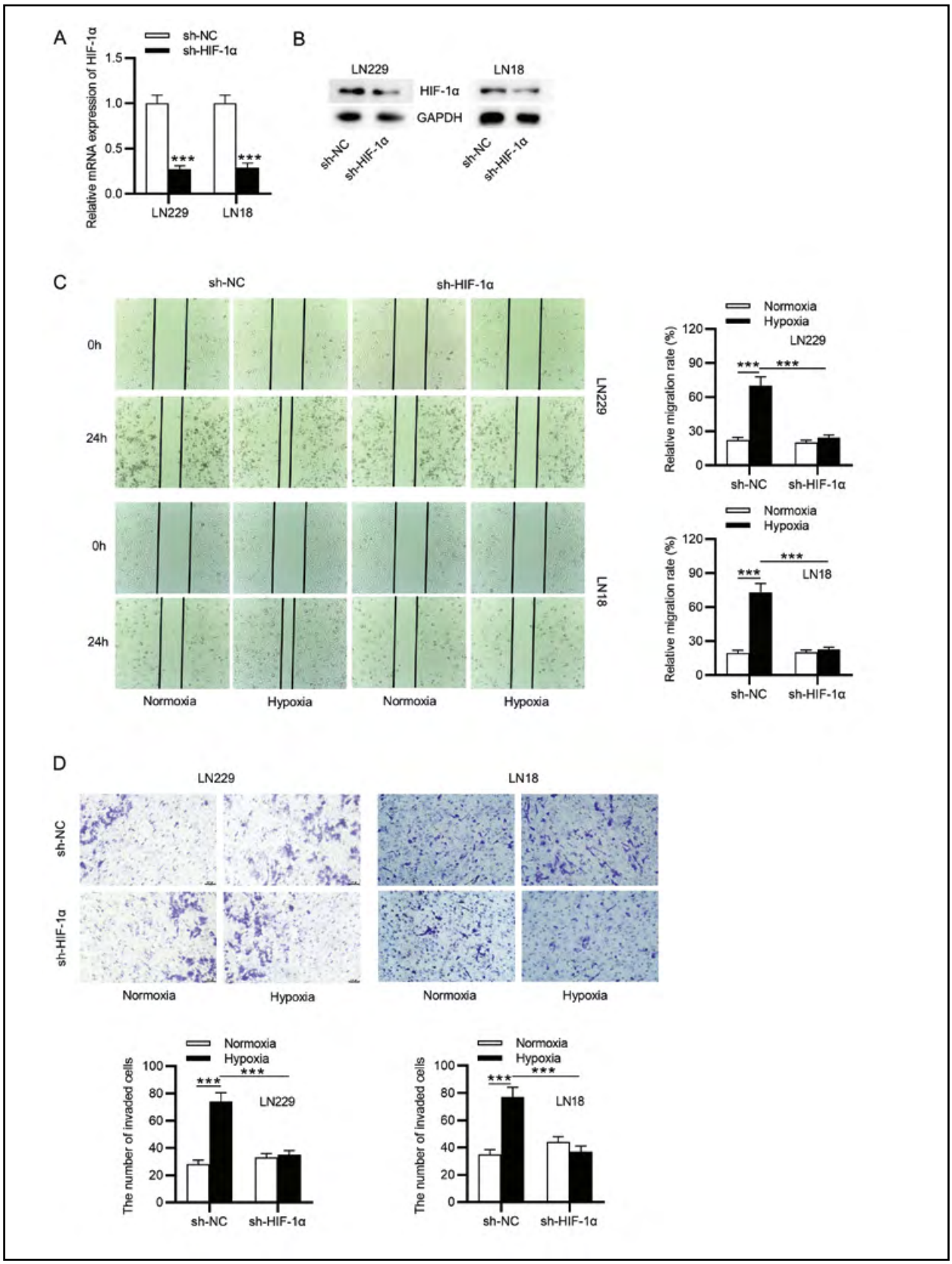

Figure 2. Silencing of HIF-1α inhibits hypoxia-induced glioma cell migration and invasion. Glioma cells were transfected with sh-NC or sh-HIF-1α for 48 h; **A, B**. HIF-1α knockdown efficiency examined by RT-qPCR and Western blot analysis. **C.** Effects of silenced HIF-1α on the migration of glioma cells cultured under hypoxic or normoxic conditions examined by wound healing assay **D.** Transwell assays of cell invasion were performed under hypoxic or normoxic conditions. ***P < 0.001.

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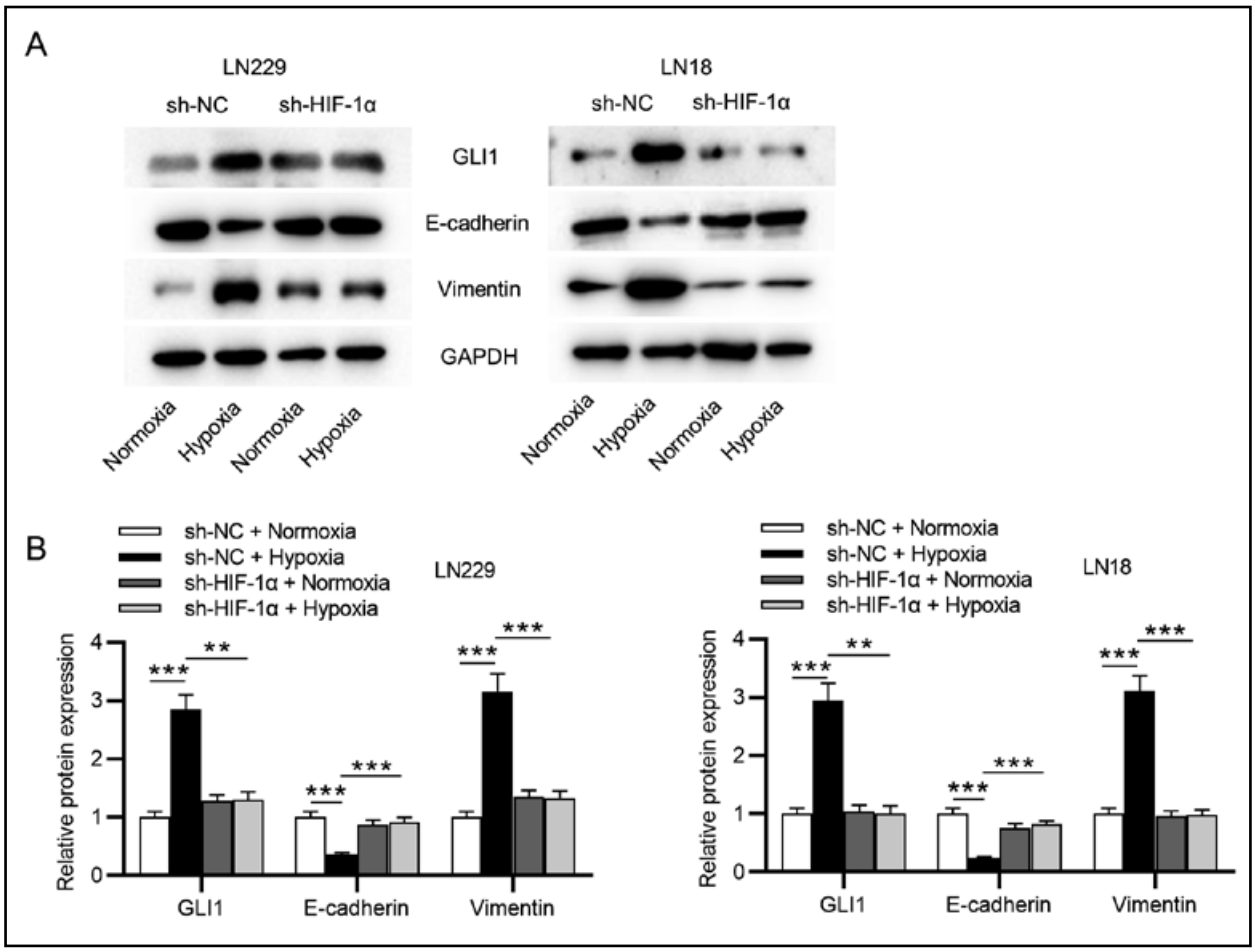

Figure 3. HIF-1α knockdown reverses hypoxia-promoted GLI1 protein level and EMT; **A, B.** Western blot analysis of the protein levels of GLI1, E-cadherin, and vimentin in sh-NC- or sh-HIF-1α-transfected glioma cells under hypoxic or normoxic conditions. All bars were normalized to normoxia control (= 1). **P < 0.01, ***P < 0.001.

discovered that hypoxia induces HIF-1α to increase the expression level of GLI1 and promote EMT and invasion of breast cancer cells and pancreatic cancer cells [26, 30]. In our current investigation, we probed into the functions of GLI1 and HIF-1α in hypoxia-induced cell migration, invasion, and EMT in glioma as well as the relationship between GLI1 and HIF-1α expression under hypoxic conditions. The obtained results showed that hypoxia strengthened the protein expression of both GLI1 and HIF-1α in glioma cells. Suppression of HIF-1α abolished hypoxia-promoted migration, invasion, GLI1 expression, and EMT of glioma cells. Additionally, depletion of GLI1 significantly mitigated hypoxia-induced-promotion of migration, invasion, and EMT. These novel findings indicate that GLI1 is involved in hypoxia-promoted migration, invasion, and EMT of glioma cells and HIF-1α promotes hypoxia-elevated GLI1 expression to accelerate cell migration, invasion, and EMT in glioma. These findings imply that GLI1 might become a novel target to intervene in glioma metastasis.

HIF-1 is composed of two subunits, one of which is HIF-1α, which is sensitive to hypoxia and has two O_2-dependent modifications at the posttranslational level [34, 35]. Under normoxic conditions, HIF-1α is hydroxylated on proline residues by O_2 sensitive prolyl hydroxylase domain (PHD) proteins [36, 37]. The hydroxylated HIF-1α is then recognized by the von Hippel–Lindau (VHL) protein for subsequent ubiquitination and degradation [38]. In contrast, under hypoxic conditions, the hydroxylation of HIF-1α is reduced due to the decreased activity of PHDs [39], resulting in HIF-1α stabilization and nuclear translocation. Interestingly, our study manifested that the protein levels of HIF-1α but not the mRNA levels of HIF-1α were elevated by hypoxia in glioma cells. This suggests that hypoxia blocks the degradation of the HIF-1α protein and increases the lifespan of the HIF-1α protein in glioma cells and thereby

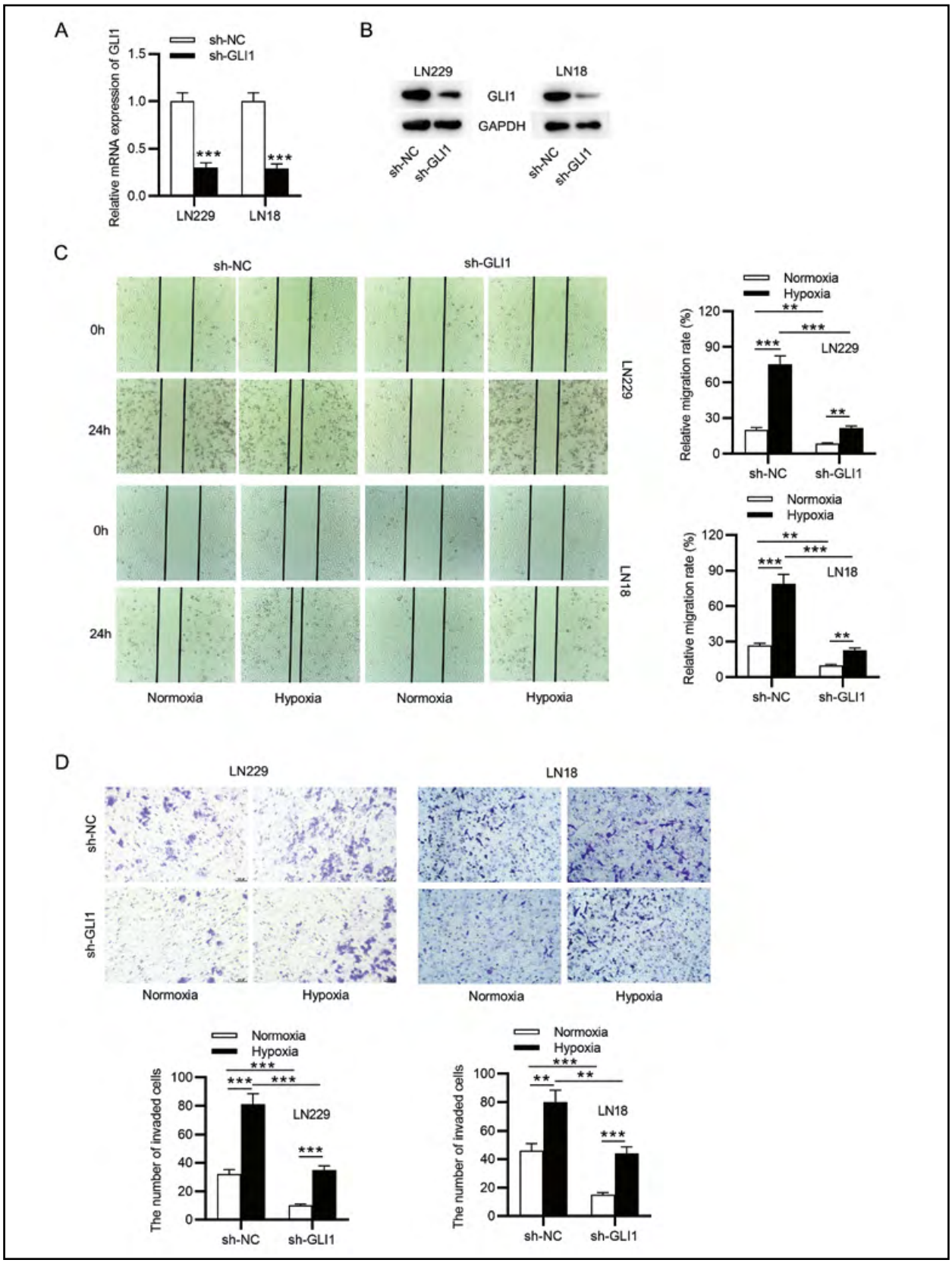

Figure 4. Silenced GLI1 suppresses hypoxia-induced glioma cell migration and invasion. Glioma cells were transfected with sh-NC or sh-GLI1 for 48 h; **A.** RT-qPCR analysis of GLI1 mRNA expression in glioma cells with the transfection of sh-NC or sh-GLI1; **B.** Western blot analysis of GLI1 protein expression in glioma cells transfected with sh-NC or sh-GLI1; **C.** Effects of silenced GLI1 on the migration of glioma cells cultured under hypoxic or normoxic conditions measured by wound healing assay; **D.** Effects of GLI1 depletion on glioma cell invasion under hypoxic or normoxic conditions measured by Transwell assays.**P < 0.01, ***P < 0.001.

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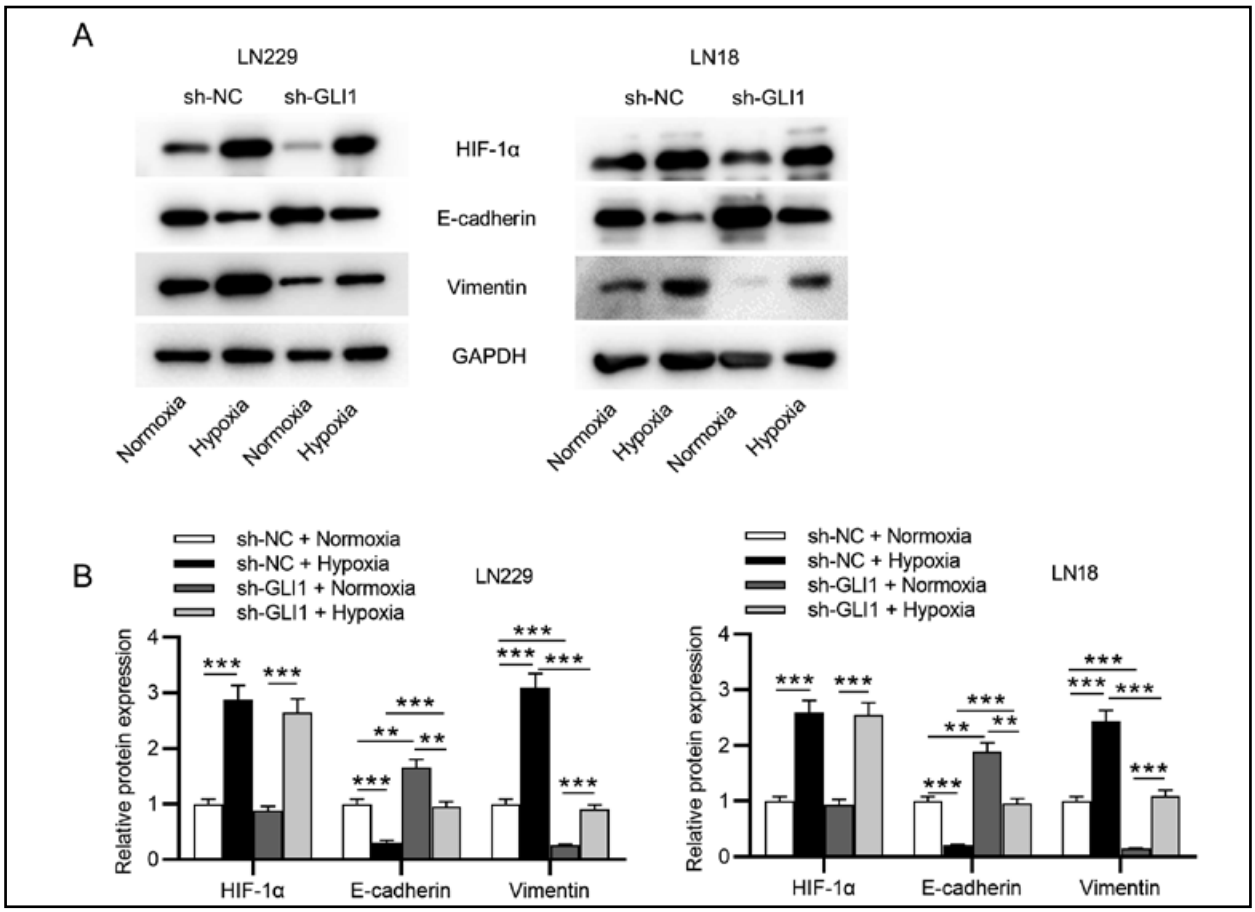

Figure 5. Silenced GLI1 reverses hypoxia-promoted EMT. (A-B). LN229 and LN18 cells transfected with sh-NC or sh-GLI1 were cultured under hypoxic or normoxic conditions. Protein levels of HIF-1α, E-cadherin, and vimentin were examined by Western blot analysis. All bars were normalized to normoxia control (= 1). **P < 0.01, ***P < 0.001.

improving the stability of HIF-1α. As demonstrated by a previous study showing that HIF-1α is a pivotal modulator of the EMT process [40], which suggests that hypoxia induces HIF-1α expression, which in turn induces SNAIL expression, leading to downregulated E-cadherin expression and upregulated vimentin expression in glioma cells. Our results manifested that hypoxia contributed to the migration, invasion, and EMT of glioma cells. Thus, HIF-1α may be a target for designing therapies to prevent and intervene in the metastasis of glioma.

It has been shown by previous studies that HIF-1α crosstalks with the NF-κB and Notch pathways to promote the EMT process of cancer cells [41, 42]. The classical Hedgehog (Hh) signaling is stimulated by hypoxia *via* upregulation of HIF-1α expression both *in vivo* and *in vitro* [43, 44]. Previous studies have revealed that HIF-1α crosstalks with the Hh signaling to enhance hypoxia-promoted cell invasion and EMT in breast cancer and pancreatic cancer [26, 30]. Our investigation shows that hypoxia blocked the breakdown of the HIF-1α protein and increased the expression of GLI1 to facilitate the migration, invasion, and the EMT of glioma cells. GLI1 plays an effector role of the Hh pathway, induction of which can occur independently of Smoothened modulation in multiple tumors such as glioma, basal cell carcinoma, prostate cancer, and medulloblastoma [45, 46]. Previous studies have illuminated that GLI1 displays a high expression level in breast cancer and it correlates with the adverse prognosis of breast cancer patients [47]. Knockdown of GLI1 eliminated hypoxia-facilitated invasion and EMT process of breast cancer cells [30]. Moreover, GANT61, a GLI inhibitor, potentiates the cytotoxic effect of temozolomide, which is a first-line chemotherapy treatment for glioma, thus becoming a promising *in vitro* strategy for glioma treatment [48, 49]. The inhibitor of the Hh pathway, cyclopamine, could potentiate the temozolomide effect in cancer stem-like cells and glioma cell lines *in vitro* [50]. Studies showed that arsenic trioxide (ATO)-mediated Hh/Notch inhibition could be used in the clinic as it represents a promising targeted therapy approach for the elimination of glioma stem-like cells [51].

These studies suggest that inhibition of GLI1 or the Hh pathway may be a possible approach for treating glioma. Consistently, our present investigation manifested that inhibition of GLI1 significantly abolished hypoxia-enhanced migration, invasion, and EMT of glioma cells *in vitro*. Given that the expression of vimentin and E-cadherin can be modulated by many chemokines and transcription factors, it is possible that GLI1 may indirectly modulate their expression to contribute to the migration, invasion, and EMT of glioma cells. Further explorations regarding how GLI1 modulates the migration, invasion, and EMT of glioma cells and how HIF-1α signaling crosstalks with the Hh pathway to upregulate the expression of GLI1 are required in the future.

In conclusion, we found that hypoxia blocks the degradation of the HIF-1α protein and GLI1 expression in glioma cells. Silencing of HIF-1α or GLI1 eliminated hypoxia-enhanced GLI1 expression, migration, invasion and EMT of glioma cells. Knockdown of GLI1 has no effect on HIF-1α expression but significantly mitigates the hypoxia-enhanced EMT and invasion of glioma cells. These findings might offer a beneficial revelation for probing into other potential targets or biomarkers for the treatment of glioma patients.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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