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Original Article

MicroRNA-1179 inhibits glioblastoma cell proliferation and cell cycle progression via directly targeting E2F transcription factor 5

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Abstract: Glioblastoma multiforme (GBM) is an extraordinary aggressive disease that requires more effective therapeutic options. In the past few years, many microRNAs (miRNAs) have been demonstrated to have important roles in promoting GBM progression. However, little is known about the role of miR-1179 in GBM. In the present study, we found that miR-1179 was significantly downregulated in glioma tissues and cell lines. Functional experiments showed that introduction of miR-1179 dramatically suppressed GBM cell proliferation and cell cycle progression. Importantly, treatment of miR-1179 strongly inhibited tumor growth in a subcutaneous GBM model. Further studies showed that E2F transcription factor 5 (E2F5), a key transcription factor that controls cell cycle transition, was a direct target of miR-1179. Silencing of E2F5 inhibited the proliferative ability of GBM cells and induces cell cycle arrest, which were consistent with the effects of miR-1179 overexpression. More importantly, reintroduction of E2F5 into GBM cells reversed the tumor-suppressive function of miR-1179. Finally, we demonstrated that miR-1179 expression was negatively correlated with E2F5 messenger RNA (mRNA) levels in high-grade gliomas. Our findings provide new insights into the role of miR-1179 in the progression of GBM, and implicate the potential application of miR-1179 in GBM therapy.

Keywords: Glioblastoma, miR-1179, proliferation, cell cycle arrest, E2F5

Introduction

Glioblastoma multiforme (GBM) is the most prevalent type of primary brain tumor in human adults and represents one of the most aggressive and lethal human cancer types [1]. Despite the introductions of multimodal treatment approaches including maximal surgical resection, postoperative radiotherapy, and chemotherapy, the prognosis of these patients still remains poor [2]. This dismal prognosis is mainly due to losing control of GBM proliferation and progression [3]. Thus, a better understanding of the molecular pathogenesis of this disease and develop effective targeted therapies are needed to improve patient outcomes.

MicroRNAs (miRNAs), are short non-coding RNAs that downregulate gene expression at post-transcriptional levels by binding to the partially complementary 3'-untranslated regions (3'-UTRs) of specific messenger RNAs (mRNAs). Emerging evidence suggests that dysregulated miRNAs are significantly associated with GBM progression and prognosis due to their roles in the regulation of oncogenes or tumor suppressors, which suggests that miRNAs are promising biomarker candidates and potential therapeutic targets for GBM [4-7]. MiRNA-1179 (miR-1179) is located on chromosome 15q26.1, which has been identified as a cancer susceptibility loci [8]. Recently, several studies have reported that the aberrant expression of miR-1179 in various human cancer. However, its role in cancer progression remains controversial. Several lines of evidence demonstrated that miR-1179 plays a role as an oncogene in colorectal cancer (CRC) [9], esophageal squamous cell carcinoma (ESCC) [10] and familial breast cancer (FBC) [11]. In contrast, miR-1179 was shown to be downregulated in follicular and papillary thyroid tumors [12]. For GBM, the expression pattern, biological roles and poten-

tial molecular mechanism are still largely unknown.

E2F transcription factor 5 (E2F5) belongs to E2F family and regulates the expression of genes involved in cell cycle progression by directly binding to the promoters of these genes [13]. The overexpression or amplification of E2F5 has been observed in various human cancers including hepatocellular carcinoma [14], gastric cancer [15], breast cancer [16], prostate cancer [17], ovarian cancer [18], CRC [19], and ESCC [20]. Furthermore, dysregulated E2F5 is associated with cancer cell proliferation and progression [16-19]. However, the expression profile and potential role of E2F5 in GBM remains to be investigated.

In the present study, we showed that decreased expression of miR-1179 in human gliomas is correlated with tumor progression, and overexpression of miR-1179 can inhibit GBM cell proliferation and cell cycle progression through directly targeting of E2F5 both *in vitro* and *in vivo*. Our data provide new insights into the molecular function of miR-1179 as well as its regulatory mechanisms in GBM.

Materials and methods

Database mining and human tissue samples

Glioma miRNA expression data were obtained from two independent datasets: Chinese Glioma Genome Atlas (CGGA) data portal (http://www.cgga.org.cn/portal.php), and the National Central for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) dataset GSE25631 (http://www.ncbi.nlm.nih.gov.geo/, accession Nos. GSE25631). Glioma gene expression data were downloaded from The Cancer Genome Atlas (TCGA) dataset (http://cancergenome.nih.gov). 10 normal brain tissues (NBTs), 25 low-grade glioma (LGG; WHO grade II) and 64 high-grade glioma (HGG; WHO grade III and IV) samples were collected from the First Affiliated Hospital of Nanjing Medical University between 2010 and 2012. Both NBTs and glioma specimens were histological confirmed. All resected samples were immediately frozen in liquid nitrogen for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The Research Ethics Committee of Nanjing Medical University approved our study. The written informed consent from each patient was received before participation.

Cell culture and transfection

Human glioma cell lines SW1783, A172, U87, U251 and H4 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (Hyclone, Chicago, IL, USA) supplemented with 10% fetal bovine serum (Hyclone). Normal human astrocytes (NHAs) were obtained from Lonza (Walkersville, MD, USA), and cultured in the provided astrocyte growth media following the manufacturer's instructions.

miR-1179 mimic, and its negative control were chemically synthesized by Ribobio (Guangzhou, China). Small interfering RNA (siRNA) against E2F5 and its negative control siRNA were purchased from GenePharma (Shanghai, China). The E2F5-overexpression plasmid was generated by inserting E2F5 cDNA into a pcDNA3.1 vector. This plasmid was sequenced confirmed by GenePharma. All oligonucleotides and plasmids were transfected into cultured cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

RNA isolation and qRT-PCR

RNA extraction and qRT-PCR were performed as our previously described [4]. PCR primers used for miR-1179 and U6 RNA were obtained from GeneCopoeia (Guangzhou, China). Primers for E2F5 and β-actin were used as described previously [18]. The relative expression of each gene was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate and repeated three times.

Protein extraction and western blot analysis

Protein isolation and western blot analysis were performed as our previously reported [4]. The antibodies used for western blots were CDK2 (1:1000), CDK4 (1:1000), CDK6 (1:1000), CCND1 (1:1000), CCND2 (1:1000), and CCNE1 (1;1000, all from Cell Signaling Technologies, Danvers, MA, USA), and E2F5 (1:500) and β-actin (1:1000, all from Abcam, Cambridge, UK).

Cell proliferation assay

GBM cells were trypsinized and seeded into 96-well culture plates 24 h after transfection at a concentration of 1000 cells per well. Cell

Counting Kit-8 (CCK-8; Beyotime) was used to detect cell proliferation at the indicated time points according to the manufacturer's instructions.

Plate colony formation

For colony formation assay, transfected cells were seeded in 60-mm cell culture plates at a density of 400 cells per plates and cultured for 14 days. Colonies were fixed with 30% formaldehyde and stained with 0.1% crystal violet. Colonies with more than 50 cells were counted.

5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

Transfected cells were seeded into 24-well plates at 5×10^3 cells per well. After 48 h incubation, Cell-light EdU imaging detection kit (Invitrogen) was used according to the manufacturer's instructions. Finally, samples were imaged under a fluorescent microscope (Leica, Wetzlar, Germany).

Cell cycle assay

At 48 h posttransfection, cells were trypsinized, washed twice with phosphate buffered saline (PBS) and fixed in 75% ethanol at -20°C overnight. Afterward, cells were washed in PBS and incubated with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) in the presence of RNase A (Sigma-Aldrich) for 20 min in the dark before analysis by flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

Dual luciferase reporter assay

Wild-type (WT) and mutated putative miR-1179-binding sites in E2F5 3'-UTR region were cloned into the downstream region of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen). For reporter assay, cells were co-transfected with WT or mutated pGL3-E2F5-3'-UTR vectors and miR-1179 mimics (RiboBio). Luciferase activities were measured with a Dual Luciferase Reporter Assay Kit (Promega, Madison, USA) following manufacturer's protocols. Data were normalized against the activity of the Renilla luciferase gene.

Glioma xenografts

Male BALB/c-A nude mice (4-5 weeks old) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). For *in vivo* tumor growth assay, xenograft tumors were generated by subcutaneous injection of 1×10^7 U87 cells. Tumors were measured with calipers to estimate their volumes. Once the volume reached 50 mm^3, the mice were randomly assigned to two treatment groups. Each group (n = 6) was treated with miR-NC or miR-1179 mimic in 15 μL Lipofectamine 2000 through local injection of the tumor at multiple sites. The treatment was performed once every three days for 27 d. Tumor volume was calculated using the formula: $V = 0.5 \times \text{Length} \times \text{Width}^2$ (in millimeters). All animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University and in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Immunohistochemistry

Immunohistochemical (IHC) analysis was conducted to study Ki-67 protein expression in glioma xenografts. The procedure was carried out similarly to our previously described methods [4]. Briefly, fresh glioma xenografts were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm-sections. Then, the sections were immunohistochemically stained using Ki-67 antibody (1:200, Cell Signaling Technologies). Slides were imaged under a light microscope (Leica).

Statistical analysis

All statistical analyses were performed using Prism GraphPad version 6.0 (GraphPad Software Inc., San Diego, USA). The relationship between miR-1179 and the clinicopathological characteristics was tested by the chi-square test. Survival analysis was carried out using the Kaplan-Meier method with the log-rank test. Correlations between miR-1179 and E2F5 mRNA levels were analyzed using Pearson's correlation coefficient. Other comparisons were analyzed using two-tailed Student's *t*-test and the results are expressed as the mean ± SD. A *p*-value less than 0.05 was considered statistically significant.

Results

Downregulation of miR-1179 is correlated with progression of primary gliomas

To identify the roles of miR-1179 in the progression of GBM, we first analyzed the expression level of miR-1179 in two independent glioma

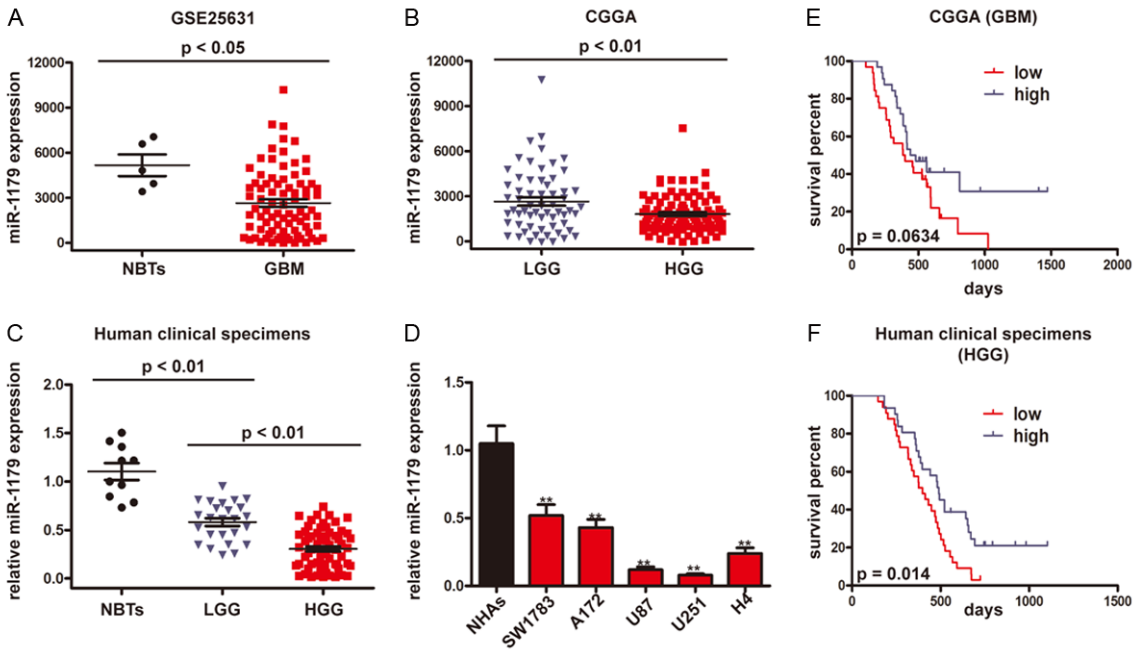

Figure 1. miR-1179 is frequently downregulated in glioma and is a promising prognostic biomarker for glioma. A. GSE25631 database showing miR-1179 expression in NBTs and GBM. B. CGGA database showing miR-1179 expression in LGG and HGG. C. qRT-PCR analysis of miR-1179 expression in 10 NBTs, 25 LGG and 64 HGG. Transcript levels were normalized by U6 expression. D. qRT-PCR analysis of miR-1179 expression in NHAs and 5 glioma cell lines (SW1783, A172, U87, U251 and H4). Transcript levels were normalized by U6 expression. **P < 0.01. E. Kaplan-Meier analysis of overall survival duration in GBM patients according to miR-1179 expression using CGGA database. F. Kaplan-Meier analysis of overall survival duration in HGG patients according to miR-1179 expression.

Table 1. Correlation of miR-1179 expression with clinicopathological features in 64 high-grade glioma patients

Variable	miR-1179 low	miR-1179 high	*P* value
Gender (F/M)	15/18	17/14	0.453
Age at diagnosis (years)	51.52 ± 4.12	49.78 ± 3.54	0.076
Location (frontal/non-frontal)	22/11	16/15	0.220
KPS (≥ 80/< 80)	20/13	13/18	0.135
Resection (total/subtotal)	24/9	25/6	0.455
Tumor size (≥ 4 cm/< 4 cm)	22/11	14/17	0.083
IDH1 mutation (WT/Mut)	28/5	24/7	0.447
MGMT promoter methylation (unmethylation/methylation)	13/20	12/19	0.955
Ki-67 (low/high)	9/24	19/12	0.006
PCNA (low/high)	10/23	17/14	0.047
PTEN (low/high)	19/13	20/11	0.674
EGFR (low/high)	21/12	15/16	0.219

Fisher's exact probability test was used for the assay. *KPS*, Karnofsky Performance Scale; *IDH1*, Isocitrate Dehydrogenase (NADP (+)) 1; *MGMT*, O-6-Methylguanine-DNA Methyltransferase; *Ki-67*, Marker Of Proliferation Ki-67; *PCNA*, Proliferating Cell Nuclear Antigen; *PTEN*, Phosphatase And Tensin Homolog; *EGFR*, Epidermal Growth Factor Receptor.

miRNA expression datasets (GSE25631 and CGGA). Compared with NBTs, significant downregulation of miR-1179 was observed in GBM tissues (**Figure 1A**). We also observed that miR-1179 levels were more reduced in high-grade glioma (HGG; WHO grades III and IV) than low-grade glioma (LGG; WHO grade II) (**Figure 1B**). To validate these observations in patient-drived

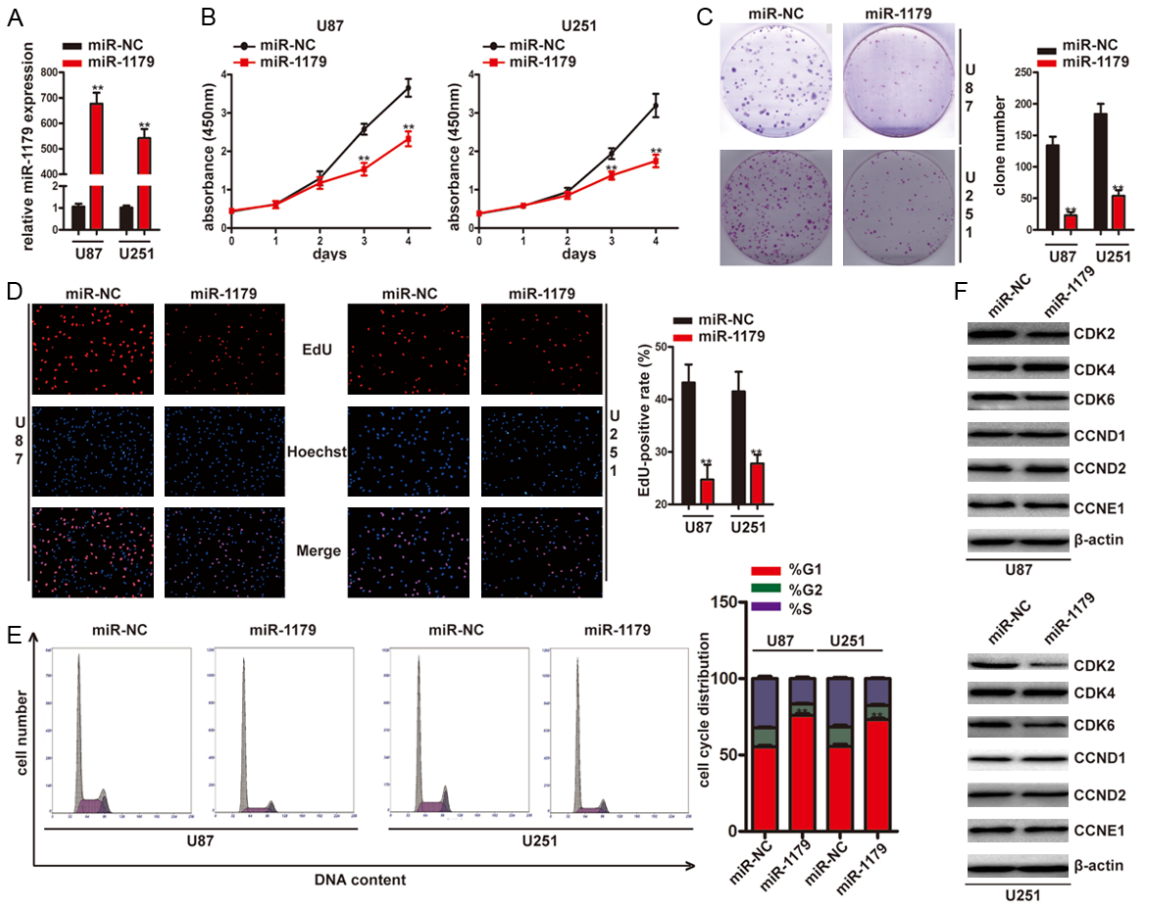

Figure 2. miR-1179 inhibits GBM cell proliferation and induces cell cycle arrest *in vitro*. A. qRT-PCR analysis of miR-1179 expression in U87 and U251 cells transfected with miR-1179 mimic and miR-NC. **P < 0.01. B. Effect of miR-1179 and miR-NC on U87 and U251 cell growth rate as measured by CCK-8 assays. **P < 0.01. C. Effect of miR-1179 and miR-NC on U87 and U251 proliferative ability as determined by colony formation assays. **P < 0.01. D. The effect of miR-1179 and miR-NC on the growth of U87 and U251 cells was examined by EdU incorporation assays (original magnification 200 X). **P < 0.01. E. Effect of miR-1179 and miR-NC on cell cycle distribution of U87 and U251 cells. **P < 0.01. F. Western blot analysis of CDK2, CDK4, CDK6, CCND1, CCND2 and CCNE1 protein levels in U87 and U251 cells after transfection with miR-1179 and miR-NC. β-actin was used as the loading control.

tissues, we next analyzed miR-1179 expression in 10 NBTs, 25 LGG and 64 HGG samples by qRT-PCR. Our results revealed that miR-1179 was significantly downregulated in glioma tissues, especially in HGG, which are consistent with the genomic datasets (**Figure 1C**). The expression of miR-1179 in glioma cell lines was also examined, NHAs cell line was used as a control. Significant downregulation of miR-1179 was observed in SW1783, A172, U87, U251 and H4 cells (**Figure 1D**).

Furthermore, we explored the relationship between miR-1179 expression and clinicopathological data. miR-1179 expression levels in HGG tissues were divided into two groups including the high expression group and the lower expression group. The data showed that low miR-1179 expression was positively related to high Ki-67 and PCNA expression (**Table 1**), which suggested that miR-1179 level was correlated with cell proliferation. More importantly, the survival analysis showed that HGG patients with high miR-1179 expression were characterized by better overall survival than patients with low miR-1179 expression (**Figure 1E** and **1F**). These results indicated that miR-1179 is involved in glioma progression.

Overexpression of miR-1179 inhibits GBM cell proliferation and cell cycle progression

To explore its biological function in GBM, miR-1179 mimic and miR-1179 negative control

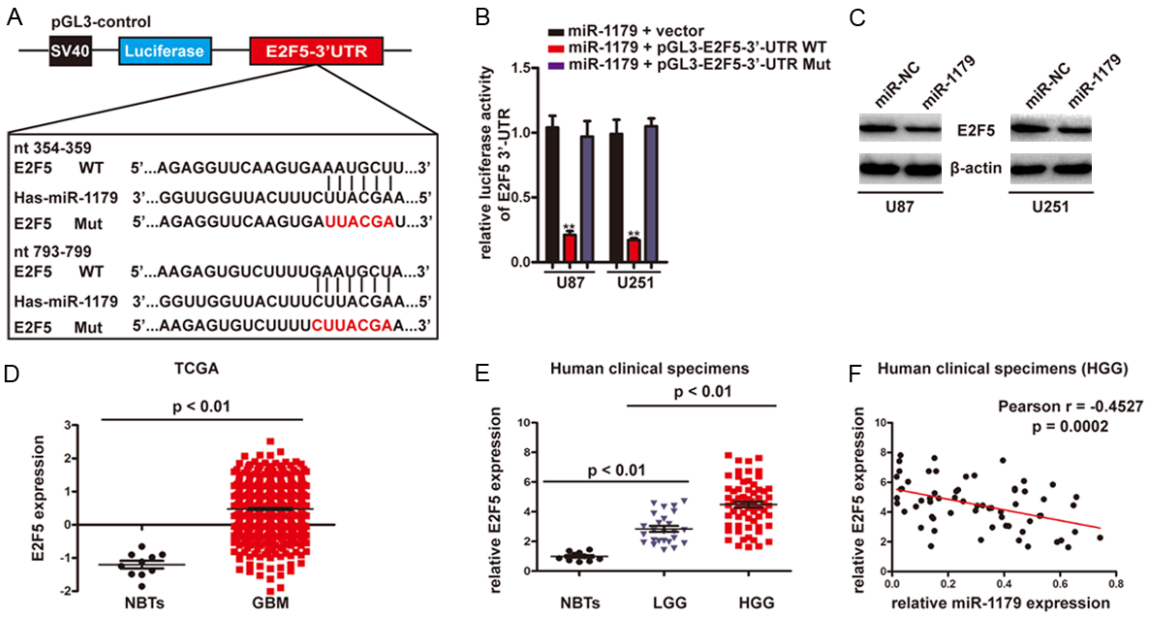

Figure 3. E2F5 is direct target of miR-1179 in U87 and U251 cells. A. Predicted binding sites of wild-type (WT) and mutated sequences of miR-1179 in the 3'-UTR of E2F5 mRNA. B. Luciferase assays of U87 and U251 cells transfected with pGL3-E2F5 3'-UTR WT or pGL3-E2F5 3'-UTR Mut reporter with miR-1179 mimic. **P < 0.01. C. Western blot analysis of E2F5 protein levels in U87 and U251 cells after transfection with miR-1179 and miR-NC. β-actin was used as the loading control. D. TCGA database showing E2F5 expression in NBTs and GBM. E. qRT-PCR analysis of E2F5 expression in 10 NBTs, 25 LGG and 64 HGG. Transcript levels were normalized by β-actin expression. F. Pearson's correlation analysis of the relative expression levels of miR-1179 and the relative E2F5 mRNA levels in HGG.

(miR-NC) were transfected into U87 and U251 cells. More than 500-fold increase in miR-1179 expression was observed in miR-1179 mimic transfected cells compared with miR-NC transfected cells by qRT-PCR (**Figure 2A**). Subsequently, GBM cell proliferation was detected *in vitro*. CCK-8 assays showed that overexpression of miR-1179 resulted in a significant decrease in growth rate than miR-NC transfected cells on day 3 after plating (**Figure 2B**). Ectopic miR-1179 expression blocked proliferation of GBM cells proliferation in colony formation assays (**Figure 2C**). Additionally, EdU incorporation assays showed that the growth of miR-1179 transfected cells were dramatically inhibited by miR-1179 relative to miR-NC (**Figure 2D**). To investigative the mechanism by which overexpression of miR-1179 blocks GBM cell proliferation, we determined whether growth inhibition was associated with cell cycle dysfunction. The effect of miR-1179 on the cell cycle distribution in U87 and U251 cells were examined by flow cytometric analysis. Compared with miR-NC transfected cells, miR-1179 transfected cells showed marked increase in the number of G0/G1 phases (**Figure 2E**). To further investigate the molecules involved in this cell cycle block, we measured the expression of Cyclin D1 (CCND1), Cyclin D2 (CCND2), Cyclin E1 (CCNE1), and cyclin-dependent kinases (CDKs; CDK2, CDK4 and CDK6) in both miR-1179 transfected cells and miR-NC transfected cells by western blot. All of these proteins have been previously reported as important regulators of G1 phase [21, 22]. As shown in **Figure 2F**, we observed that overexpression of miR-1179 markedly inhibited CDK2 and CDK6 expression in U87 and U251 cells, while the expression levels of CCND1, CCND2, CCNE1 and CDk4 were unchanged. Collectively, these results demonstrated that ectopic expression of miR-1179 inhibits GBM cell proliferation and induces G0/G1 cell cycle arrest.

miR-1179 directly targets E2F5 3'-UTR

To delineate the mechanism by which miR-1179 inhibited cell proliferation and cell cycle progression in GBM cells, miR-1179 target genes were searched using the micorna.org algorithm. We identified that miR-1179 could potentially target E2F5 (**Figure 3A**), which has been reported to be involved in the promotion of cell proliferation and cell cycle progression

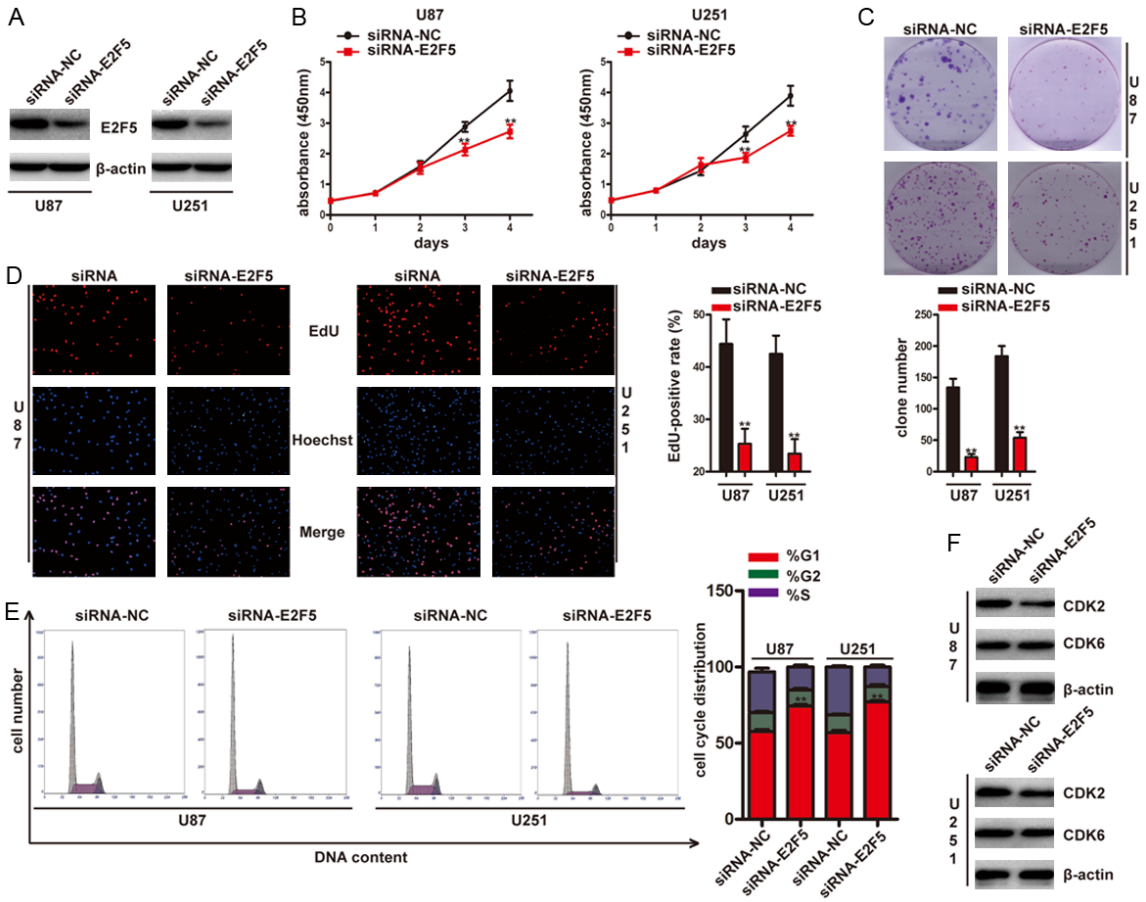

Figure 4. E2F5 knockdown suppresses GBM cells proliferation and cell cycle progression *in vitro*. A. Western blot analysis of E2F5 protein levels in U87 and U251 cells after knockdown E2F5. β-actin was used as the loading control. B. Effect of E2F5 knockdown on U87 and U251 growth rates as measured by CCK-8 assays. **P < 0.01. C. Effect of E2F5 knockdown on U87 and U251 proliferative ability as determined by colony formation assays. **P < 0.01. D. The effect of E2F5 knockdown on the growth of U87 and U251 cells was examined by EdU incorporation assays (original magnification 200 X). **P < 0.01. E. Effect of E2F5 knockdown on cell cycle distribution of U87 and U251 cells. **P < 0.01. F. Western blot analysis of CDK2 and CDK6 protein levels in U87 and U251 cells after E2F5 knockdown. β-actin was used as the loading control.

[14, 16-18]. To confirm whether miR-1179 mediated the expression of E2F5, a dual-luciferase reporter system was employed. We subcloned 3'-UTR of E2F5 mRNA including the predicted miR-1179 recognition site (WT) or the mutated sequences (mutate type) into the pGL3 vector, downstream of the luciferase open reading frame. Our results showed that the pGL3 vector with 3'-UTR WT of E2F5 resulted in a significant decrease in luciferase activity after transfection with miR-1179 mimic, whereas the vector with mutated 3'-UTR of E2F5 had no change in luciferase activity (**Figure 3B**). Subsequently, protein levels of E2F5 in miR-1179 transfected cells were detected by western blot. Overexpression of miR-1179 significantly decreased E2F5 protein levels compared with miR-NC transfected cells (**Figure 3C**).

We further examined E2F5 mRNA expression pattern in TCGA database. As shown in **Figure 3D**, the mRNA levels of E2F5 in GBM were significantly upregulated compared with those in NBTs. To determine whether the expression of E2F5 was associated with miR-1179 in glioma or not, the expression of E2F5 mRNA in 10 NBTs, 25 LGG and 64 HGG samples were also measured. We found that E2F5 mRNA levels were higher in glioma samples than NBTs and increased with ascending pathological grade (**Figure 3E**). Pearson's correlation analysis revealed a significant negative correlation between miR-1179 and E2F5 in HGG (**Figure**

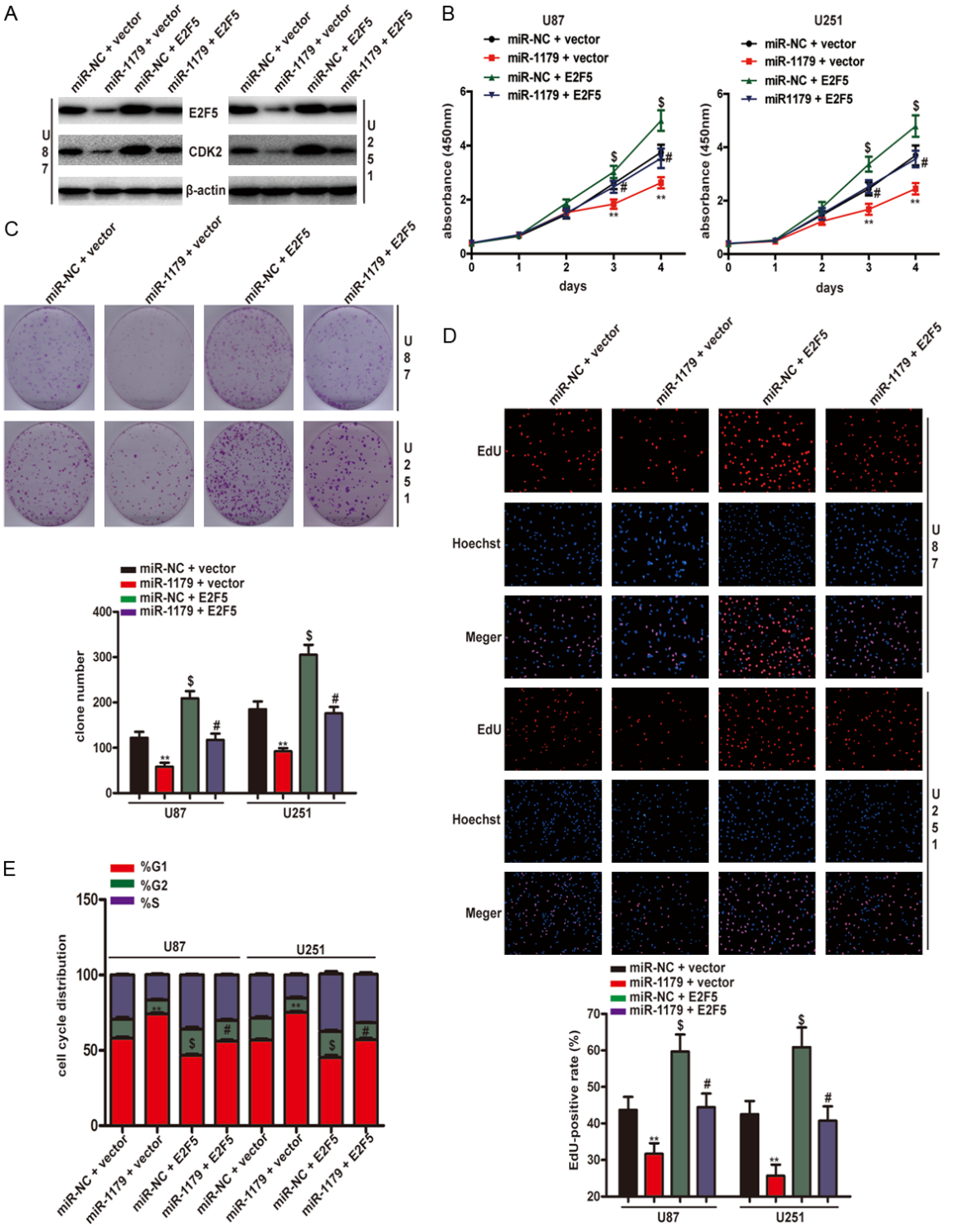

Figure 5. Reintroduction of miR-1179 abrogated the inhibitory effects of miR-1179 on GBM cell proliferation and cell cycle transition. A. Western blot analysis of E2F5 and CDK2 protein levels in cells transfected with vector or E2F5 in the presence of miR-NC or miR-1179. β-actin was used as the loading control. B. Effect of E2F5 reintroduction on miR-NC or miR-1179 transfected U87 and U251 growth rates as measured by CCK-8 assays. C. Effect of E2F5 reintroduction on miR-NC or miR-1179 transfected on U87 and U251 proliferative ability as determined by colony formation assays. D. The effect of E2F5 reintroduction on the growth of miR-NC or miR-1179 transfected U87 and U251 cells was examined by EdU incorporation assays (original magnification 200 X). E. Effect of E2F5 reintroduction on cell cycle distribution of miR-NC or miR-1179 transfected U87 and U251 cells. **indicates a statistically significant

difference (P < 0.01) between miR-NC + vector group and miR-1179 + vector group. $ indicates a statistically significant difference (P < 0.01) between miR-NC + vector group and miR-NC + E2F5 group. # indicates a statistically significant difference (P < 0.01) between miR-NC + E2F5 group and miR-1179 + E2F5 group.

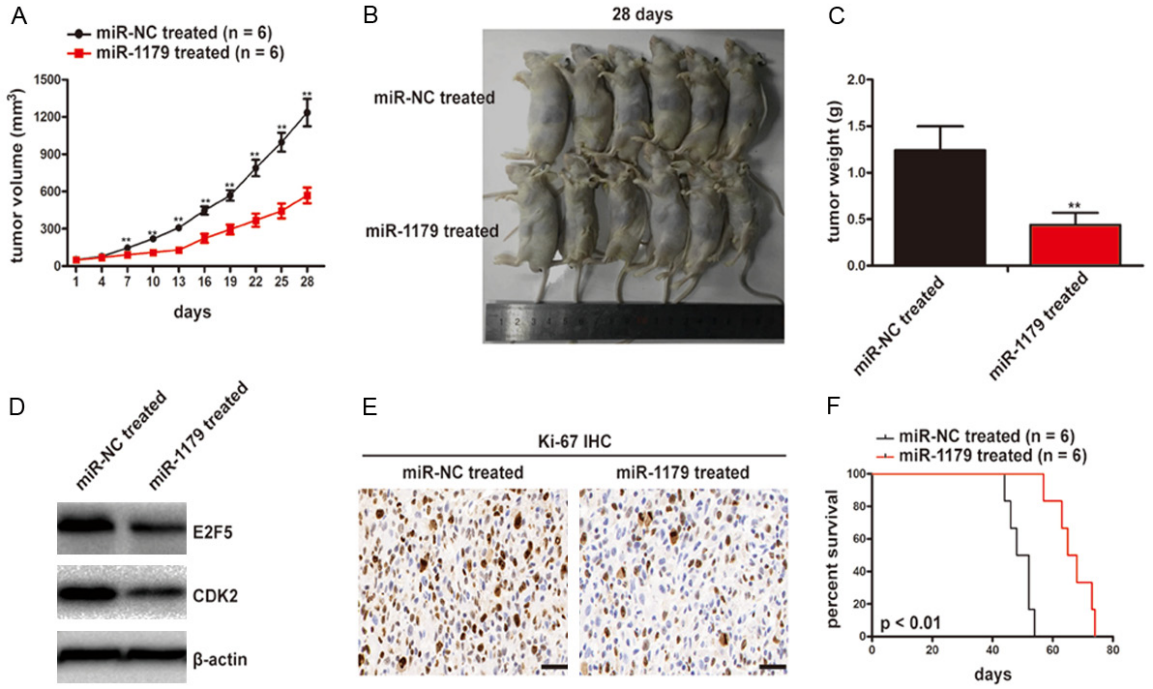

Figure 6. miR-1179 mimic treatment inhibits GBM growth *in vivo*. A. U87 cells were subcutaneously implemented into nude mice. Treatment started when the tumor volume reached 50 mm^3. miR-NC or miR-1179 was injected intratumorally into each subcutaneous tumor every 3 d. Tumor volume was measured using a vernier caliper on the indicated days. **P < 0.01. B. Images of mice in the miR-NC and miR-1179 treated groups. C. Tumor weight in the miR-NC and miR-1179 treated groups. **P < 0.01. D. Western blot analysis of E2F5 and CDK2 protein levels in miR-NC and miR-1179 treated groups. β-actin was used as the loading control. E. Ki-67 staining of subcutaneous tumors in miR-NC and miR-1179 treated groups. Scale bar = 50 μm. F. Kaplan-Meier curves were drawn to measure overall survival in miR-NC treated and miR-1179 treated groups.

3F). These results suggested that E2F5 may be a target of miR-1179 in primary gliomas.

E2F5 functions to promote GBM cell proliferation and cell cycle progression

To determine whether the biological functions of E2F5 and miR-1179 were same or not, E2F5 was knocked down using siRNA and then the cell proliferation and cell cycle distribution were detected. After 48 h, the expression of E2F5 protein decreased more than 60% in cells transfected with siRNA-E2F5 (**Figure 4A**). The results of CCK-8 assays showed that the proliferation capacity of U87 and U251 cells was significantly reduced after treatment with siRNA-E2F5 (**Figure 4B**). Colony formation was also significantly reduced following E2F5 silencing (**Figure 4C**). Additionally, EdU incorporation assays revealed that E2F5 knockdown dramatically reduced the EdU positive rates compared with control groups (**Figure 4D**). Furthermore, E2F5 downregulation induced GBM cell cycle arrest at G0/G1 phases (**Figure 4E**). To explore the molecules involved in this cell cycle block, we measured the expression of CDK2 and CDK6 protein levels in siRNA-E2F5 and siRNA-NC transfected cells by western blot. As shown in **Figure 4F**, we observed that knockdown of E2F5 markedly inhibited CDK2 expression in U87 and U251 cells, while the expression levels of CDK6 remained unchanged. Collectively, these results demonstrated that silencing of E2F5 inhibits GBM cell proliferation and induces G0/G1 cell cycle arrest as effectively as miR-1179 overexpression.

Ectopic expression of E2F5 reverses miR-1179 suppression of GBM cell proliferation and cell cycle progression

To determine whether miR-1179 targeting E2F5 was responsible for inhibition of the prolifera-

tion and cell cycle progression of GBM cells. We constructed an expression vector that encode the entire E2F5 coding sequence but lacks the 3'-UTR. Then we cotransfected this vector or its control with miR-1179 mimic or miR-NC into U87 and U251 cells. Both cell proliferation and cell cycle assay data showed that concomitant overexpression of miR-1179 and E2F5 abrogated the inhibitory effects of miR-1179 (**Figure 5B-E**). Meanwhile, the expression levels of CDK2 also recovered after exogenous introduction of E2F5 (**Figure 5A**). These results suggest that E2F5 is a functional target of miR-1179 in GBM cells.

miR-1179 suppresses tumorigenicity in vivo

To assess the therapeutic potential of miR-1179 *in vivo*, an U87 xenograft model was employed. Xenograft tumors from the miR-1179-treated group exhibited a dramatic reduction in tumor volume compared with the miR-NC-treated group (**Figure 6A** and **6B**). Tumor weights were also significantly reduced in the siRNA-E2F5-treated group (**Figure 6C**). Western blot confirmed the E2F5 and CDK2 downregulation in miR-1179-treated group (**Figure 6D**), which was consistent with the *in vitro* results. Ki-67 staining showed that tumors of miR-1179-treated group had fewer proliferative cells than miR-NC-treated group (**Figure 6E**). Furthermore, miR-1179-treated mice showed increased survival compared with miR-NC-treated mice (**Figure 6F**). These results demonstrated that miR-1179 could exert a significant inhibitory effect on tumorigenesis of GBM cells *in vivo.*

Discussion

The rapid proliferative ability of GBM is a critical contributing factor leading to dismal prognoses, but the involved molecular mechanisms remain largely unknown. Thus, identification of key genes that are dysregulated in GBM tissues and elucidation of mechanisms that lead to aberrant expression of genes promoting GBM progression are essential to develop successful management of GBM [5, 6]. Emerging evidence suggests that miRNAs play important role in tumorigenesis of human cancers and function as molecular biomarker for cancer diagnosis, prognosis and treatment [7]. In this study, we explored the biological functions of miR-1179 and its target gene E2F5 in GBM cell proliferation and cell cycle progression.

miR-1179 has dual functions: either as tumor suppressor or oncogene dependent on the specific cancer type. It has been reported that miR-1179 was upregulated in a number of solid tumors, including CRC [9], ESCC [10] and FBC [11] and plays an oncogenic role by directly targeting slit guidance ligand 2 [10]. Inversely, miR-1179 might play a tumor suppressive role in follicular and papillary thyroid tumors [12]. In this study, we found that miR-1179 was decreased in miRNA expression datasets (GSE25631 and CGGA), glioma tissue samples as well as glioma cell lines (**Figure 1A-D**). Further analysis showed that low miR-1179 levels were correlated with high Ki-67 and PCNA in HGG patients (**Table 1**). More importantly, low miR-1179 was associated with decreased survival in participants with HGG (**Figure 1E** and **1F**). These data suggested that miR-1179 may serve as a potential diagnostic biomarker and therapeutic target for glioma patients.

Mounting evidence has demonstrated that dysregulated miRNAs are usually correlated with malignant biological behaviors including proliferation, cell cycle progression, chemoresistance, angiogenesis, apoptosis, migration and invasion [7]. To test the biological function of miR-1179 in glioma, we overexpressed miR-1179 in U87 and U251 cells by transfecting them with miR-1179 mimics (**Figure 2A**). Results showed that restoration of miR-1179 can decrease GBM cell proliferation, induce cell cycle arrest at G0/G1 phases and inhibit CDK2 and CDK6 expression (**Figure 2B-F**). Importantly, *in vivo* studies revealed a marked decrease in subcutaneous xenograft tumor growth following miR-1179 mimics treatment (**Figure 6A-C**), indicating its therapeutic potential for GBM patients. Although we focused on the biological functions of miR-1179 in GBM, little is known about the mechanism contributing to the downregulation of miR-1179 in GBM. Previous studies have demonstrated that impaired miRNA biogenesis process, misexpressed transcriptional factors and aberrant epigenetic regulation are major factors that induce dysregulated miRNAs in human cancers [23-27]. Therefore, elucidating the key mechanism that contributes to downregulation of miR-1179 are essential for development of miR-1179-based treatments.

Several studies have demonstrated that E2F5 is a crucial transcriptional factor that exerts

various biological functions [13]. A variety of human cancers have recently been reported to overexpress E2F5 protein. E2F5 protein is well-known for its role in cell proliferation and cell cycle progression by binding with pocket proteins in the G1 phase [13, 28, 29]. Furthermore, patients with high E2F5 expression are correlated with worse prognoses [20]. However, the molecular mechanism responsible for the observed E2F5 upregulation in human cancers remains largely unknown. Previous studies have shown that E2F5 is negatively regulated by multiple miRNAs, such as miR-34a [19], miR-613 [30], miR-98 [31], miR-154-5p [17], miR-128-2 [32], and miR-106 [33]. These studies demonstrated that miRNA-mediated repressing mechanism play a key role in regulating E2F5 expression in human cancers. In this study, we showed for the first time that miR-1179 inhibits GBM cell proliferation and induces cell cycle arrest by specifically targeting E2F5, which was demonstrated by luciferase activity assays (**Figure 3A** and **3B**). Moreover, upregulation of miR-1179 in U87 and U251 cells can decrease E2F5 protein level (**Figure 3C**). Furthermore, we found that E2F5 is upregulated in human gliomas (**Figure 3D** and **3E**) and inversely correlated with miR-1179 levels in HGG (**Figure 3F**). Thus, it is reasonable to conclude that overexpression of E2F5 in GBM may be the result of decreased levels of miR-1179.

To determine whether E2F5 is a functional target of miR-1179 in U87 and U251 cells, we knocked down E2F5 by using specific siRNA (**Figure 4A**). Results showed that inhibition of E2F5 expression decreased GBM cell proliferation and induced cell cycle arrest as effectively as miR-1179 overexpression (**Figure 4B-E**), whereas overexpression of E2F5 antagonizes the effects of miR-1179 overexpression. Our findings provide the first evidence that E2F5 is a crucial mediator of the miR-1179-induced GBM-suppressive function. Thus, we conclude that upregulated E2F5 induced by decreased miR-1179, may facilitate GBM cell proliferation and cell cycle progression, which ultimately drives GBM progression and induces patients death.

Although we have demonstrated tumor promoting role of E2F5 in GBM, the underlying mechanisms remain ambiguous. It has been reported that E2F5 overexpression activates p38/SMAD3 signaling axis [29], a key regulator of GBM progression [34]. Whether E2F5 promotes GBM progression via activating p38/SMAD3 pathway remains to be determined. Additionally, E2F5 directly interacts with pocket proteins and then promotes cell cycle transition [13]. The interaction of E2F5 with pocket proteins and their functions in GBM proliferation and cell cycle progression need to be investigated in the further.

In summary, our results demonstrated that the expression of miR-1179 is significantly downregulated in glioma tissues and cell lines and is negatively associated with E2F5 expression levels. Overexpression of miR-1179 is able to inhibit GBM cell proliferation and induce cell cycle arrest by directly targeting E2F5. Thus, miR-1179/E2F5 axis may serve as a promising therapeutic target for GBM treatment.

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Disclosure of conflict of interest

None.

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