

MicroRNA-181b Inhibits Cellular Proliferation and Invasion of Glioma Cells via Targeting Sal-Like Protein 4

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MicroRNAs (miRs), a class of noncoding RNAs that are 18–25 nucleotides in length, are able to suppress gene expression by targeting complementary regions of mRNAs and inhibiting protein translation. Recently, miR-181b was found to play a suppressive role in glioma, but the regulatory mechanism of miR-181b in the malignant phenotypes of glioma cells remains largely unclear. In this study, we found that miR-181b was significantly downregulated in glioma tissues when compared with normal brain tissues, and decreased miR-181b levels were significantly associated with high-grade pathology and a poor prognosis for patients with glioma. Moreover, miR-181b was downregulated in glioma cell lines (U87, SHG44, U373, and U251) compared to normal astrocytes. Overexpression of miR-181b significantly decreased the proliferation, migration, and invasion of glioma U251 cells. Sal-like protein 4 (SALL4) was identified as a novel target gene of miR-181b in U251 cells. The expression of SALL4 was significantly upregulated in glioma tissues and cell lines, and an inverse correlation was observed between the miR-181b and SALL4 expression levels in glioma. Further investigation showed that the protein expression of SALL4 was negatively regulated by miR-181b in U251 cells. Knockdown of SALL4 significantly inhibited the proliferation, migration, and invasion of U251 cells, while overexpression of SALL4 effectively reversed the suppressive effects of miR-181b on these malignant phenotypes of U251 cells. In conclusion, our study demonstrates that miR-181b has a suppressive effect on the malignant phenotypes of glioma cells, at least partly, by directly targeting SALL4. Therefore, the miR-181b/SALL4 axis may become a potential therapeutic target for glioma.

Key words: Glioma; MicroRNAs (miRs); Sal-like protein 4 (SALL4); Proliferation; Migration; Invasion

INTRODUCTION

Glioma is the most common malignant brain tumor, accounting for about 80% of all malignant tumors in the brain^{1,2}. Despite great efforts that have been made with surgical resection combined with radiotherapy and chemotherapy, the overall survival for patients with advanced glioma is still not satisfactory^{1,2}. In recent years, the dysfunction of oncogenes or tumor suppressors has been demonstrated in glioma, and exploring the regulatory mechanism underlying glioma growth and metastasis may be of benefit in the identification of novel therapeutic targets for its treatment^{3,4}.

MicroRNAs (miRs) are a class of noncoding RNAs that are 18–25 nucleotides in length and have been demonstrated to negatively regulate gene expression by directly binding to the complementary regions of their target mRNAs, causing protein translation inhibition or mRNA degradation^{5,6}. In recent decades, miRs have been found to participate in various physiological and pathological biological processes through mediating their target genes, such as cell

proliferation, differentiation, apoptosis, motility, angiogenesis, and tumorigenesis^{6–8}. Moreover, some deregulated miRs have been found to play key roles in the development and progression of glioma^{9,10}. For instance, miR-27b promotes cell invasion in glioma U251 cells through inhibiting Spry2 expression¹¹. miR-503 inhibits cell proliferation and invasion in glioma by targeting L1CAM¹². Previous studies suggest that some glioma-related miRs may become potential molecular targets or reagents for the treatment of glioma^{13,14}. However, the regulatory mechanism of miRs in glioma needs to be fully uncovered.

miR-181b has been reported to play an oncogenic or tumor-suppressive role in different human cancers. For instance, miR-181b-3p enhances the epithelial–mesenchymal transition (EMT) in breast cancer cells through SNAIL stabilization by targeting YWHAG, suggesting that miR-181b may promote breast cancer metastasis¹⁵. On the contrary, miR-181b inhibits glycolysis in gastric cancer cells via inhibition of hexokinase 2 expression, suggesting that it may repress gastric cancer

growth¹⁶. Recently, miR-181b was found to function as a tumor suppressor in glioma¹⁷. For instance, Shi et al. reported that miR-181b could inhibit glioma cell growth and invasion and induce cell apoptosis¹⁷. However, the underlying mechanism of miR-181b in glioma growth and metastasis remains largely unclear.

In our study, we aimed to investigate the clinical significance of miR-181b expression in glioma, as well as its regulatory mechanism underlying glioma growth and metastasis.

MATERIALS AND METHODS

Tissue Collection

The study was approved by the ethics committee of the Second Xiangya Hospital of Central South University, Changsha, P.R. China. Written informed consent was obtained from participants involved in this study. Glioma tissues ($n=74$) and normal brain tissues ($n=18$) were obtained from our hospital between June 2009 and July 2011. Patients with glioma received neither radiation therapy nor chemotherapy before surgical resection. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until use. The clinical information of the patients is summarized in Table 1.

Cell Culture

Human glioma cell lines (U87, SHG44, U373, and U251) were obtained from the Cell Bank of Central South University (Changsha, P.R. China) and cultured in DMEM (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Life Technologies)

at 37°C in a humidified incubator containing 5% CO_2 . Normal human astrocytes were purchased from the IBS Cell Bank of Fudan University (Shanghai, P.R. China) and cultured in astrocyte media (Science Cell, Carlsbad, CA, USA) with 10% FBS at 37°C in a humidified incubator containing 5% CO_2 .

Cell Transfection

Scramble miR (miR-NC), miR-181b mimic, negative control (NC) inhibitor, miR-181b inhibitor, non-specific siRNA, and Sal-like protein 4 (SALL4) siRNA were all obtained from GeneCopoeia (Rockville, MD, USA). pc-DNA3.1-SALL4 expression plasmid was transfected into U251 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. After incubation at 37°C , 5% CO_2 for 6 h, the transfection mixture was replaced by DMEM with 10% FBS. Cells were then cultured for 48 h before the following assays.

RT-PCR Analysis

Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. A total of 800 ng of RNA was converted into cDNA using the Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. Real-time (RT)-PCR was then performed using a Q-PCR detection kit (Life Technologies) on an ABI 7500 thermocycler. The PCR steps were 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s and annealing/elongation step at 60°C for 60 s. GAPDH was used as an internal

Table 1. Association Between miR-181b Expression and Clinicopathological Characteristics in Glioma

Variables	Cases	Low miR-181b ($n=36$)	High miR-181b ($n=38$)	<i>p</i> Value
Age				0.332
<50	25	10 (40%)	15 (60%)	
≥ 50	49	26 (53.1%)	23 (46.9%)	
Gender				0.812
Male	45	21 (46.7%)	24 (53.3%)	
Female	29	15 (51.7%)	14 (48.3%)	
WHO grade				0.012*
I-II	23	6 (26.1%)	17 (73.9%)	
III-IV	51	30 (58.8%)	21 (41.2%)	
Surgery type				0.245
Gross total resection	37	15 (40.5%)	22 (59.5%)	
Partial resection	37	21 (56.8%)	16 (43.2%)	
Karnofsky performance scale				0.012*
>90	34	11 (32.4%)	23 (67.6%)	
≤ 90	40	25 (62.5%)	15 (37.5%)	

* $p < 0.05$.

control. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). Protein was separated with 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Life Technologies). The PVDF membrane was incubated with PBS containing 5% milk overnight at 4°C. After washing with PBS three times, the PVDF membrane was incubated with primary antibodies (Abcam, Cambridge, MA, USA) at room temperature for 3 h. After washing with PBS three times, the PVDF membrane was incubated with secondary antibody (Abcam) at room temperature for 1 h. Super Signal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA) was then used to detect

signals according to the manufacturer's instructions. The relative protein expression was analyzed by Image-Pro Plus software 6.0, represented as the density ratio versus GAPDH.

MTT Assay

U251 cells were plated at a density of 10,000 cells per well in 96-well plates. After being cultured for 0, 24, 48, and 72 h, the cells were incubated with MTT (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.5 mg/ml for 4 h at 37°C. After the removal of the medium, 150 mM DMSO solution (Sigma-Aldrich) was added. The absorbance was read at 570 nm using a Bio-Tek™ ELx800™ Absorbance Microplate Reader.

Wound Healing Assay

U251 cells were cultured to full confluence. Wounds of approximately 1-mm width were created with a plastic

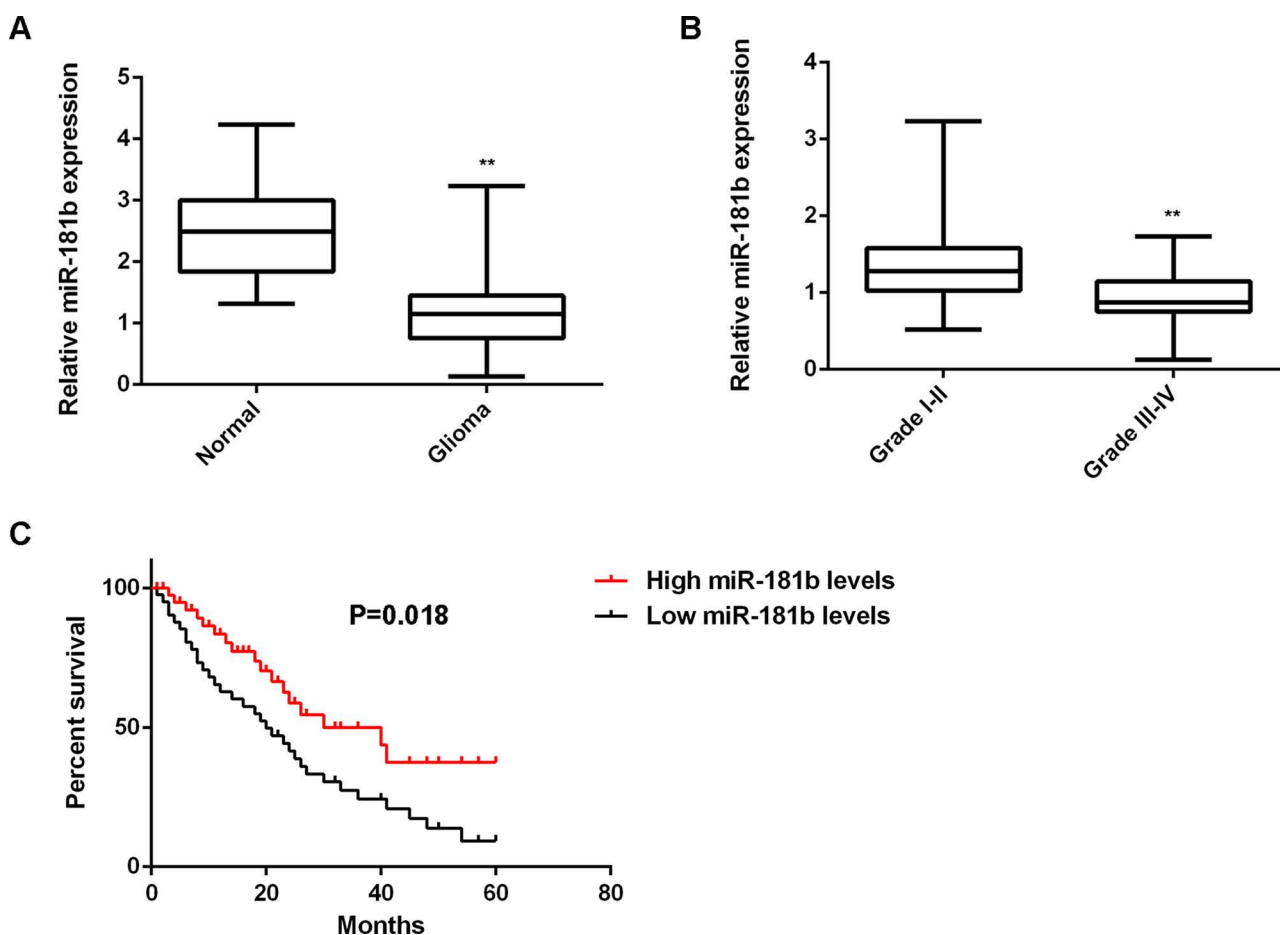
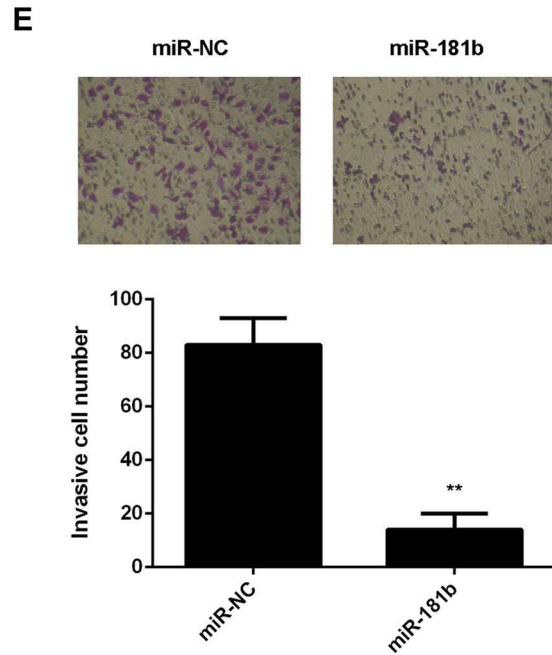
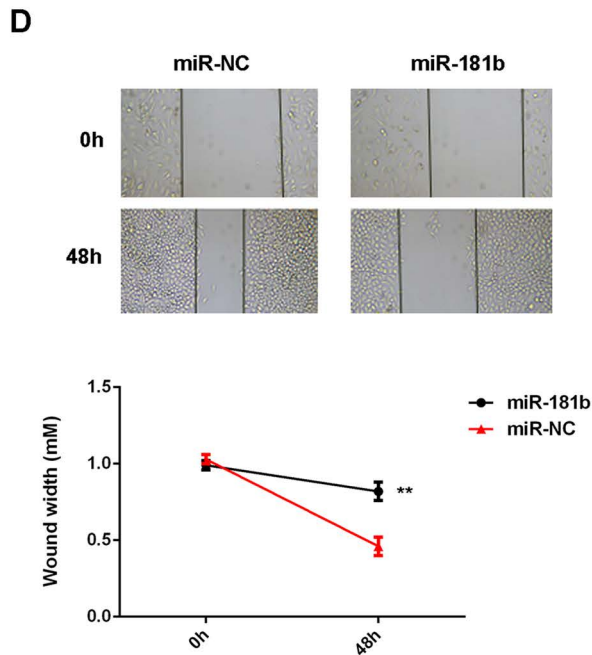
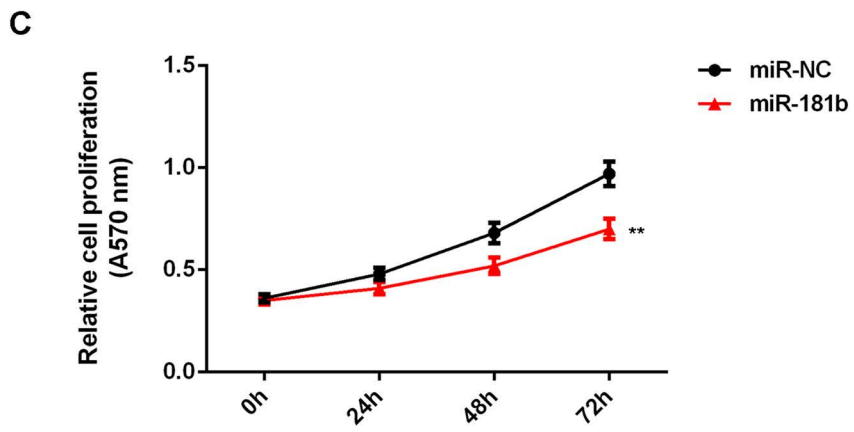
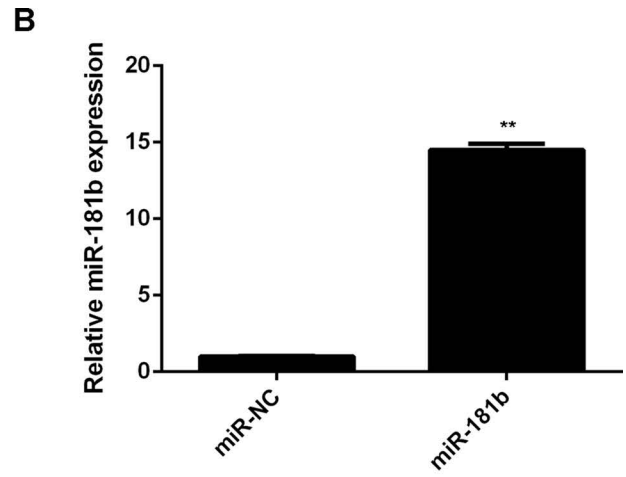
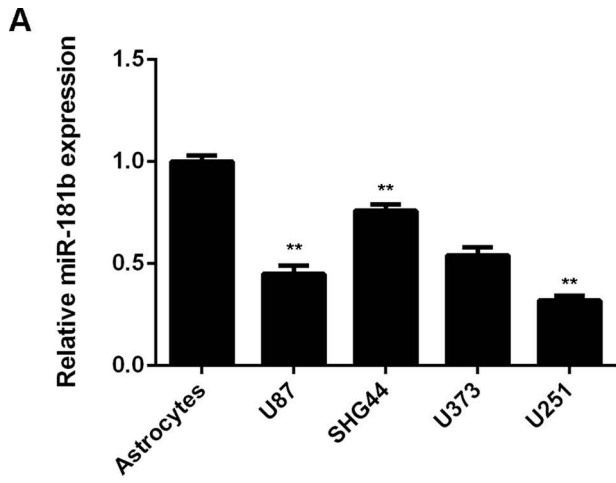


Figure 1. Downregulation of miR-181b is associated with malignant progression and poor prognosis in glioma. (A) RT-PCR was used to determine the miR-181b levels in glioma tissues ($n=74$) and normal brain tissues ($n=18$). $**p<0.01$ versus Normal. (B) RT-PCR was used to determine the miR-181b levels in high- or low-grade glioma tissues. $**p<0.01$ versus grades I–II. (C) Glioma patients with low expression of miR-181b had shorter overall survival when compared with those with high expression of miR-181b.



scriber. Cells were washed and then cultured in DMEM containing 10% FBS for 48 h. Cells were then observed and photographed under a microscope.

Transwell Assay

Transwell assay was performed to examine cell invasion using Transwell chambers (BD Biosciences, San Jose, CA, USA). U251 cell suspension (5×10^5 cells/ml) was prepared in a serum-free medium, and 300 μ l of cell suspension was added into the upper chamber. Then 500 μ l of DMEM with 10% FBS was added into the lower chamber. Cells were incubated for 24 h. A cotton-tipped swab was then used to carefully wipe off the cells that did not migrate through the pores. The filters were fixed in 90% alcohol, stained with crystal violet, and observed under an inverted microscope (Olympus, Tokyo, Japan).

Bioinformatics Prediction and Dual-Luciferase Reporter Assay

TargetScan software (www.targetscan.org) was used to predict the putative target of miR-181b. The wild-type (WT) or mutant-type (MT) 3'-UTR of SALL4 was constructed and inserted into the multiple cloning sites in the psiCHECK2 luciferase reporter vector. U251 cells were cotransfected with WT-SALL4-3'-UTR or MT-SALL4-3'-UTR reporter plasmid plus miR-181b mimics or miR-NC using Lipofectamine 2000 according to the manufacturer's instructions. The dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to determine the luciferase activities 48 h after cotransfection. *Renilla* luciferase activity was normalized to firefly luciferase activity.

Statistical Analysis

Data are expressed as the mean \pm SD. The differences between the two groups were analyzed using Student's *t*-test. Statistical analysis was performed using SPSS 19.0 (SPSS, Armonk, NY, USA). A value of $p < 0.05$ was considered significantly different.

RESULTS

Downregulation of miR-181b Is Associated With Malignant Progression and Poor Prognosis in Glioma

In our study, we first conducted RT-PCR to determine the miR-181b levels in glioma tissues ($n = 74$) and normal brain tissues ($n = 18$). Our data showed that the

expression of miR-181b was significantly reduced in glioma tissues compared to normal brain tissues (Fig. 1A). Moreover, low expression of miR-181b was significantly associated with a high pathological grade and a low Karnofsky performance scale (KPS) in glioma, but not associated with age, gender, or surgery type (gross total resection or partial resection) (Table 1), suggesting that downregulation of miR-181b may contribute to the malignant progression of glioma. Indeed, the high-grade gliomas (grades I–II) showed lower expression levels of miR-181b when compared with the low-grade gliomas (grades III–IV) (Fig. 1B). Furthermore, glioma patients with a low expression of miR-181b had shorter overall survival when compared with those with a high expression of miR-181b (Fig. 1C), suggesting that the reduced miR-181b expression is associated with a poor prognosis for patients with glioma.

miR-181b Overexpression Inhibits the Proliferation, Migration, and Invasion of Glioma Cells

To further reveal the role of miR-181b in glioma, we examined its expression in several common glioma cell lines including U87, SHG44, U373, and U251. Normal human astrocytes were used as control. Our data showed that miR-181b was significantly downregulated in glioma cell lines when compared with that in normal astrocytes (Fig. 2A). Because U251 cells showed the lowest expression of miR-181b, we used this cell line in the following experiments. U251 cells were then transfected with the miR-181b mimic or with miR-NC as the control group. RT-PCR data showed that the miR-181b levels were significantly upregulated in the miR-181b group when compared to the miR-NC group (Fig. 2B). The proliferation, migration, and invasion of U251 cells were then detected using the MTT assay, wound healing assay, and Transwell assay. Our data indicated that overexpression of miR-181b led to a significant decrease in U251 cell proliferation, migration, and invasion (Fig. 2C–E). These findings suggest that miR-181b plays a suppressive role in the malignant phenotypes of glioma cells.

SALL4 Is a Target Gene of miR-181b in Glioma Cells

Because miRs function through regulating the protein expression of their targets, the potential targets of miR-181b were further investigated. Bioinformatics prediction indicated that SALL4 was a putative target of miR-181b

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Figure 2. miR-181b overexpression inhibits the proliferation, migration, and invasion of glioma cells. (A) RT-PCR was used to determine the miR-181b expression in glioma cell lines (U87, SHG44, U373, and U251) compared with normal human astrocytes. $**p < 0.01$ versus astrocytes. U251 cells were transfected with miR-181b mimic or scramble miR (miR-NC). (B) RT-PCR was used to determine the miR-181b expression. (C) MTT, (D) wound healing, and (E) Transwell assays were conducted to examine cell proliferation, migration, and invasion. $**p < 0.01$ versus miR-NC (B–E).

(Fig. 3A). To confirm their targeting relationship, the luciferase reporter plasmids containing the WT or MT of SALL4 3'-UTR were generated (Fig. 3B). The luciferase activity was significantly decreased in U251 cells cotransfected with the WT-SALL4-3'-UTR luciferase reporter plasmid and the miR-181b mimic when compared to the control group, which was eliminated by transfection with the MT-SALL4-3'-UTR luciferase reporter plasmid (Fig. 3C). This indicated that miR-181b can directly bind to the 3'-UTR of SALL4 mRNA. Therefore, SALL4 is a target gene of miR-181b in U251 cells.

SALL4 Is Upregulated in Glioma, With an Inverse Correlation to miR-181b Levels

The mRNA levels of SALL4 were then detected in glioma tissues and normal brain tissues. Our data showed that SALL4 was significantly upregulated in glioma tissues compared to normal brain tissues (Fig. 4A). Interestingly, the SALL4 mRNA levels were found to be inversely correlated to the miR-181b levels in glioma tissues (Fig. 4B), suggesting that the upregulation of SALL4 in glioma may be due to the decreased expression of miR-181b. In addition, the mRNA and protein levels of SALL4 were upregulated in glioma cell lines compared to normal astrocytes (Fig. 4C and D). These findings

further support the targeting relationship between miR-181b and SALL4 in glioma tissues.

SALL4 Is Negatively Regulated by miR-181b in Glioma Cells

The effects of miR-181b on SALL4 expression were further investigated in U251 cells. We found that overexpression of miR-181b led to a significant decrease in the protein levels of SALL4 (Fig. 5A). To further confirm the data, U251 cells were transfected with the miR-181b inhibitor or with the NC inhibitor as the control group. Western blot data indicated that the miR-181 levels were significantly reduced in the miR-181b inhibitor group compared with the NC inhibitor group (Fig. 5B). We then found that knockdown of miR-181b markedly upregulated the SALL4 protein levels in U251 cells (Fig. 5C). Therefore, the protein expression of SALL4 is negatively regulated by miR-181b in U251 cells.

SALL4 Acts as a Downstream Effector in miR-181b-Mediated Malignant Phenotypes of U251 Cells

Upregulation of SALL4 was reported to be associated with glioma progression, and SALL4 was negatively regulated by miR-181b. Therefore, we speculated that SALL4 might play a role in the miR-181b-mediated malignant

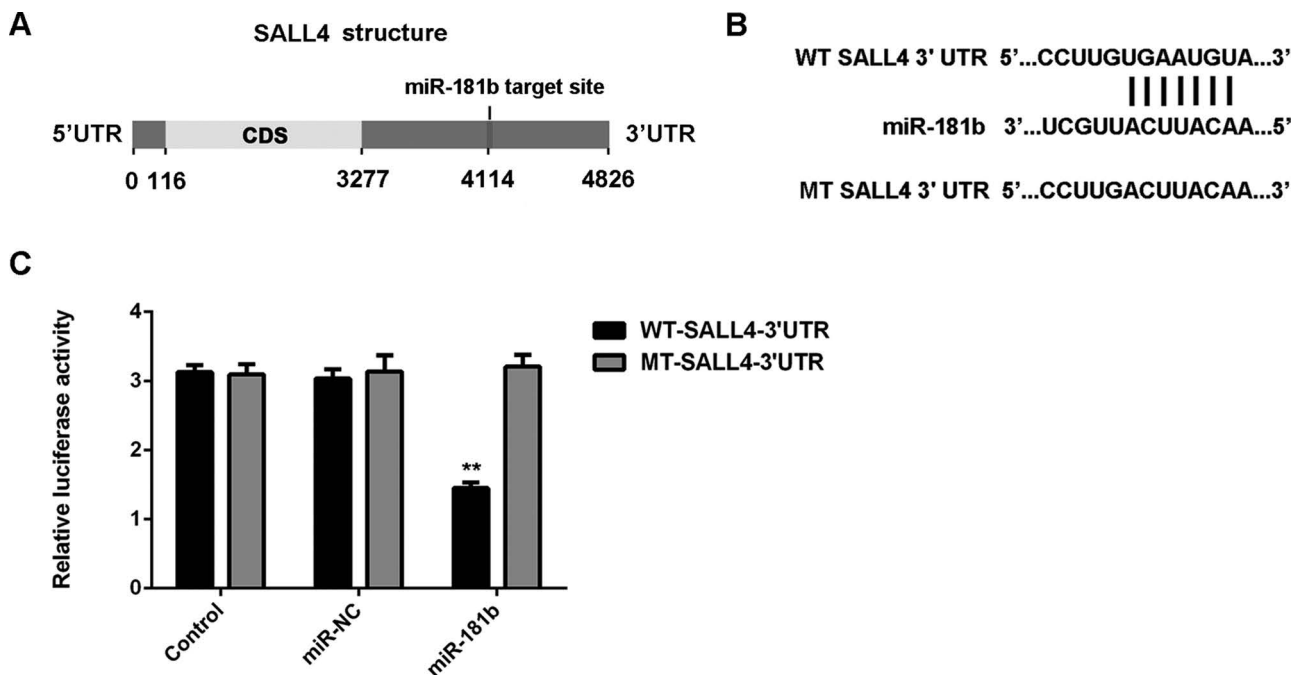


Figure 3. SALL4 is a target gene of miR-181b in glioma cells. (A) The TargetScan software indicated that SALL4 was a putative target of miR-181b, and their targeting relationship was evolutionally conserved. (B) The luciferase reporter plasmids containing the wild type (WT) or mutant type (MT) of SALL4 3'-UTR were constructed. (C) The luciferase activity was significantly decreased in U251 cells cotransfected with the WT-SALL4-3'-UTR luciferase reporter plasmid and miR-181b mimic, when compared to the control group, which was eliminated by transfection with MT-SALL4-3'-UTR luciferase reporter plasmid. ** $p < 0.01$ versus control.

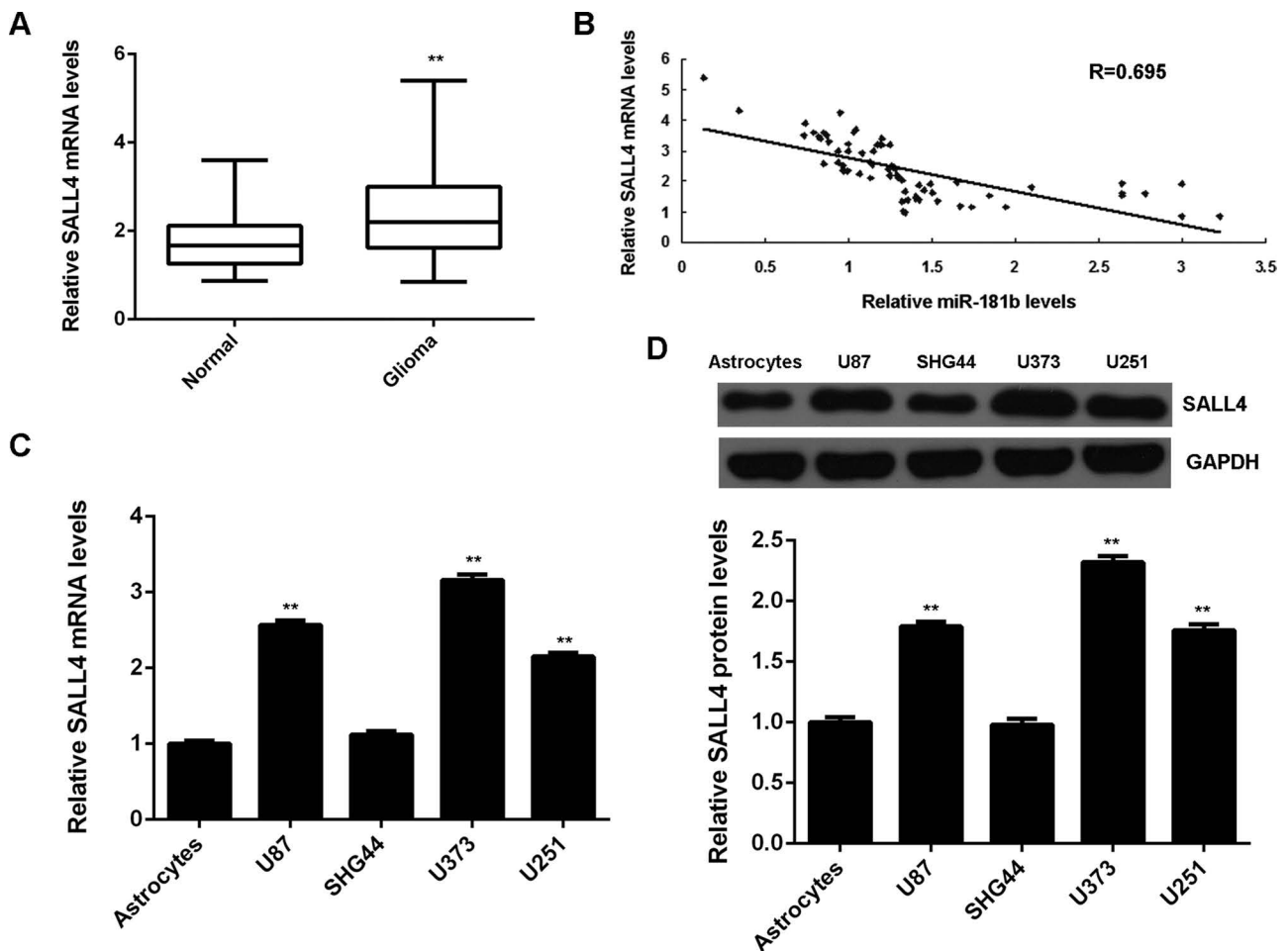


Figure 4. SALL4 is upregulated in glioma, with an inverse correlation to miR-181b levels. (A) RT-PCR was used to determine the mRNA levels of SALL4 in glioma tissues and normal brain tissues. $**p < 0.01$ versus Normal. (B) The SALL4 mRNA levels were inversely correlated to the miR-181b levels in glioma tissues. (C) RT-PCR and (D) Western blot were used to examine the mRNA and protein levels of SALL4 in glioma cell lines (U87, SHG44, U373, and U251) compared with normal human astrocytes. $**p < 0.01$ versus astrocytes.

phenotypes of U251 cells. To clarify this speculation, miR-181b-overexpressing U251 cells were further transfected with SALL4 expression plasmid or blank vector. Western blot data showed that the protein levels of SALL4 were significantly increased in the miR-181b+SALL4 group when compared to the miR-181b+blank group (Fig. 6A and B). Further investigation showed that cell proliferation, migration, and invasion were significantly upregulated in the miR-181b+SALL4 group when compared to the miR-181b+blank group (Fig. 6C–E). These findings suggest that miR-181b inhibits the malignant phenotypes of U251 cells via directly targeting SALL4.

DISCUSSION

Recently, miR-181b was reported to play a suppressive role in glioma, but its regulatory mechanism underlying

glioma growth and metastasis is still obscure. In this study, we found that miR-181b was significantly downregulated in glioma tissues and cell lines, and low expression of miR-181b was significantly associated with malignant progression and a poor prognosis in glioma. Overexpression of miR-181b significantly decreased the proliferation, migration, and invasion of glioma cells. SALL4, upregulated in glioma, was then identified as a novel target of miR-181b in glioma cells, and its expression levels were inversely correlated to miR-181b levels in glioma tissues. Further investigation revealed that SALL4 acted as a downstream effector in the miR-181b-mediated malignant phenotypes of glioma cells.

Recently, miR-181b was reported to play a suppressive role in glioma. Conti et al. reported that miR-181b was downregulated in glioma tissues compared with

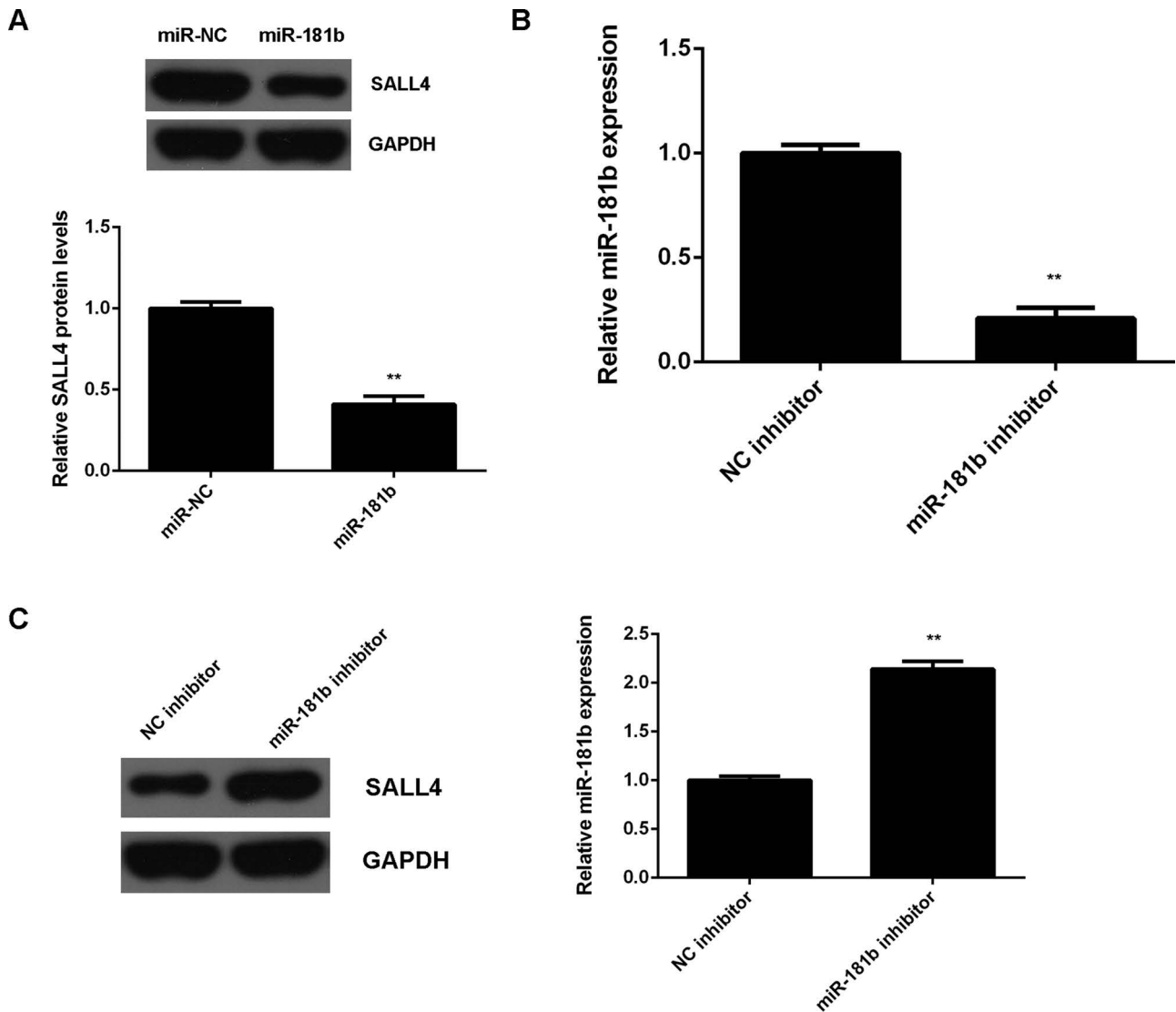


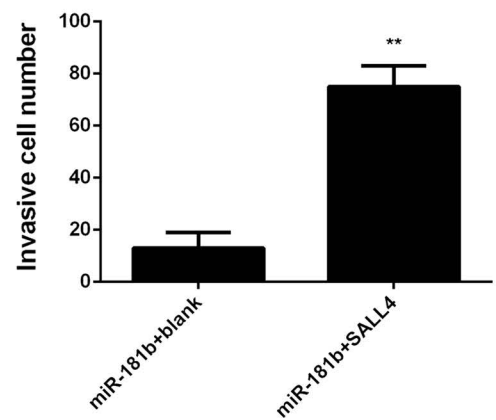
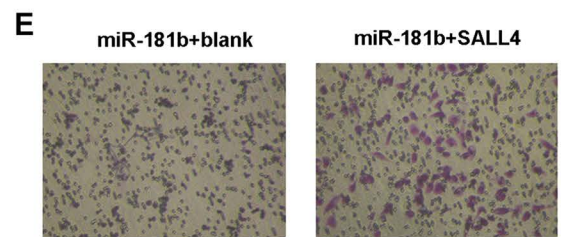
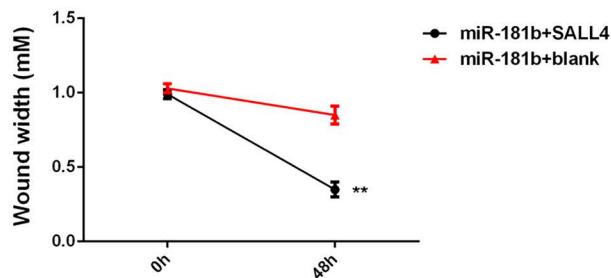
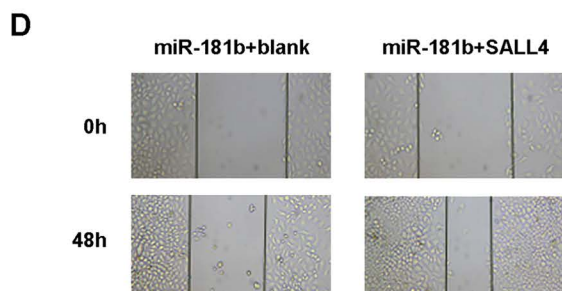
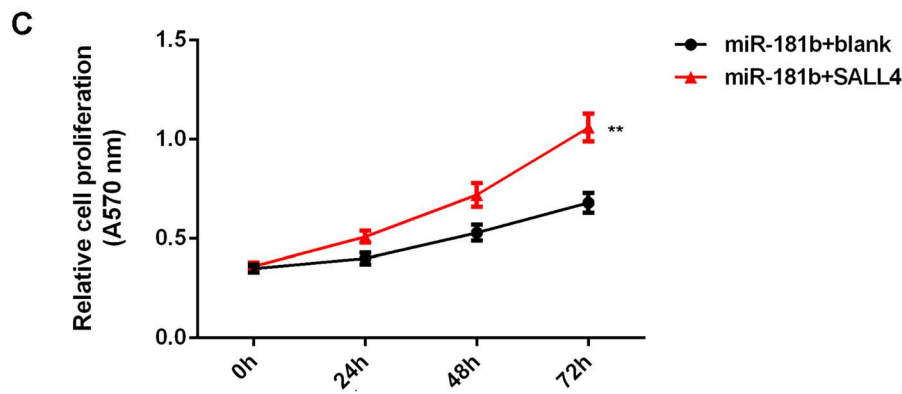
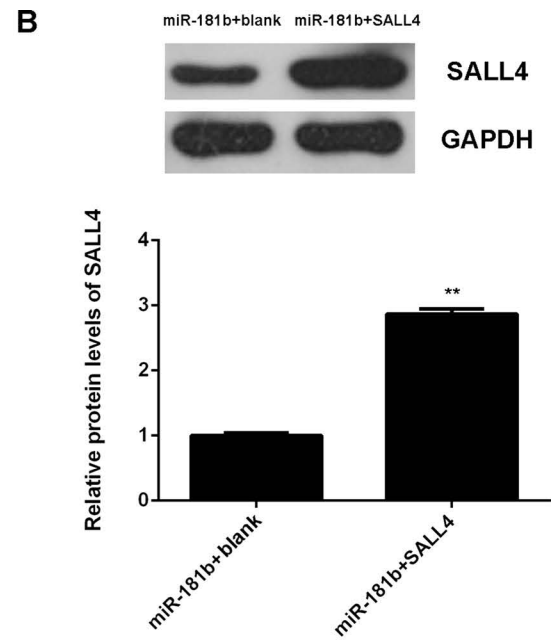
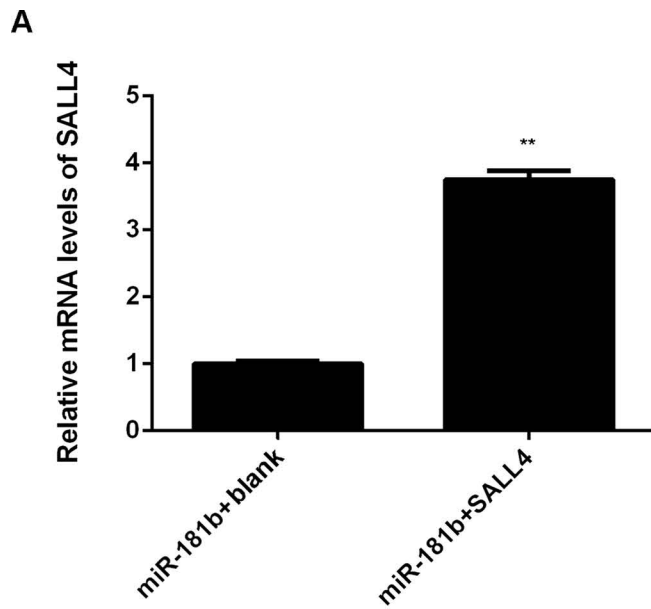
Figure 5. SALL4 is negatively regulated by miR-181b in glioma cells. (A) Western blot was used to examine the protein levels of SALL4 in U251 cells transfected with miR-181b mimic or scramble miR (miR-NC). ** $p < 0.01$ versus miR-NC. U251 cells were transfected with the miR-181b inhibitor or negative control (NC) inhibitor. (B) RT-PCR was used to determine the miR-181b levels. (C) Western blot was used to examine the protein levels of SALL4. ** $p < 0.01$ versus NC inhibitor (B–C).

normal brain tissue¹⁸. Shi et al. showed that miR-181a and miR-181b function as tumor suppressors, which trigger growth inhibition, inducing apoptosis and inhibiting invasion in glioma cells, and the tumor-suppressive effects of miR-181b were more apparent than miR-181a¹⁷. Li et al. reported that miR-181b suppressed the proliferation of and the reduced chemoresistance to temozolomide in glioma

U87 cells¹⁹. In the present study, we found that miR-181b was downregulated in glioma tissues and cell lines when compared with that in normal brain tissues and astrocytes. Moreover, the decreased miR-181b levels were significantly associated with advanced WHO grade, low KPS, and a poor prognosis in glioma, suggesting that downregulation of miR-181b may contribute to glioma progression.

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Figure 6. SALL4 acts as a downstream effector in miR-181b-mediated malignant phenotypes of U251 cells. miR-181b-overexpressing U251 cells were transfected with SALL4 expression plasmid or blank vector. (A) RT-PCR and (B) Western blot were used to examine the mRNA and protein levels of SALL4. (C) MTT, (D) wound healing, and (E) Transwell assays were conducted to examine cell proliferation, migration, and invasion. ** $p < 0.01$ versus miR-181b+blank.



For the first time, we identified SALL4 as a novel target gene of miR-181b, and its protein expression was negatively regulated by miR-181b in glioma cells. SALL4, a zinc finger transcription factor, is generally used as biomarker for stem cells and is involved in maintaining the self-renewal of embryonic stem cells²⁰. Recently, deregulations of SALL4 have been implicated in several human cancers, such as hepatocellular carcinoma (HCC)²¹, esophageal squamous cell carcinoma²², gastric cancer²³, and intrahepatic cholangiocarcinoma²⁴. For instance, SALL4 is upregulated in intrahepatic cholangiocarcinoma, and its high expression is associated with malignant progression and a poor prognosis²⁴. In our study, we found that SALL4 was also significantly upregulated in glioma tissues and cell lines when compared with that in normal brain tissues and astrocytes. Recently, Zhang et al. also found that SALL4 was upregulated in glioma tissues, consistent with our data, and that high SALL4 expression was correlated with a high-grade pathology and a poor outcome in patients with glioma²⁵. We further observed an inverse correlation between the miR-181b and SALL4 levels in glioma tissues, suggesting that downregulation of miR-181b may contribute to the upregulation of SALL4, which further promotes glioma progression.

Moreover, SALL4 was reported to play a promoting role in tumor cell proliferation, migration, invasion, angiogenesis, and drug resistance in different human cancers^{22–24}. For instance, knockdown of SALL4 expression by siRNA represses the proliferation of lung cancer cells by inducing a cell cycle arrest at the G₁ phase²⁶. Overexpression of SALL4 promoted the proliferation of HCC cells in vitro, correlating with increased expression of CK19, EpCAM, and ABCG2, while downregulation of SALL4 led to a significant decrease in growth inhibition in vitro and in vivo²⁷. In this study, we also found that siRNA-induced inhibition of SALL4 significantly decreased the proliferation, migration, and invasion of glioma cells. As SALL4 was found to be a target gene of miR-181b, we speculated that SALL4 might be involved in the miR-181b-mediated proliferation, migration, and invasion of glioma cells. Therefore, miR-181b-overexpressing U251 cells were then transfected with SALL4 expression plasmid to upregulate its expression, and we found that overexpression of SALL4 significantly eliminated the suppressive effects of miR-181b on the malignant phenotypes of U251 cells, which supports the hypothesis that the inhibitory role of miR-181b on the malignant phenotypes of glioma cells is through directly targeting SALL4. In addition to miR-181b, miR-107 was found to directly target SALL4 in glioma and play a tumor-suppressive role by inhibiting tumor cell proliferation and inducing cell apoptosis²⁸, and thus our study expands the understanding of the epigenetic regulatory mechanism of SALL4 expression in glioma. In addition,

several other targets of miR-181b in glioma have been reported, such as MDM2²⁹, KPNA4³⁰, NOVA1³¹, and MEK1³². Therefore, we also expand the understanding of the miR-related molecular mechanism underlying glioma progression.

In conclusion, our study demonstrates that miR-181b could inhibit the proliferation, migration, and invasion of glioma cells, at least partly, through directly targeting SALL4, highlighting the clinical importance of miR-181b/SALL4 in the treatment of glioma in the future.

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