miR-423-5p contributes to a malignant phenotype and temozolomide chemoresistance in glioblastomas

Shouwei Li[†], Ailiang Zeng[†], Qi Hu[†], Wei Yan, Yanwei Liu, and Yongping You

Department of Neurosurgery, Sanbo Brain Hospital, Capital Medical University, Beijing, China (S.L.); Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China (A.Z., Q.H., W.Y., Y.Y.); Beijing Neurosurgical Institute, Capital Medical University, Beijing, China (Y.L.)

Corresponding Author: Shouwei Li, MD, Department of Neurosurgery, Sanbo Brain Hospital, Capital Medical University, Xiangshanyikesong 50#, HaiDian District, Beijing 100093, China (lishouweisanbo@sina.com).

[†]These authors contributed equally to this work.

Abstract

Background Gliomas are based on a genetic abnormality and present with a dismal prognosis. MicroRNAs (miR-NAs) are considered to be important mediators of gene expression in glioma tissues.

Methods Real-time PCR was used to analyze the expression of microRNA-423-5p (miR-423-5p) in human glioma samples and normal brain tissue. Apoptosis, cell cycle, proliferation, immunostaining, transwell, in vitro 2D and 3D migration, and chemosensitivity assays were performed to assess the phenotypic changes in glioma cells overexpressing miRNA-423-5p. Western blotting was used to determine the expression of inhibitor of growth 4 (ING-4)in glioma tissues, and a luciferase reporter assay was conducted to confirm whether ING-4 is a direct target of miR-423-5p. Western blotting was used to identify the potential signaling pathways that are affected in glioma cell growth by miR-423-5p. Xenograft tumors were examined in vivo for the carcinogenic effects of miR-423-5p in glioma tissues.

Results We first reported that miR-423-5p expression was increased in gliomas and was a potential tumor promoter via targeting ING-4. The overexpression of miR-423-5p resulted in upregulation of important signaling molecules such as p-AKT and p-ERK1/2. In clinical samples, miR-423-5p was dysregulated, and a corresponding alteration in ING-4 expression was observed (P = .0207). Furthermore, the overexpression of miR-423-5p strengthened glioma cell proliferation, angiogenesis, and invasion. Finally, miR-423-5p overexpression also strengthened GBM neurosphere formation and rendered glioma cells resistant to temozolomide (TMZ).

Conclusion This study establishes that miR-423-5p functions as an oncogene in glioma tissues by suppressing ING-4 and suggests that it has therapeutic potential for glioma.

Glioblastoma (GBM), the most common and aggressive primary brain tumor, carries an invariably poor clinical outcome.[1] GBM, a grade IV diffuse glioma according to the World Health Organization (WHO) scale, is characterized by highly infiltrative growth with invariably aggressive biological features, requires definitive surgical resection, and leads to a dismal prognosis for the patient.[2] Despite treatment involving surgery, ionizing radiation, and chemotherapy with the alkylating agent temozolomide (TMZ), patients with GBM only have a median survival rate of approximately 14 months.[3] Therefore, it is essential to find novel and effective therapeutic methods for treating glioma.

MicroRNAs (miRNAs) are small, endogenous noncoding RNAs that act as regulators of gene expression by posttranscriptionally reducing target mRNAs with partial complementarity in their 3′-untranslated regions (UTRs).[4] miRNAs are found to function as tumor suppressors or oncogenes involved in the pathogenesis of tumors.[5]

Recently, many studies have reported the observation of upregulated and downregulated microRNAs in several tumors, including glioma and its aggressive glioblastoma subtype.[6] Among them, miR-21 is highly overexpressed in glioblastoma cells and contributes to their malignant phenotype by blocking the expression of critical apoptosis-related genes.[7] In contrast to miR-21, miR-7 expression is decreased in glioblastomas and suppresses AKT pathway activation and EGFR expression via its 3′-UTR. Increased expression of miR-7 inhibits the viability and invasiveness of glioblastoma cells.[8] Our previous studies demonstrated that overexpressed miR-423-5p is involved with chemoresistance to TMZ ("TCR subtype") when compared with TMZ chemosensitivity ("TCS subtype") and indicated a significantly poorer clinical outcome.[9] However, further research needs to be performed to clarify the role of miR-423-5p in the development of glioma.

The inhibitor of growth (ING) gene is a recently discovered tumor suppressor gene family, and the known member, ING-4, is a candidate tumor suppressor with a leading role in gene regulation, cell-cycle control, apoptosis induction, and angiogenesis.[10,11] ING-4 is implicated and functionally altered in various cancers including glioma.[12] ING-4 is significantly downregulated in gliomas compared with normal human brain tissue, and the extent of reduction is associated with the progression of tumors from lower to higher grades.[13] Evidence demonstrates that ING-4 interacts physically with NF-kB by forming a transcriptional complex that suppresses NF-kB targeted genes.[13] Many of the genes that are transcriptionally regulated by NF-kB (eg, ,matrix metalloproteases MMP-2, MMP-9 and urokinase plasminogen activator [u-PA]), are already known to be involved in the course of invasion.[14] However, it is unclear which of the miRNAs directly regulate ING-4 in glioma tissues.

In the present study, we showed that miR-423-5p is upregulated in human glioma tissues. Then, we sought to understand: (i) the potential direct target of miR-423-5p that may be involved in glioma development; (ii) the roles of miR-423-5p in tumor growth, invasiveness, angiogenesis, GBM neurosphere formation, and migration; (iii) whether miR-423-5p overexpression inactivates AKT and ERK1/2 signaling pathways via its direct target; (iv) whether miR-423-5p overexpression confers GBM cell resistance to TMZ; and (v) the role miR-423-5p has in glioma cell growth in nude mice. Taken together, these insights will help us understand the underlying mechanisms in glioma and develop a unique miRNA-based therapy for GBM management.

Methods

Human Tissue Samples

Human glioma samples and normal tissues were obtained from the Department of Neurosurgery at the First Affiliated Hospital of Nanjing Medical University. This study was approved by the hospital institutional review board, and written informed consent was obtained from all patients. Tissue samples were collected at surgery and immediately frozen in liquid nitrogen and stored until total RNAs or proteins were extracted.

Cell Culture

The human glioma cell lines U87 and U251 were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose and sodium pyruvate supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin). Primary human N3 GBM cells were obtained from Beijing Neurosurgical Institute, Capital Medical University. Human brain microvessel endothelial cells (HBMVEC) were cultured in endothelial cell basal medium supplemented with 1% endothelial cell growth supplement and 5% fetal bovine serum. Neurosphere culture cells were grown in stem cell medium consisting of DMEM/F12 (Gibco) supplemented with 1% N2, 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor, and fibroblast growth factor–2 (Invitrogen).

Lentiviral Packaging and Stable Cell Line Establishment

The lentiviral packaging kit was purchased from Open Biosystems. Lentivirus-carrying hsa-miR-423-5p or hsa-miR-negative control (miR-NC) was packaged in human embryonic kidney 293T cells and collected from the supernatant, as instructed by the manufacturer's manual. Stable cell lines were established by infecting lentivirus into U87, U251, and N3 GBM cells, followed by puromycin selection.

RNA Extraction, Reverse Transcription PCR, and Quantitative Real Time-PCR

RNA was isolated from harvested cells or human tissues with Trizol reagent according to the manufacturer's instructions (Invitrogen). A stem-loop–specific primer method was used to measure the expression levels of miR-423-5p, as described previously.[15,16] Expression of U6 was used as an endogenous control.[17] The cDNAs were amplified by qRT-PCR using SYBR Premix DimerEraser (Takara) on a 7900HT system, and the fold changes were calculated by relative quantification ($2^{-\triangle\triangle Ct}$).

Protein Extraction and Immunoblotting

Protein extraction and Western blot analysis were performed as previously described.[18] Representative images from 2 or 3 independent experiments are shown. The antibodies that were used included ING-4, MMP9, p53, BCL-2, BAX (Abcam), CD133 (Amersham), Nestin (BD), cyclin D1, cyclin E1, p-RB Ser780, GFAP, BMI1, MMP2, EGFR, PDGFR, Akt and pAkt Ser473 (Cell Signaling), ERK and Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187), Sox2 (R&D Systems), and GAPDH (Sigma Aldrich).

Luciferase Reporter Assay

The 3′-UTR of ING-4 was synthesized, annealed, and then inserted into the SacI and HindIII sites of the pMIR-reporter luciferase vector (Ambion) downstream of the stop codon of the gene for luciferase. To induce mutagenesis, the

sequences complementary to the binding site of miR-423-5p in the 3'-UTR (ING-4: GCUGUCAUCCCUUCUCCUCCCCUCU) were replaced by GCUGACAACCGUUGUCCUACGGCGU. These constructs were validated by sequencing. U87 cells were seeded into a 24-well plate to perform the luciferase assay. After being cultured overnight, cells were cotransfected with the wild-type or mutated plasmid, pRLTK plasmid, and equal amounts of miR-143 or miR-NC. Luciferase assays were performed 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega).

Cell Proliferation and Apoptosis Assay

Cells in the logarithmic phase of growth were seeded at 3000 cells per well and cultured in 96-well plates. Cell proliferation was assayed using the Cell-Counting Kit 8 (CCK8; Dojindo Laboratories) according to the manufacturer's instructions at indicated time points. The apoptosis assay was tested in U87 and U251 cells after transfection using the annexin V–fluorescein isothiocyanate Apoptosis Detection Kit I (BD Biosciences) and analyzed by fluorescence-activated cell sorting. (FACS).

Immunostaining

For immunostaining undifferentiated neurospheres, the cells were fixed with 4% paraformaldehyde and incubated with antibodies against CD133/1 (Miltenyi Biotec) and Nestin (Abcam). Appropriate secondary antibodies (FITC Green goat anti-mouse and Cy3 Red goat anti-rabbit; Molecular Probes) were used. Cells were washed with PBS and stained with 4', 6-diamidino-2-phenylindole. The staining was detected by microscopy (Leica DMI3000B).

Wound Healing Assay

The cells were cultured until they reached 90% confluence in 6-well plates. Cell layers were scratched using a 20 µL tip to form wounded gaps and were then washed with PBS twice and cultured.

In Vitro 2D and 3D Assays

3D spheroid migration assay in a collagen matrix and its quantification were performed.[19] The vessel-forming ability of HBMVECs was characterized in vitro using a Matrigel assay. To pretreat 8-chamber polystyrene vessel tissue culture-treated glass slides, growth-factor–reduced Matrigel (200 µL) (BD Biosciences) was thawed on ice, added to each well, and allowed to gelatinize for 20 minutes at 37°C. Then, HBMVECs (5×10^4) cells were seeded into each well of the culture slides in MCDB-131 medium. Next, tube formation was analyzed by phase contrast microscopy after 16 hours and recorded by a microscope (Leica DMI3000B). Flow cytometry-based cell-cycle analysis was conducted using FACScan (Beckman Gallios) with ModFit software and presented as a percentage of cells in a particular phase. Experiments were performed in triplicate.

In Vitro Chemosensitivity Assay

Cancer cells were seeded at a density of 4000 cells per well in a 96-well plate overnight. Freshly prepared TMZ (Sigma-Aldrich) was added with the final concentration ranging from 25 to 400 µM. Forty-eight hours later, cell viability was assayed using a CCK8 kit.

In Vivo Studies

Male BALB/c nude mice (6 weeks old) were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences) and maintained in a special pathogen-free (SPF) condition for one week. Animal handling and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethics Committee of Nanjing Medical University. In vivo studies were performed as previously described.[20] Tumors were formalin-fixed, paraffin-embedded, and sectioned at 5 µm for Ki-67 (Abcam) and CD31 (Santa Cruz) immunohistochemical staining using the standard procedure, as previously described.[21]

Statistical Analysis

All experiments were performed 3 times, and the data were analyzed with GraphPad Prism 5. The correlations between miR-423-5p expression and ING-4 levels in glioma tissues were analyzed using the Spearman rank test. Statistical evaluation for data analysis was determined using the *t* test. The differences were considered to be statistically significant at $P < .05$.

Results

miR-423-5p Expression Is Upregulated in Glioblastomas

Our previous study showed that overexpressed miR-423-5p is associated with TMZ chemoresistance ("TCR subtype") when compared with TMZ chemosensitivity ("TCS subtype"), thereby correlating with a significantly poorer prognosis.[9] Thus, to fully assess the expression of miR-423-5p in glioma, miRNA profiling of 491 glioma samples was downloaded from the The Cancer Genome Atlas (TCGA) data portal and analyzed. As shown in Fig. 1 A, miR-423-5p expression of glioblastomas was significantly higher than that of the normal human brain. We validated the expression levels of miR-423-5p in 6 normal brain tissues and 24 glioma tumor samples by TaqMan microRNA assay-based real-time RT-PCR (Fig. 1 B). By categorizing all glioma samples into grade II, grade III, and grade IV (8 glioma tissues each group) using the WHO classification, we found that miR-423-5p levels were upregulated in these 3 groups when compared with the normal brain group (Fig. 1 C). Moreover, the level of miR-423-5p expression in grade IV cases was much higher than that of grade II cases, thereby indicating that miR-423-5p expression is involved in glioma malignancy progression (Fig. 1 C, $P < .05$). Our results indicated that miR-423-5p may act as a tumor promoter in glioma tissues.
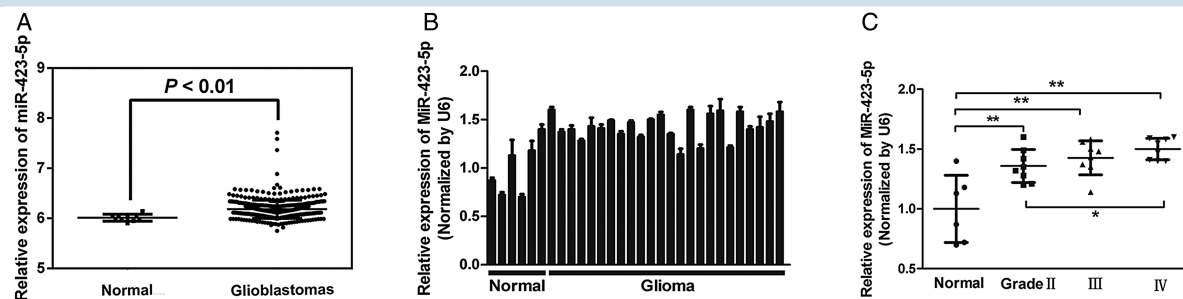
Fig. 1   miR-423-5p is upregulated in patient glioblastoma (GBM) specimens. (A) Relative expression of miR-423-5p was analyzed by The Cancer Genome Atlas in GBM brain tumor specimens versus healthy brain tissue. (B) Relative miR-423-5p expression levels were validated in 6 healthy brain tissues and 24 glioma tissues. (C) Relative expression levels of miR-423-5p in normal brain tissues and 3 different grades of glioma samples. The 24 glioma tissues were divided into 3 groups (8 glioma tissues in each group): WHO grade II, grade III, and grade IV. The Student t test was used to analyze the significant differences among the 3 groups; * and **indicate significant differences at $P <$ .05 and $P <$ .01, respectively.

ING-4 Is a Direct Target of miR-423-5p

To further understand the molecular action of miR-423-5p in glioma tissues, we searched for potential targets of miR-423-5p using TargetScan and checked the potential targets using bioinformatic databases. ING-4 has been thought to be a putative target of miR-423-5p (Fig. 2A). Thus, we cotransfected GBM cells with the wild-type ING-4 luciferase reporter vector along with miR-423-5p or miR-NC for 24 hours and then evaluated the luciferase activities of those cells. miR-423-5p transfected cells showed a remarkable reduction of luciferase activities of the ING-4 reporter in both U87 and U251 cells (Fig. 2 B). A similar assay was performed using the mutant reporters containing the ING-4, which was mutated at its miR-423-5p binding sites as indicated. In doing so, the luciferase activities of the cells transfected with wild-type 3′UTR showed significant reduction compared with those with mutated 3′UTR (Fig. 2 B). Next, immunoblotting analysis was conducted to evaluate ING-4 protein expression. We found that the expression of ING-4 was downregulated in miR-423-5p overexpressed cells but increased in cells that were transfected with miR-423-5p inhibitor (Fig. 2 C).

To further determine whether the elevated miR-423-5p expression correlates with the levels of ING-4 expression in tumor tissues, protein expression of ING-4 in glioma and normal brain tissues was analyzed. The results showed that the average expression level of ING-4 was significantly lower in tumor tissues than in normal brain tissues (Fig. 2 D). mRNA profiling of 220 glioma samples was downloaded from the Chinese Glioma Genome Atlas(CGGA) data portal, and the expression level of ING-4 was analyzed (Supplementary material, Fig. 1). As shown in Fig. 2 E, Spearman correlation analysis indicated that the ING-4 level in glioma tissues was inversely associated with miR-423-5p expression levels (Spearman correlation r = −0.4692 and P = .0207, respectively). These observations confirmed that higher expression levels of miR-423-5p are significantly correlated with lower levels of ING-4 protein expression in the same set of glioma tissues.

miR-423-5p Overexpression Promotes Cell Proliferation, Angiogenesis, and Invasion

We became interested in exploring the effect of miR-423-5p on GBM cell behavior. U87 and U251 cells were transfected with miR-423-5p or miR-NC and evaluated for cell growth. The results showed that cell proliferation was enhanced in the miR-423-5p–overexpressing cells compared with the glioma cells expressing miR-NC (Fig. 3 A). A cell-cycle assay following miR-423-5p transfection indicated a marked decrease in the G1 fraction, an increase in the S fraction, and an increase in G2 arrest (Fig. 3 B). Additionally, to explore the potential mechanism of miR-423-5p–mediated glioma cell proliferation, we detected the expression of cell-cycle–regulated proteins cyclin D1, cyclin E1, and phosphor-RB(p-RB) in miR-423-5p–enhanced U87 cells. The expression of these proteins was significantly increased in the miR-423-5p–transfected glioma cells relative to the miR-NC group (Fig. 3 C). Similar to the impacts on cell-cycle control, there was also a significant effect of miR-423-5p on cell apoptosis (Fig. 3 D). Moreover, a tube formation assay showed that the miR-423-5p–transfected group HBMVECs displayed a longer tube length, indicating that angiogenesis was activated (Fig. 3 E). In addition, miR-423-5p overexpression notably enhanced the invasive capacity of U87 cells (Fig. 3 F). As expected, the forced expression of ING-4 reversed miR-423-5pmediated promotion of cell proliferation, angiogenesis, and invasion. These results thus indicated that miR-423-5p overexpression facilitated glioma cell proliferation, angiogenesis, and invasion in vitro.

Overexpression of miR-423-5p in GBM Cells Strengthens Neurosphere Formation and Migration Through Multiple Effectors

We enriched a CD133-positive "stem-like" neurosphere from U87 glioma cell lines and primary human GBM cells N3 by growing them in stem-like conditions (Fig. 4 A). The neurosphere formation assay was employed to assess the
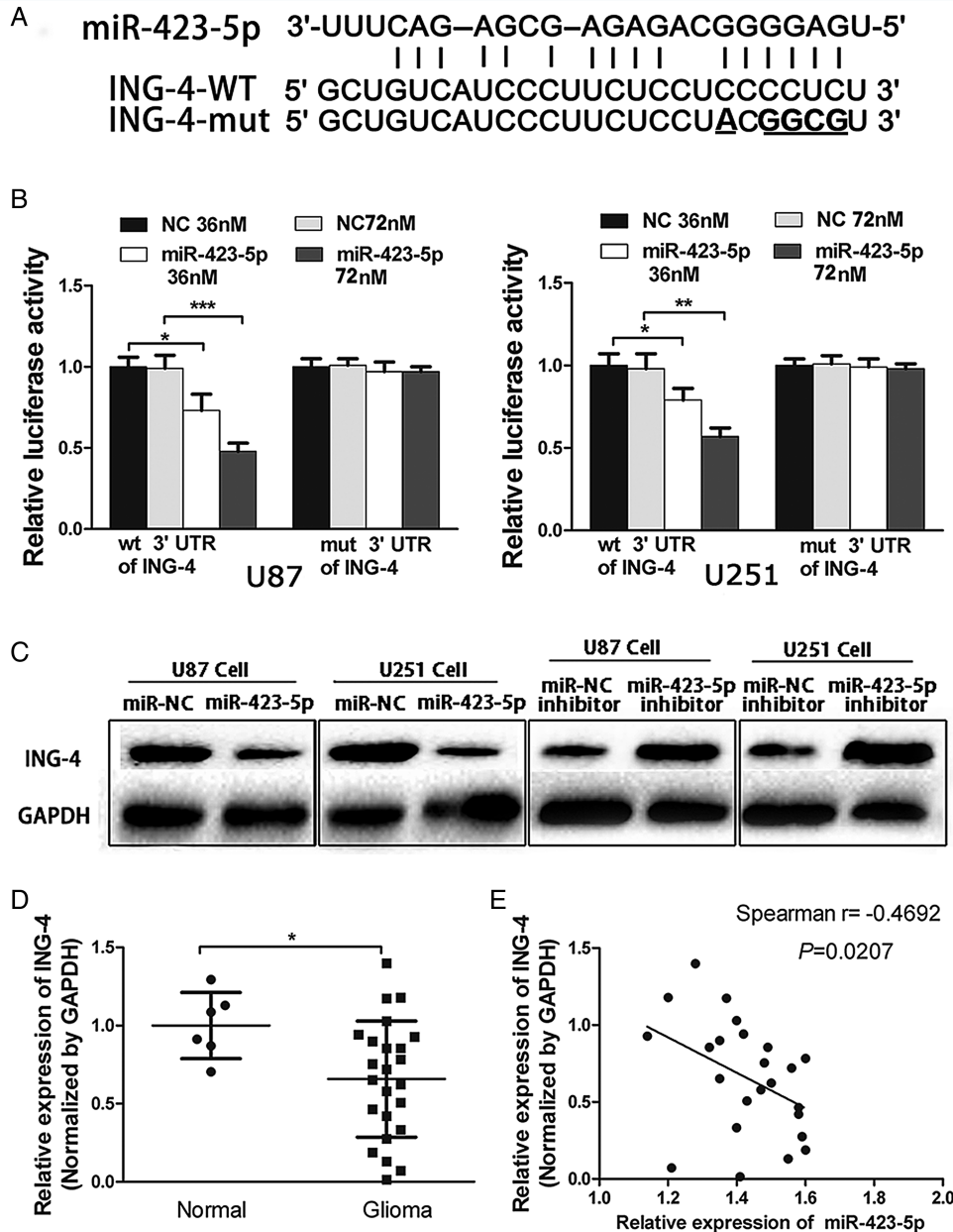
Fig. 2 (A) Inhibitor of growth in the ING-4 3′-UTR and a schematic diagram of the reporter construct showed the mutated ING-4 3′-UTR sequence. (B) Direct targeting of the ING-4 3′-UTR by miR-423-5p was validated using a luciferase/3′-UTR reporter assay. U87 and U251 cells were cotransfected with a luciferase/ING-4 wild-type 3′-UTR reporter vector (wt) and 36 nM or 72 nM negative control miR (NC) or miR-423-5p. A reporter vector with a mutated miR-423-5p binding site in the ING-4 3′-UTR (mut) was used as a control. (C) Western blotting analysis showed that the expression levels of ING-4 were decreased in cells with miR-423-5p overexpression but increased in cells with an miR-423-5p inhibitor. (D) Relative expression of ING-4 protein levels was validated by Western blotting analysis in glioma specimens ($n = 24$) versus normal human brain tissues ($n = 6$). Fold changes were obtained from the ratio of ING-4-to-GAPDH levels. (E) Spearman correlation analysis was used to determine the correlations between the levels of ING-4 expression and miR-423-5p in human glioma specimens. (Spearman correlation analysis, r = −0.4692, $P = .0207$). *$P < .05$, **$P < .01$ and ***$P < .001$.

impact of miR-423-5p overexpression on GBM self-renewal ability. As shown in Fig. 4 B, miR-423-5p overexpression promoted neurosphere formation of both U87 and N3 cells in stem-like conditions. As expected, the forced expression of ING-4 reversed miR-423-5p–mediated promotion of

GBM self-renewability. Immunofluorescence of the putative glioma stem cell marker CD133 and Nestin also showed that miR-423-5p overexpression significantly increased CD133 and Nestin protein levels when the U87 cell lines were grown as stem-like neurospheres (Fig. 4 A). Forced
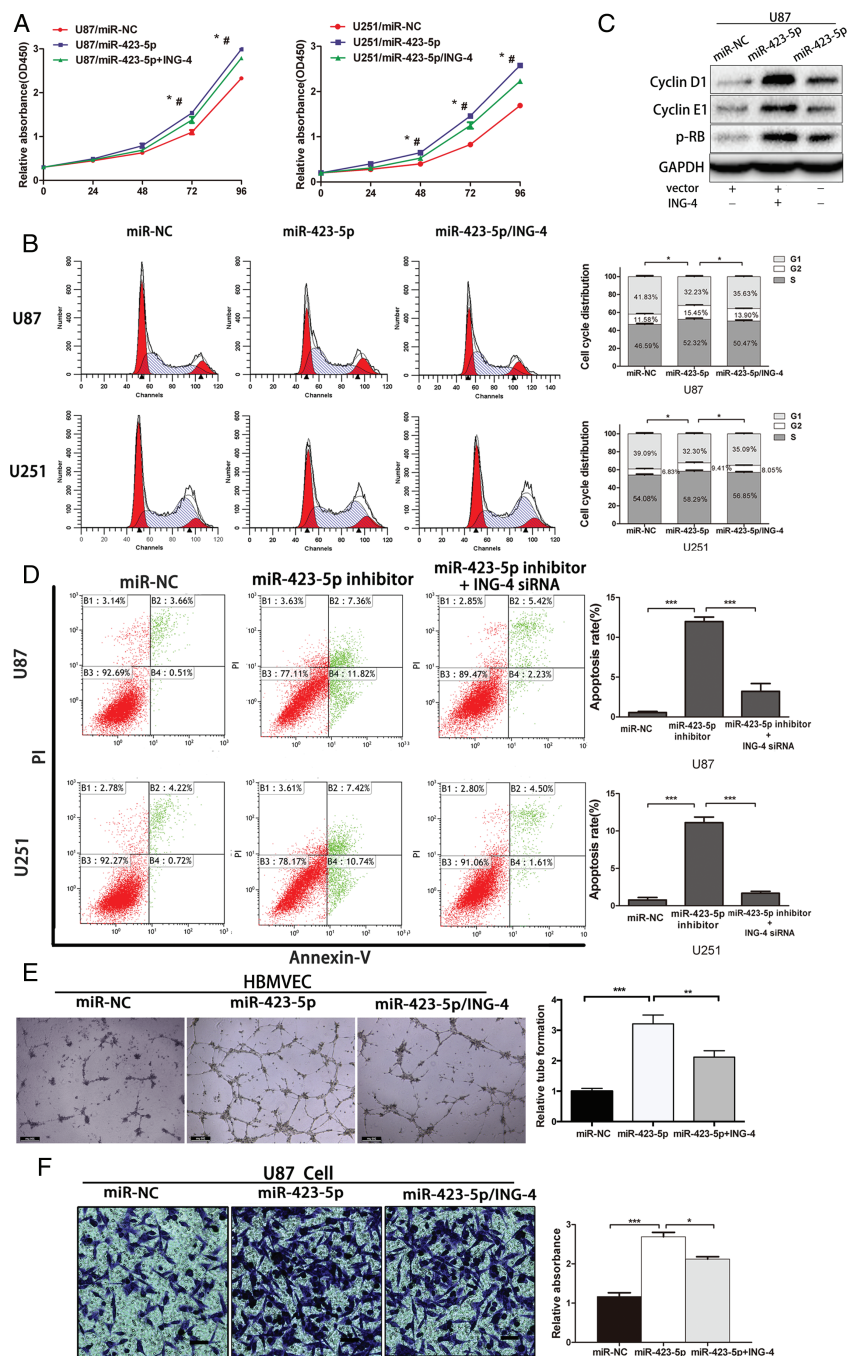
Fig. 3 miR-423-5p overexpression promotes cell proliferation, angiogenesis, and invasion. (A) The overexpression of miR-423-5p promoted cell proliferation, which was rescued upon induction of the expression of exogenous ING-4 in both U87 and U251 cells. *indicates significant difference compared with control, #indicates significant difference compared with miR-423-5p/ING-4 treatment at $P < .01$. (B) Cell-cycle assay results of U87 and U251 glioma cells 3 days after transfection with miR-423-5p or control miR (monitored by flow cytometry). (C) Western blot analysis was performed to indicate the regulation of cell-cycle–regulated proteins, cyclin D1, cyclin E1, and p-RB by miR-423-5p in U87 cells. (D) Apoptosis assay results of U87 and U251 glioma cells 3 days after transfection with the miR-423-5p inhibitor or the control miR were monitored by flow cytometry. (E) Tube formation of human brain microvessel endothelial cells (HBMVECs) transfected with miR-423-5p or control miR was monitored by Matrigel-coated plates with conditioned medium from the indicated cells. Scale bar = 300 μm. (F) miR-423-5p overexpression promoted cell invasion in U87 cells. Cells were transfected with miR-423-5p, which was followed by ING-4 transfection. Scale bar = 100 μm. $*P < .05$ and $***P < .001$.
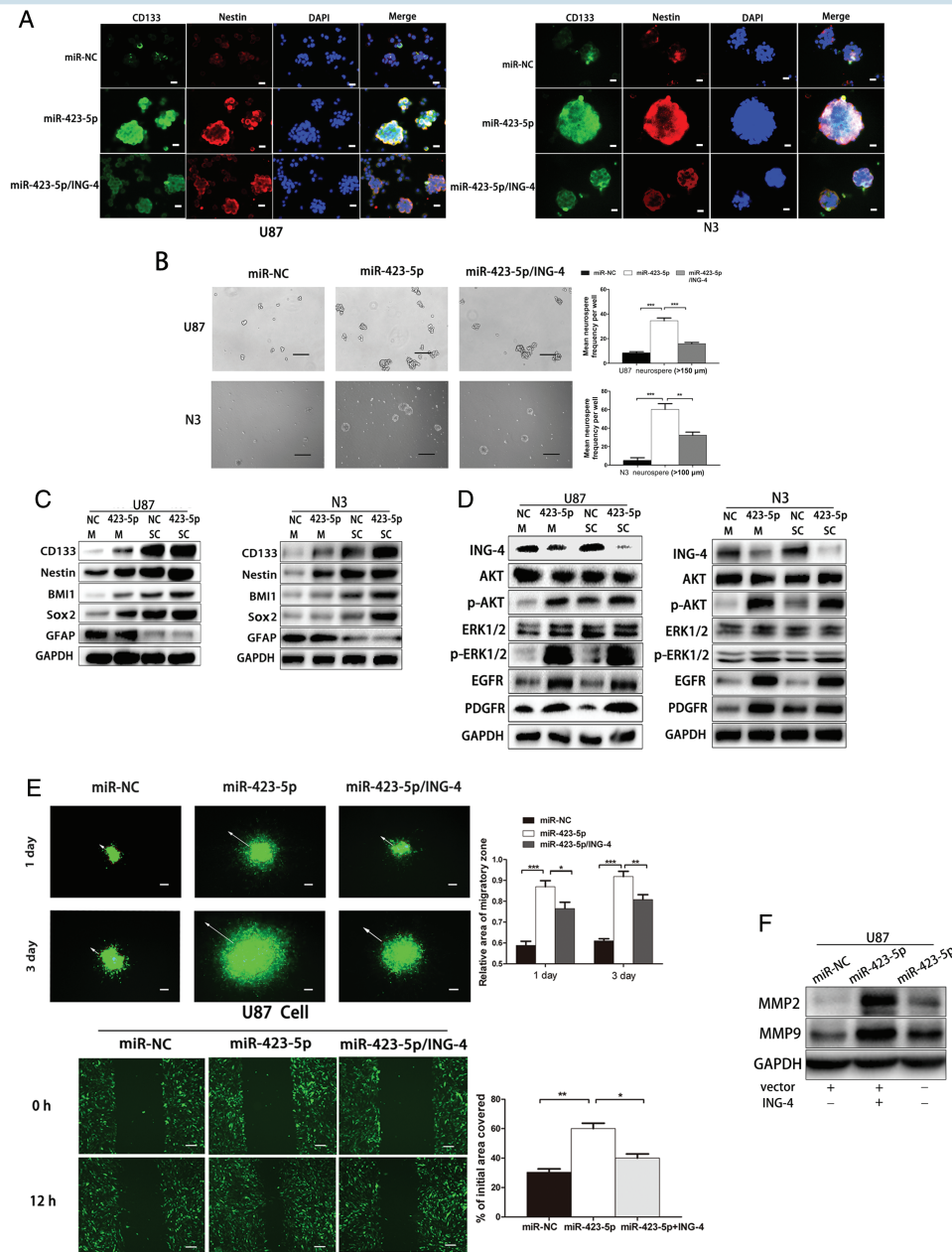
Fig. 4 Overexpression of miR-423-5p in glioblastoma (GBM) cells strengthens neurosphere formation and migration through multiple effectors. (A) CD133 marker, Nestin, and DAPI staining shown by immunostaining in neurosphere from U87 and N3 cells. Scale bar = 20 μm (B) Neurosphere formation capacity in the presence of miR-423-5p overexpression was determined by a self-renewal assay. miR-423-5p overexpression promoted neurosphere formation of both U87 and N3 cells. Scale bar = 150 μm. (C) The stemness of U87 and N3 cell lines, which were cultured as monolayer (M) or stem cell-like neurospheres (SCs), was determined by Western blot analysis. (D) Cellular signaling and the cellular receptors EGFR and PDGFR were monitored by Western blotting analysis of U87 and N3 cell lines that were cultured as monolayer (M) or stem cell-like neurospheres (SC). Cells were transfected with either negative control miR (NC) or miR-423-5p. (E) The migration of GBM cells was monitored by a spheroid dispersal assay. Representative images and quantification of spheroid migration of U87 GBM cells transfected with either negative control miR (NC) or miR-423-5p. miR-423-5p mediated GBM migration was rescued upon forced coexpression of ING-4. Scale bar = 200 μm. The same results were validated by wound-healing assay. Scale bar = 200 μm. (F) Western blot analysis to demonstrate the regulation of invasion-related proteins, MMP-2 and MMP-9, by miR-423-5p in U87 cells. *$P < .05$, **$P < .01$ and ***$P < .001$.

expression of ING-4 also reversed the miR-423-5p–mediated increase of GBM CD133 and Nestin expression (Fig. 4 A). Stem-like culture conditions caused not only a significantly increased level of CD133 and Nestin but also an increase in stem-cell self-renewal factors BMI1 and Sox2 of both U87 and N3 cells in stem-like conditions (Fig. 4 C). We also found that the astrocyte lineage marker GFAP was significantly decreased under neurosphere culture conditions (Fig. 4 C). Interestingly, miR-423-5p transfection enhanced these effects of neurosphere culture and elevated levels of BMI1 and Sox2 (Fig. 4 C). The above results showed that miR-423-5p expression resulted in a variety of cell-behavior changes. We thus speculated that miR-423-5p could upregulate certain tumor-promoting signaling pathways that are involved in GBM progression (Fig. 4 D). The condition of oncogenic signaling in GBM cells that were transfected with miR-423-5p and cultured either as monolayers or in stem-like neurosphere cultures was investigated. When compared with miR-NC, cellular levels of p-AKT and p-ERK1/2 were significantly increased in both U87 and N3 cells that overexpressed miR-423-5p, while no statistically significant increase in AKT and ERK1/2 was detected (Fig. 4 D). Furthermore, EGFR and PDGFR levels were elevated in miR-423-5p–overexpressing U87 and N3 cells (Fig. 4 D).

We next explored the effect of miR-423-5p on GBM phenotypic changes in migration. miR-423-5p expression-enhanced infiltration in a 3D collagen matrix and miR-423-5p–overexpressing cells showed a more invasive morphology (Fig. 4 E). As shown in Fig. 4 E, the same results were validated using a wound healing assay by growing U87 cells in serum cultures. To study the biological mechanism of miR-423-5p–mediated glioma cell migration, we examined the expression of invasion-associated molecules MMP2 and MMP9 and found that their protein levels increased significantly after miR-423-5p overexpression in U87 cells (Fig. 4 F). These results demonstrated that miR-423-5p promoted GBM migration and activated multiple signaling pathways that were related to "stemness" of GBM stem-like cells, strengthening neurosphere formation.

miR-423-5p Confers Resistance to Temozolomide by Suppressing its Target ING-4

Our previous study demonstrated that miR-423-5p was 1 of 4 miRNAs that have been identified as being overexpressed in the TMZ-chemoresistant subtype in Chinese primary glioblastoma.[9] As expected, our results showed that the overexpression of miR-423-5p in U87 and U251 cells significantly decreased chemosensitivity to treatment with TMZ (Fig. 5 A). Furthermore, to assess whether the forced expression of ING-4 reversed miR-423-5p–mediated resistance to TMZ, the viability of cells that were treated with TMZ (100 mM) was assayed by CCK8 at different time points (Fig. 5 B). The results showed that the forced expression of ING-4 reversed miR-423-5p–induced glioma chemoresistance to TMZ (Fig. 5 B). To test whether miR-423-5p and its target ING-4 have a role in cell apoptosis in the presence of TMZ treatment, we performed FACS analysis to detect cell apoptosis rates (Fig. 5 C). The combination treatment of miR-423-5p +TMZ significantly showed less cell apoptosis when compared with that of miR-NC + TMZ (Fig. 5 C). As expected, the forced expression of ING-4 partially abolished the effect that was induced by miR-423-5p

+TMZ treatment (Fig. 5 C). Similarly, we also found that the expression of ING-4 in U87/423-5p cells treated with TMZ was higher than the untreated cells using Western blotting (Fig. 5 D). Our results also showed that U87/423-5p cells expressed less p53, more inhibition of apoptosis protein BCL-2, and less proapoptosis protein Bax than U87/SC cells treated with TMZ (100 μM) (Fig. 5 E). The forced expression of ING-4 reversed these miR-423-5p–induced effects. These results indicated that miR-423-5p rendered glioma cells to be more resistant to TMZ treatment and that miR-423-5p suppressed its target ING-4 to rescue GBM cell apoptosis induced by TMZ treatment.

miR-423-5p Promotes Tumorigenicity, Angiogenesis, and Invasion In Vivo

Considering the remarkable glioblastoma-promoting effects of miR-423-5p in vitro, we extended our investigation to examine if miR-423-5p could accelerate glioblastoma growth in vivo using nude mice. GBM cell tumorigenicity was evaluated by intracranial implantation of U87 cells that stably expressed miR-423-5p. Bioluminescence imaging showed the promotion of tumor growth in the miR-423-5p–overexpressing group compared with the control group (Fig. 6 A). Survival analysis also demonstrated significantly worse outcome for the animals injected with miR-423-5p–expressing U87 cells (Fig. 6 B). We analyzed neovascularization of symptomatic mice at 6 weeks after tumor implantation. miR-423-5p–expressing tumors showed elevated CD-31 staining with a longer average branch length (Fig. 6 C). We also found that control U87 cells almost localized intratumorally, while many U87 cells that expressed miR-423-5p migrated out of the tumor core (Fig. 6 D). Consistent with in vitro studies, an immunoblotting assay indicated that the level of ING-4 from miR-423-5p–overexpressing tumor tissues was lower than that of the control group (Fig. 6 E). Moreover, some important pathway proteins, such as p-AKT, p-ERK1/2, EGFR, and PDGFR, were significantly elevated by miR-423-5p expression in glioma tissues (Fig. 6 E). When considered together, these results suggest that miR-423-5p promotes tumor growth, angiogenesis, and invasion in vivo by targeting ING-4.

Discussion

miRNAs act as posttranscriptional gene regulators in modulating various physiological and pathological events. miRNA abnormalities are thought to be involved in cancer development.[22,23] Several studies have reported that miR-423-5p functions as a biomarker and has important roles in many types of cancers.[24–26] However, there is no research to date that refers to the role of miR-423-5p in glioma tissues. In this study, we report that miR-423-5p is overexpressed in GBMs and is a novel tumor promoter in GBM. We describe tumorigenic effects of miR-423-5p overexpression by suppressing its target ING-4.

The ING gene family has aroused significant interest because of its putative roles as tumor suppressors. ING-associated proteins have well-established effects in numerous cell processes including cell growth, survival, and tumor suppression.[27] ING-4 has also been found to bind with and modulate p53, NF-κB, and HIF-1α activity, thereby
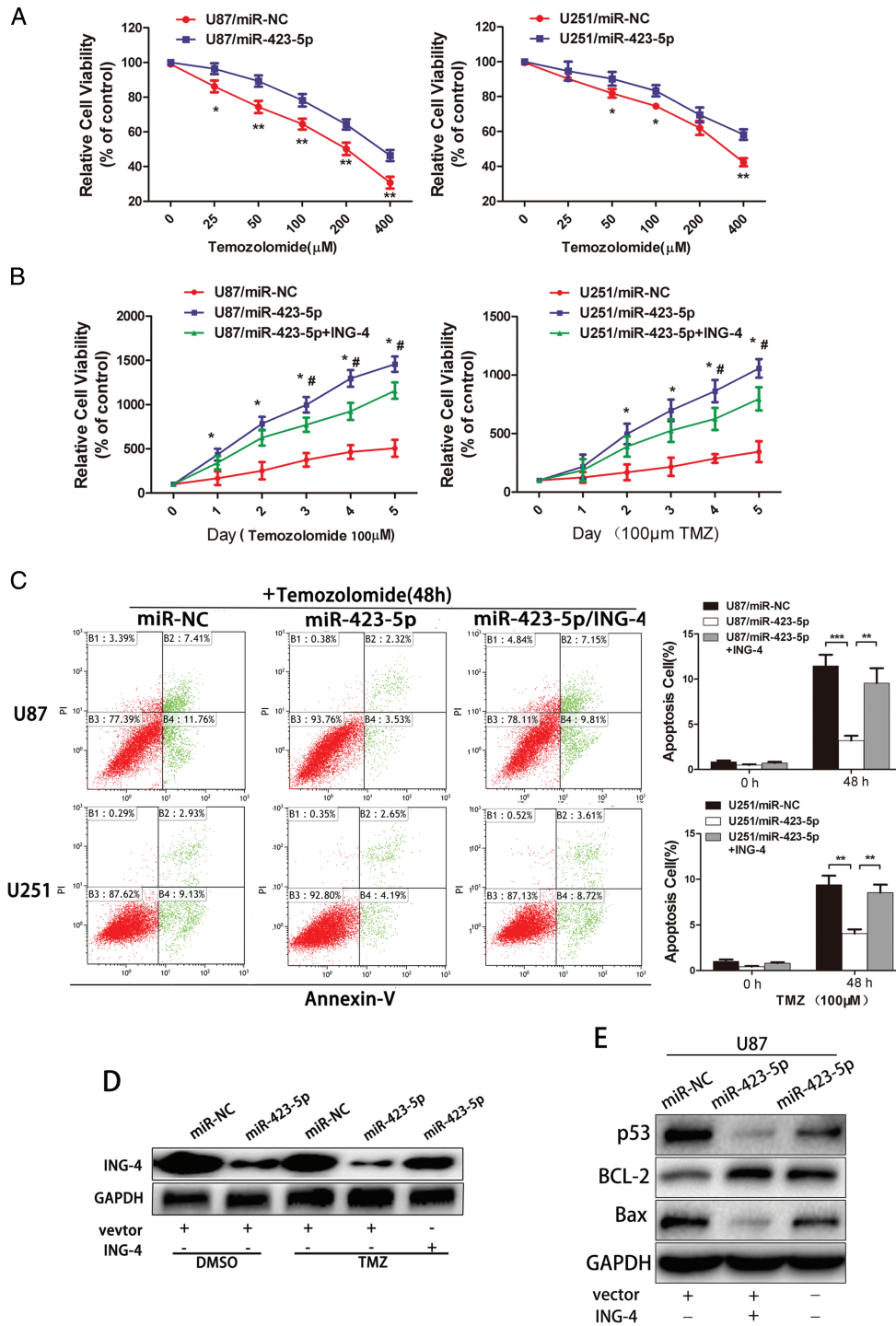
Fig. 5 miR-423-5p confers resistance to temozolomide (TMZ) by suppressing its target ING-4. (A) Cell proliferation was examined in U87 and U251 cells that were stably expressing miR-NC or miR-423-5p following TMZ treatments at different doses. The CCK8 assay was conducted 48 hours after TMZ treatment. (B) The cell proliferation of U87 and U251 cells, which were treated with 100 μM TMZ, were tested every 24 hours. The overexpression of miR-423-5p renders glioblastoma (GBM) cells resistant to TMZ, while inducing the expression of ING-4 rescued the effects of miR-423-5p. (C) U87 and U251 cells were transfected with miR-423-5p or miR-NC and cultured in 100 μM TMZ. The cells were then determined by apoptosis analysis using flow cytometry 48 hours later. (D) Western blotting. (E) Western blot analysis was performed to assess the regulation of apoptosis-related proteins p53, BCL-2, and Bax by miR-423-5p in U87 cells cultured in 100 μM TMZ. *indicates significant difference compared with control, #indicates significant difference compared with miR-423-5p/ING-4 treatment at *P* < .01. **P* < .01 and ***P* < .001.
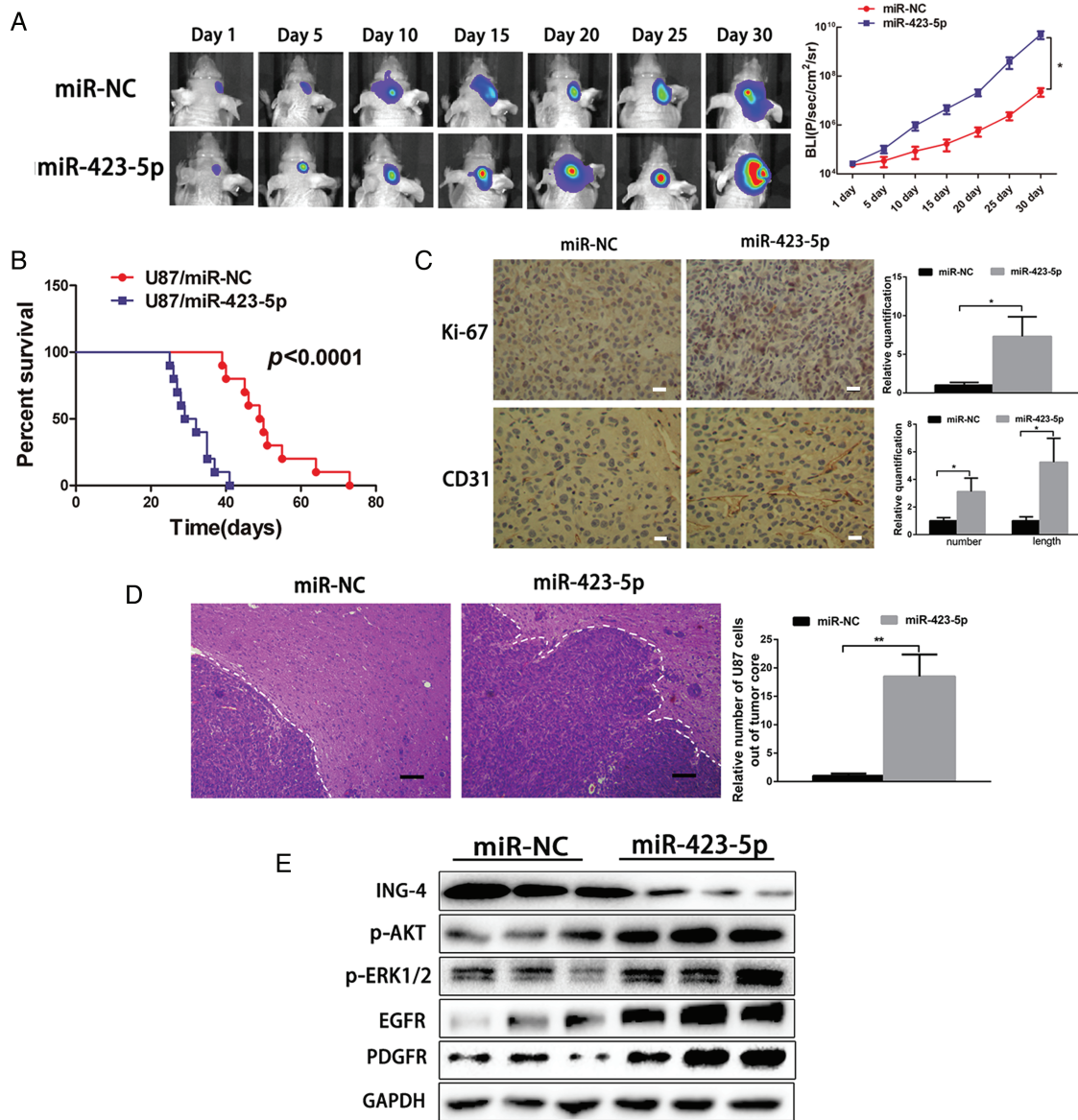
Fig. 6 miR-423-5p promotes tumorigenicity, angiogenesis, and invasion in vivo. (A) Representative images of mice implanted with intracranial tumors formed by U87 on days 1, 5, 10, 15, 20, 25, and 30. Plot of the Fluc activity by bioluminescence imaging for intracranial tumors. (B) Kaplan–Meier survival curve of animals injected with glioblastoma (GBM) tumor cells U87 transfected with miR-423-5p (N = 10) or miR-NC (N = 10). (C) Cell proliferation and angiogenesis in vivo were determined by Ki-67 and CD31, respectively, using immunohistochemistry. Scale bar = 20 μm. (D) Invasiveness in vivo was determined by hematoxylin & eosin stain. Scale bar = 50 μm. (E) The expression levels of pathway proteins and of EGFR or PDGFR in the miR-423-5p–overexpressing tumor tissues were significantly higher than were those of the miR-NC group according to Western blotting. $*P < .05$ and $**P < .01$.

regulating brain tumor growth and angiogenesis.[13,27,28] In our study, we determined that the expression of miR-423-5p and ING-4 was inversely correlated in human GBM samples and that miR-423-5p directly binds to the site on the ING-4 3′-UTR, which results in the suppression of ING-4 protein levels both in vitro and in vivo.

Our results show that miR-423-5p acts as a tumor promoter through various mechanisms including enhancement of tumor cell growth, invasion, angiogenesis, and activation of the AKT and ERK1/2 signaling pathways. The cellular and molecular mechanisms of tumor angiogenesis are major focuses in almost all current concepts of both tumor biology and targeted cancer therapy.[29] Here, we report that miR-423-5p is an angiogenic promoter in glioma tissues and that the overexpression of miR-423-5p in glioma cells results in an increased number of microvessels and promotes tumorigenicity in a tumor xenograft. GBMs display striking cellular heterogeneity and have been shown to include

subpopulations of tumorigenic cells with stem cell-like features called cancer stem cells (CSCs). The existence of cellular populations (ie, CSCs) is thought to maintain tumor growth through self-renewal ability and the generation of larger tumor bulk. CSCs in glioma tissues display capacities of multilineage differentiation potential and sphere-forming growth in serum-free conditions.[30] Our results demonstrate that miR-423-5p elevates stem cell factors that are known to have an important role in GBM stem cell maintenance such as CD133, Nestin, BMI1, and Sox2. Multiple signaling pathways are activated by miR-423-5p and are related to the stemness of GBM stem-like cells. miR-423-5p significantly strengthens the capabilities of neurosphere formation and self-renewal. Meanwhile, miR-423-5p renders glioma cells more resistant to TMZ treatment and rescues the GBM cell from apoptosis induced by TMZ treatment via suppression of its target gene, ING-4. Thus, the inhibition of miR-423-5p in combination with TMZ treatment could be a useful therapeutic strategy for suppressing glioma growth.

In summary, our results show for the first time that miR-423-5p is frequently upregulated in glioma tissues. We confirmed that miR- 423-5p acts as an oncogene by targeting ING-4 through various mechanisms including the promotion of tumor cell growth, migration, invasion, angiogenesis, and neurosphere formation along with the activation of AKT and ERK1/2 pathways. We also demonstrated that miR-423-5p renders glioma cells more resistant to TMZ treatment. These findings shed new light to better understand the molecular events governing development and the progression of glioma and open new possibilities for therapeutic intervention.

Conflict of interest statement. None declared.

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