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Let-7f Inhibits Glioma Cell Proliferation, Migration, and Invasion by Targeting Periostin

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ABSTRACT

Glioma is one of the most aggressive and malignant tumor types. Despite advances in surgery, imaging, chemotherapy, and radiation, glioma patient prognosis remains poor. Glioma pathogenesis is an urgent problem that must be solved. MicroRNAs (miRNAs) are endogenous small non-coding RNAs that are key post-transcriptional regulators of gene expression. miRNA deregulation commonly occurs in human tumorigenesis. In the present study, the expression levels of Let-7f were down-regulated in both glioma tissues and glioma cells. The enhanced expression of Let-7f suppressed glioma cells proliferation, migration, and invasion via direct targeting perisotin oncogenic activity. Experiments with periostin siRNA or over-expression further suggest that Let-7f may serve as tumor suppressors through perisotin signal. These findings provide insights regarding the role and mechanism of Let-7f in regulating biological behavior of glioma cells via the Let-7f/periostin axis, and Let-7f may serve as a potential therapeutic target in glioma. J. Cell. Biochem. 116: 1680–1692, 2015.

KEY WORDS: GLIOMA; Let-7f; PERIOSTIN; PROLIFERATION; MIGRATION; INVASION

Glioma is one of the most common deadly brain tumors of the adult central nervous system [Yue et al., 2012]. The median life expectancy for glioma patients is approximately 14 months. A maximum of 5% of patients are alive 5 years after diagnosis despite multiple advanced treatments [Shen et al., 2010]. The morbidity of glioma is primarily due to its rapid growth, angiogenesis, and invasion throughout the brain [Furnari et al., 2007]. New therapies are required; however, understanding the biological and molecular mechanisms causing glioma morbidity is required first.

MicroRNAs (miRNAs) are small RNA molecules that regulate target protein expression by repressing translation or by degrading the target mRNA [Chen et al., 2013]. Many studies have suggested that miRNAs can serve as oncogenes and tumor suppressors [Chen et al., 2013] and that miRNAs play important roles in tumor cell differentiation, proliferation, migration, and invasion [Manikandan et al., 2008; Qian et al., 2013]. Many miRNAs are abnormally expressed in gliomas and are involved in the origin and development of glioma [Nikaki et al., 2012; Li et al., 2013a,b]; miR-7, miR-124, miR-221/222, and miR-21 are dysregulated in glioma [Lin et al., 2013; Quintavalle et al., 2013; Liu et al., 2014; Shi et al., 2014]. Let-7f, which is a member of the Let-7 family, is located at 9q22.3 [Kuehbacher et al., 2007], and its expression varies in different tumor types. Let-7f down-regulation has been reported in various human malignancies, including nasopharyngeal carcinoma, ovarian cancer, and human gastric cancer [Liang et al., 2011; Zheng et al., 2013]. However, Let-7f is up-regulated in primary breast cancer and in hepatocellular carcinoma [Liang et al., 2011; Zheng et al., 2013; Ge et al., 2014]. Furthermore, Let-7f regulates kallikrein-related peptidases (KLKs) to control cell proliferation [White et al., 2010]. In addition, a recent study demonstrated that Let-7f inhibits tumor invasion and metastasis by targeting MYH9 in human gastric cancer [Liang et al., 2011]. These studies indicate that Let-7f is involved in cancer initiation and progression. More importantly, recent studies have indicated that Let-7f expression is decreased in glioma tissue specimens compared with normal brain tissue [Loftus et al., 2012; Wang et al., 2013c]. However, the biological role and the molecular mechanisms of Let-7f in malignant glioma pathogenesis remain unclear.

Lin Deng and Gang Li contributed equally to this work.
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The primary aim of this study was to investigate the effect of Let-7f on glioma cells and to identify the target genes for Let-7f that might mediate its biological functions in glioma cells. Let-7f was down-regulated in glioma tissues and cell lines. Let-7f over-expression inhibited glioma cell proliferation, invasion, and migration and promoted apoptosis. In addition, bioinformatic analysis indicated that periostin was the direct target of Let-7f in glioma cells. These findings contribute to a better understanding of the mechanism of glioma development and suggest that Let-7f may serve as an attractive new target for glioma treatment.

MATERIALS AND METHODS

TISSUE SAMPLES AND CELL LINES

Human glioma cell lines (U87, T98G, and A172) were purchased from the Chinese Academy of Sciences Cell Bank. Normal human astrocytes (NHAs) were purchased from Lonza (USA). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone) supplemented with 10% FBS (TSB, Tianjin, China) in a humidified chamber with 5% CO_2 at 37°C. Twenty-five normal brain tissues were collected from patients undergoing internal decompression surgery following severe traumatic brain injury. One hundred forty human glioma tissues, including 55 low-grade glioma (20 grade I tumors and 35 grade II tumors) and 85 high-grade glioma (30 grade III tumors and 55 grade IV tumors) were obtained from the Department of Neurosurgery, Qilu Hospital of Shandong University. Glioma specimens were verified and classified by two experienced clinical pathologists according to the World Health Organization (WHO) standard classification of tumors. This study was approved by the Institutional Review Board of Shandong University. Written informed consent was obtained from all patients, and the hospital ethics committee approved the experiments.

REAGENTS AND CELL CULTURE

Cell transfection. Mature Let-7f mimics and scrambled control (NC) were designed and synthesized by RiboBio (Guangzhou, China). Cell transfection and co-transfection experiments were performed with oligonucleotides (40 nmol) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions when the cells reached 70% confluence.

Periostin knockdown was achieved by transient transfection with periostin-specific siRNA (100 pmol) using Lipofectamine 2000. Periostin-specific siRNA and control siRNA were purchased from GenePharm (Shanghai, China). The siRNA sequences were as follows:

Periostin-siRNA-1: 5′-GGUCCUAAUUCCUGAUUCTT-3′ and 5′-AGAAUCAGGAAUUAGGACCTT-3′; Negative scramble control sequences: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

Stable clones of A172 and U87 cells over-expressing periostin were transfected with a periostin cDNA plasmid (2.5 μg/ml) containing a mutated (MUT) 3′ untranslated region (UTR; GenePharm, Shanghai, China) and with a blank plasmid (2.5 μg/ml), which were generated as previously described [Lee et al., 2013].

Untransfected cells were used as the blank control, while cells transfected with scrambled NC or empty vectors were used as the negative control.

Transfection efficiency was verified by qRT-PCR or Western blot analysis. At 48 h after transfection, glioma cells were harvested for subsequent experiments.

Cell proliferation assay. A172 and U87 cells were seeded in 96-well culture plates at a density of 3,000 cells/well, and cell proliferation was analyzed using a Cell Counting Kit-8 (Beyotime) at 24, 48, 72, and 96 h after transfection. Then, 10 μl of CCK-8 solution was added to each well, and the cells were incubated for another 1 h in a humidified incubator at 37°C. Optical density was measured at 450 nm using a microplate reader (Bio-Rad, USA). Each assay was performed in triplicate.

5-Ethynyl-20-deoxyuridine (EdU) incorporation assay. Cell proliferation was also measured using an EdU assay kit (RiboBio, China). At 48 h after transfection, 5×10^3 cells per well were seeded into 96-well plates and cultured for 24 h. The EdU assay was performed as described previously [Jiang et al., 2014]. Each assay was performed in triplicate.

Colony formation assay. Colony formation was examined as previously described [Jiang et al., 2014]. Cells were seeded into 6-well-plates with a density of 500 cells/well at 48 h after transfection. DMEM containing 10% FBS was changed every third day. After 15 days, the colonies were fixed and stained with crystal violet for 15 min, and representative colonies were imaged and quantified. Each assay was performed in triplicate.

FLOW CYTOMETRY ASSAY

The cell cycle distribution and apoptosis levels were assayed using flow cytometry (FCM). For cell cycle analysis, transfected glioma cells were collected by trypsinization, washed three times with cold PBS, and fixed in 70% ethanol for 2 h at 4°C. After washing with PBS, the transfected cells were incubated with propidium iodide and RNase (Beyotime) for 30 min at 37°C in the dark. Finally, the cells were analyzed using a FACScan flow cytometer (BD, USA).

An annexin V-FITC apoptosis detection kit was used (Invitrogen Life Technologies, CA) according to the manufacturer's instructions to analyze apoptosis. The transfected cells were collected and incubated with annexin V-FITC and PI in the dark for 15 min at room temperature. Then, these cells were analyzed using a FACScan flow cytometer (BD, USA), and the data were analyzed using FlowJo software. Each experiment was performed in triplicate.

IN VITRO CELL MIGRATION AND INVASION ASSAYS

Cell migration and invasion were evaluated using a transwell chamber that was 6.5 mm in diameter (8-μm pore size, Corning). In total, 5×10^4 transfected cells in FBS-free medium were seeded into the upper chamber of an uncoated transwell chamber (BD; for the migration assay) or a Matrigel-coated transwell chamber (for the invasion assay). Medium containing 10% FBS was added to the lower chamber. After 24 h, the cells that did not migrate or invade were removed by cotton swabs, while the cells that did migrate or invade on the lower surface were fixed and stained with eosin for 15 min and counted under a microscope (Olympus, Japan). Five random views were used to count the cells, and the independent experiments were repeated three times.

BIOINFORMATICS PREDICTION AND LUCIFERASE REPORTER ASSAY

The common targets of Let-7f that were predicted by computer-aided algorithms were obtained from the following target prediction programs: TargetScan (http://www.targetscan.org); microRNA (http://www.microrna.org); and miRBase (http://www.mirbase.org). The pGL3 reporter constructs containing the target sequences of periostin and mutant periostin were obtained from BioAsia (Jinan, Shandong Province, China). Glioma cells were co-transfected with the luciferase reporters together with Let-7f mimics or NC using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, luciferase assays were performed using a luciferase assay kit (Promega) according to the manufacturer's instructions. Each experiment was performed in triplicate.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from the tissues and cell lines using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (50 ng) was reverse-transcribed with Let-7f stem-loop RT primers or U6 RT primers (RiboBio Guangzhou, China) using a ReverTra Ace qRT-PCR kit (Toyobo, Tokyo, Japan) according to the manufacturer's protocol to synthesize cDNA. Real-time PCR was performed using a SYBR Premix Ex Taq^{TM} Kit (TaKaRa, Japan) with Let-7f or U6 PCR primers (RiboBio) as previously described [Qian et al., 2013; Wang et al., 2013b; Song et al., 2014]. The reactions were performed using a Lightcycler 2.0 instrument (Roche Applied Science). U6 expression was used as the endogenous control.

All data for each sample were collected in triplicate, and the expression level of Let-7f was normalized to the expression level of U6. The fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$).

WESTERN BLOT ANALYSIS

Total protein was extracted from tissues and cells using RIPA buffer (Beyotime, Haimen, Jiangsu Province, China) with 1% phenylmethyl sulfonylfluoride, and the protein concentration was assayed using the bicinchoninic acid (BCA method) (Beyotime). Equal amounts of protein were loaded into 10% sodium dodecyl sulfate polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore). Then, the blots were incubated with primary antibodies overnight at 4°C. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Finally, protein bands were visualized by enhanced chemiluminescence (ECL; Millipore) and detected using an ECL detection system (Thermo Scientific, Beijing, China).

The following primary antibodies were used: periostin (Abcam); Bax (CST); Bcl-2 (CST); MMP2/9 (CST); cyclin D1 (BioWorld); cyclin E (BioWorld); P27 (BioWorld); and P21 (BioWorld).

The relative integrated density values were measured based on the actin protein as the control.

XENOGRAFT TUMOR MODELS

A subcutaneous implantation assay was performed as previously described [Cui et al., 2012; Ge et al., 2013]. Four-week-old BALB/c nude mice were obtained from the Chinese Academy of Sciences (Beijing, China). In total, 5×10^6 U87 cells were subcutaneously implanted in the left groin of the nude mice. When the subcutaneous tumor volume reached approximately 200 mm^3, 24 mice were randomly divided into three groups (eight mice per group). Then, 10 nmol Let-7f mimics, NC (90 μl PBS and 10 μl Lipofectamine 2000) or PBS (90 μl PBS and 10 μl Lipofectamine 2000; control) was injected into the xenograft tumor model in a multi-site injection manner every 3 days for 4 weeks. The tumor was measured with a caliper every 4 days, and the volume was calculated using the following formula: volume = (length × width^2)/2. Tumor growth was monitored every 4 days for 4 weeks. When the experiment was complete, the mice were anesthetized and sacrificed, and the tumors were removed for imaging, weighing, and miRNA extraction. The tumors were formalin-fixed, paraffin-embedded, and cut into 4-μm sections for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Millipore), periostin (Abcam), Ki67 (CST), MMP2/9 (CST), and immunohistochemical staining following standard procedures as previously described [Jiang et al., 2014]. The proliferation index was determined by the proportion of Ki67-positive cells, and the apoptotic index was determined as the percentage of TUNEL-positive cells.

Intracranial implantations were performed as described previously [Zhu et al., 2013]. Nude mice were anesthetized and mounted on a stereotactic device. U87 cells (5×10^5 cells/3 μl of DMEM) were injected into the left striatum of the brain to a depth of 3 mm. The craniotomy was covered with bone wax, and the incision was closed with sutures. Then, 10 nmol Let-7f mimics, 10 nmol NC in PBS (90 μl of PBS and 10 μl of Lipofectamine 2000) or PBS (90 μl of PBS and 10 μl of Lipofectamine 2000; control) was injected into the xenograft tumor model mice via the tail vain every 2 days. Every day, the animals were weighed, and any symptoms of neurological deficits were noted. In accordance with ethical practices, the mice were sacrificed when they showed negligible limb movement and a loss of 20% body weight compared with their maximal weight during the experiment. After the mice were anesthetized, the whole brains were removed and fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Then, 5-μm thick serial sections were cut and stained with hematoxylin and eosin (H&E) and evaluated using a microscope. Data were collected from six mice per condition.

The experiments conformed to the Animal Management Rule of the Chinese Ministry of Health (Documentation 55, 2001), and the experimental protocol was approved by the Animal Care and Use Ethics Committee of Shandong University.

STATISTICAL ANALYSES

All of the above experiments were independently repeated three times. All results are expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test or one-way ANOVA, followed by Dunnett's test for multiple comparisons of the means. $P < 0.05$ was considered significant.

RESULTS

LET-7F IS DOWN-REGULATED IN HUMAN GLIOMA TISSUES AND CELL LINES

First, we analyzed Let-7f expression in normal brain tissue, glioma tissues, and cell lines using real-time PCR to assess the role of Let-7f

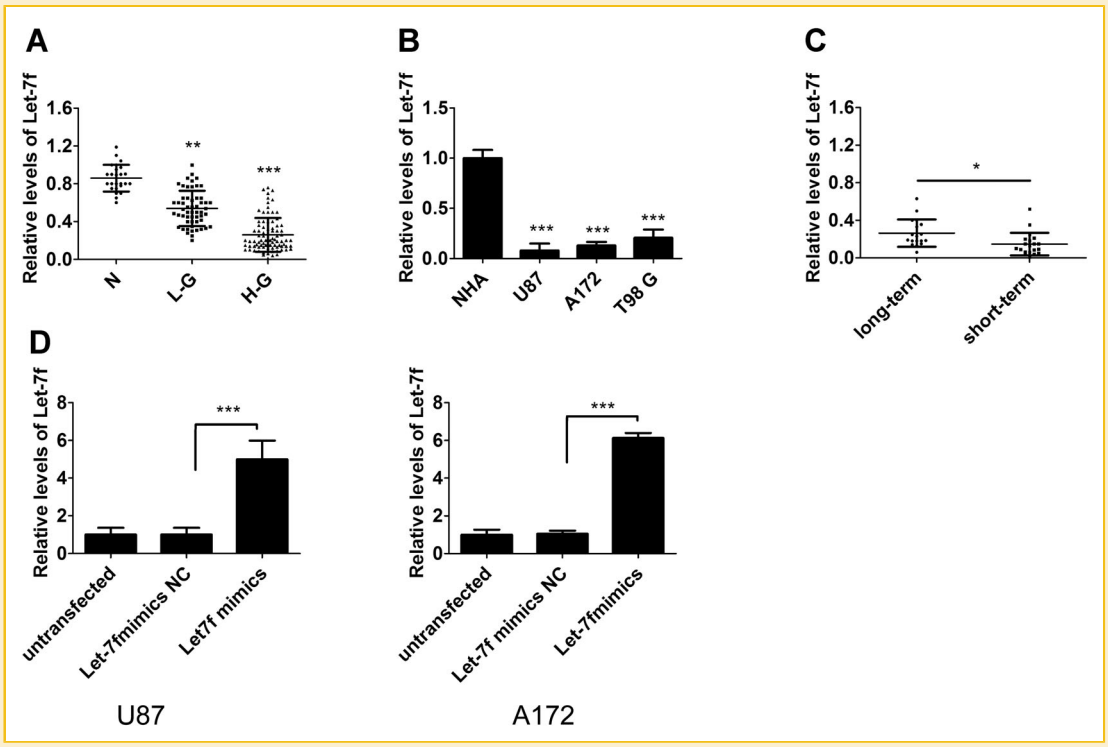

Fig. 1. Let-7f expression is deregulated in glioma tissues and cell lines. A: Quantitative RT-PCR revealed that Let-7f expression was down-regulated in low-grade and high-grade glioma tissues compared with normal brain tissues samples. B: Quantitative PCR analysis of Let-7f expression in NHAs and in U87, A172, and T98G cells. C: RNA extracted from GBM patient biopsies was assayed for Let-7f gene expression by qPCR, and Let-7f gene expression was plotted as a function of patient survival after diagnosis (long-term: >2 yr, n = 18; short term: <1 yr, n = 20). D: The expression level of Let-7f was evaluated by qPCR in transfected U87 and A172 cells. The data showed that expression of Let-7f was increased in the Let-7f mimics-transfected group compared with its negative control group, and the U6 RNA level was used as an internal control. The data were normalized to the U6 control and are represented as the mean ± standard deviation (SD) from three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). N: normal brain; L-G: low-grade glioma; H-G: high-grade glioma; NHA: normal human astrocyte.

expression in the malignant progression of glioma. As shown in Figure 1A, the Let-7f level in normal brain tissue was relatively high compared with those levels in low-grade and high-grade glioma tissues. Let-7f expression levels were also down-regulated in U87, A172, and T98 G glioma cell lines compared with those in NHAs (Fig. 1B).

We also compared Lef-7f expression levels in glioblastoma (GBM) tumors from patients with short-term survival (<1 yr) and long-term survival (>2 yr) to determine whether a relation between patient survival and Let-7f expression in GBM existed. We found significantly lower Let-7f expression levels in tumors from patients with short-term survival (Fig. 1C). The significant down-regulation of Let-7f in glioma tissues and cell lines indicated that Let-7f might function as a tumor suppressor in aggressive glioma.

Next, Let-7f mimics or NC was transiently transfected into U87 and A172 glioma cells for the following experiments. The real-time PCR results showed a significant increase in Let-7f expression in both U87 and A172 cells in the Let-7f mimics-transfected group compared with the NC-transfected group or the untransfected group (Fig. 1D, $P < 0.001$).

LET-7F INHIBITS GLIOMA CELL PROLIFERATION IN VITRO

Cell proliferation ability was analyzed using CCK-8 at 24, 48, 72, and 96 h after transfection in both U87 and A172 cells. The results showed that cell proliferation was reduced in the Let-7f mimics-transfected group compared with the NC-transfected group or the untransfected group in both U87 and A172 cells (Fig. 2A). Then, an EdU incorporation assay was used to further study the effect of Let-7f on cell proliferation. The data showed that the number of EdU-positive cells in the Let7f mimics-transfected group was significantly decreased compared with the NC-transfected group or with the untransfected group (Fig. 2B). The colony formation assay was used to evaluate the long-term effects of Let-7f on cell proliferation. The results showed that Let-7f mimics-transfected cells formed significantly fewer colonies compared with NC-transfected or untransfected cells (Fig. 2C).

These results indicated that Let-7f inhibits glioma cell growth in vitro.

EFFECT OF LET-7F ON THE CELL CYCLE AND ON APOPTOSIS

Propidium iodide staining for cell cycle progression was analyzed by flow cytometric analysis to further examine the mechanism of Let-7f-modulated glioma cell growth. The results showed that Let-7f mimics-transfected U87 and A172 cells resulted in increased cell populations at the G1 phase and decreased cell populations at the S and M phase compared with the NC-transfected group or untransfected group (Fig. 3A). The effect of Let-7f on apoptosis in glioma cells

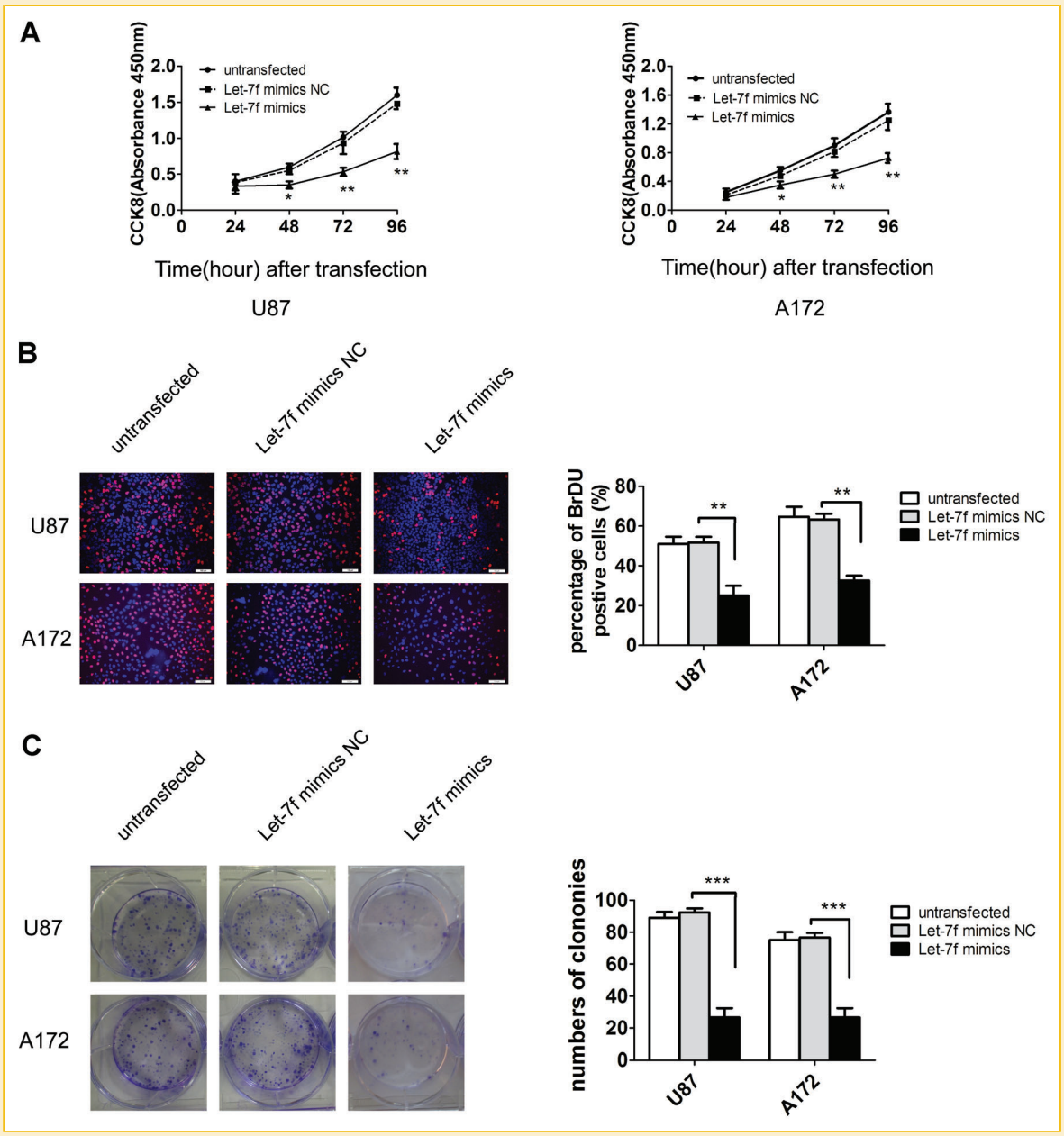

Fig. 2. Over-expression of Let-7f suppresses glioma cell proliferation in vitro. A: CCK-8 assays showed that the proliferation of cells transfected with Let-7f mimics was reduced at certain time points compared with NC-transfected cells. No obvious difference between the untransfected group and the NC-transfected group was observed. B: Edu-positive cells when Let-7f was over-expressed in U87 and A172 cells are shown. The scrambled NC was used as the control. C: Colony formation assay was performed and analyzed in glioma cells 15 d after transfection. The scrambled NC was used as the control. Scale bar, 100 μm. All experiments were performed in triplicate. The data are shown as the mean ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

was evaluated by flow cytometry using annexin V-FITC/PI double staining. The results demonstrated that the apoptosis rates in both Let-7f mimics-transfected cell lines were significantly increased compared with the NC-transfected group or untransfected group (Fig. 3B).

Cyclin D1 and cyclin E are cell cycle regulators that prevent cell cycle arrest in the G0/G1 phase and accelerate S phase progression. Immunoblot analysis showed that the expression levels of cyclin D1 and cyclin E in mimics-transfected cells were down-regulated compared with the NC-transfected group or untransfected group (Fig. 3C). The levels of the cyclin-dependent kinase inhibitors P21 and P27 also increased in glioma U87 and A172 cells that over-expressed Let-7f (Fig. 3C). Apoptosis regulators were further examined, as shown in Figure 3C. Bax expression increased; however, Bcl-2 expression levels were decreased in both cell lines that over-expressed Let-7f (Fig. 3C). Additionally, caspase-3 activity was significantly up-regulated in Let-7f mimics-transfected glioma cells compared with the control (Fig. 3C).

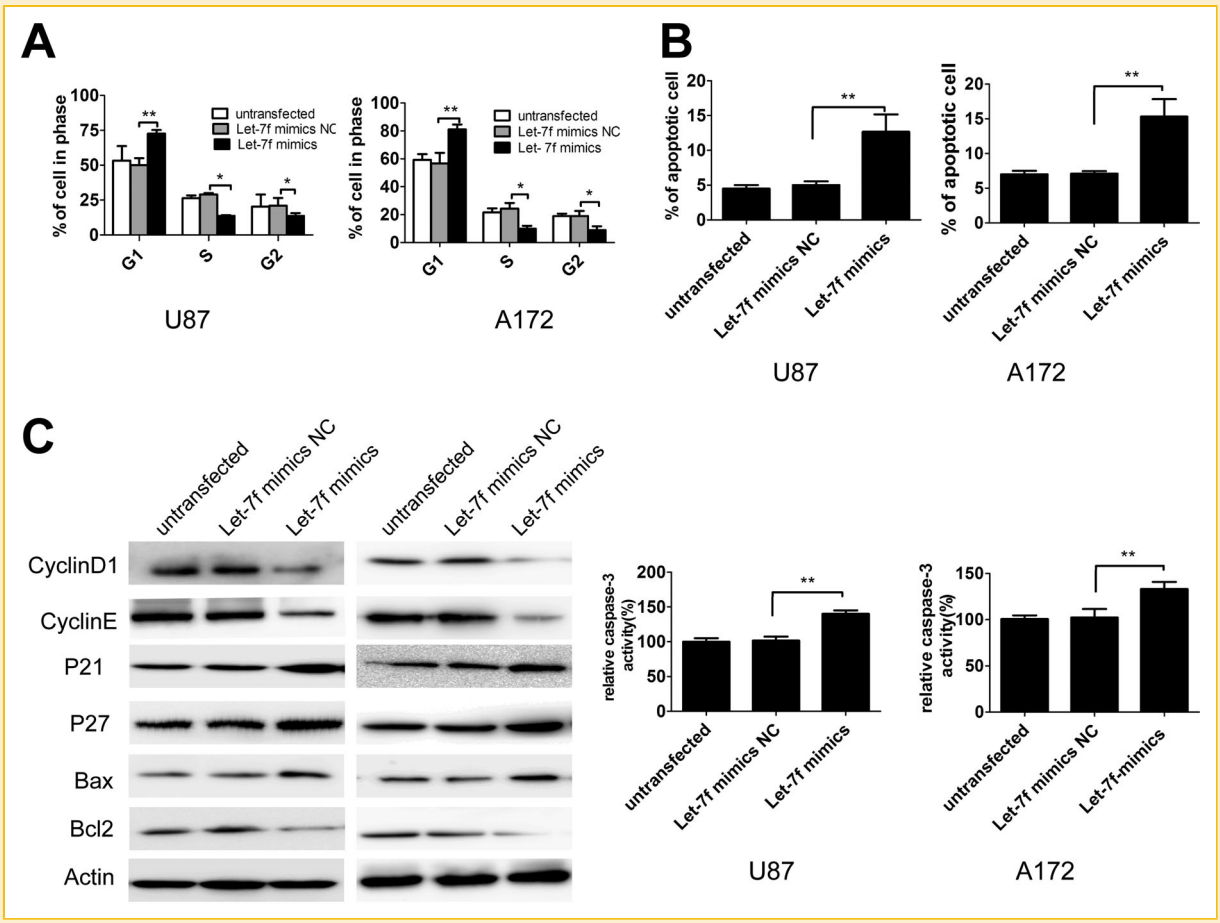

Fig. 3. Let-7f induces G1 phase arrest and promotes apoptosis in glioma cells. A: Cell cycle distributions of transfected U87 and A172 cells were detected by flow cytometry after 48 h. The results showed that Let-7f over-expression in U87 and A172 cells resulted in an increase in the cell population at the G1 phase and a decrease in the cell population at the S and M phases. B: Apoptosis of Let-7f mimics-transfected cells was analyzed by flow cytometry. The results showed that apoptosis levels were increased in the Let-7f mimics-transfected group compared with the untransfected group and NC-transfected group. C: Western blot analysis of the relative protein levels of cyclin D1, cyclin E, p21, p27, Bcl-2, and Bax in the Let-7f over-expressed group and in the NC and blank groups of A172 and U87 cells. Actin was used as a whole-cell protein loading control (left). Caspase-3 activities of glioma cells were assayed in the Let-7f mimics-transfected, NC-transfected and or untransfected groups (right). The data are presented as the mean ± SD of three independent experiments $^{*}P < 0.05$, ($^{**}P < 0.01$).

These data suggested that Let-7f affects the growth of glioma cells through cell cycle arrest and apoptosis, which are mediated by regulators of the cell cycle and apoptosis-related signaling pathways.

LET-7F INHIBITS GLIOMA CELL MIGRATION AND INVASION

To evaluate the effect of Let-7f on glioma cell mobility, transwell migration and invasion assays were performed in U87 and A172 cells at 48 h after transfection. The results of the cell migration assay showed that the migration ability of Let-7f mimics-transfected A172 and U87 cells decreased by 49% and 47%, respectively (Fig. 4A). Furthermore, the invasion ability of Let-7f mimics-transfected A172 and U87 cells was inhibited compared with the NC-transfected group (Fig. 4A). Because MMP2 and MMP9 play important roles in tumor cell migration and invasion, the effect of Let-7f on the expression levels of MMP2 and MMP9 was examined. As shown in Figure 4C and D, Let-7f over-expression induced the down-regulation of MMP-2 and MMP-9 proteins in U87 and A172 cells. These results suggested that Let-7f significantly inhibits glioma cell migration and invasion in vitro.

PERIOSTIN IS A POTENTIAL TARGET OF LET-7F IN GLIOMA CELLS

We identified the target genes of Let-7f by microRNAs, TargetScan and miRBase to explore the molecular mechanisms by which Let-7f inhibited glioma cell growth and mobility and found that the 3′ UTR of periostin contains highly conserved putative Let-7f binding sites (Fig. 5A).

The expression level of periostin was analyzed following Let-7f mimics transfection in glioma cells to determine whether periostin expression was regulated by Let-7f. Western blotting analysis showed that periostin was down-regulated in glioma cells after Let-7f over-expression compared with NC-transfected or untransfected cells (Fig. 5B). pGL3-WT-periostin-A-3′ UTR and pGL3-MUT-periostin-A-3′ UTR luciferase reporter plasmids were generated to determine whether periostin is a direct target of Let-7f. The reporter assay

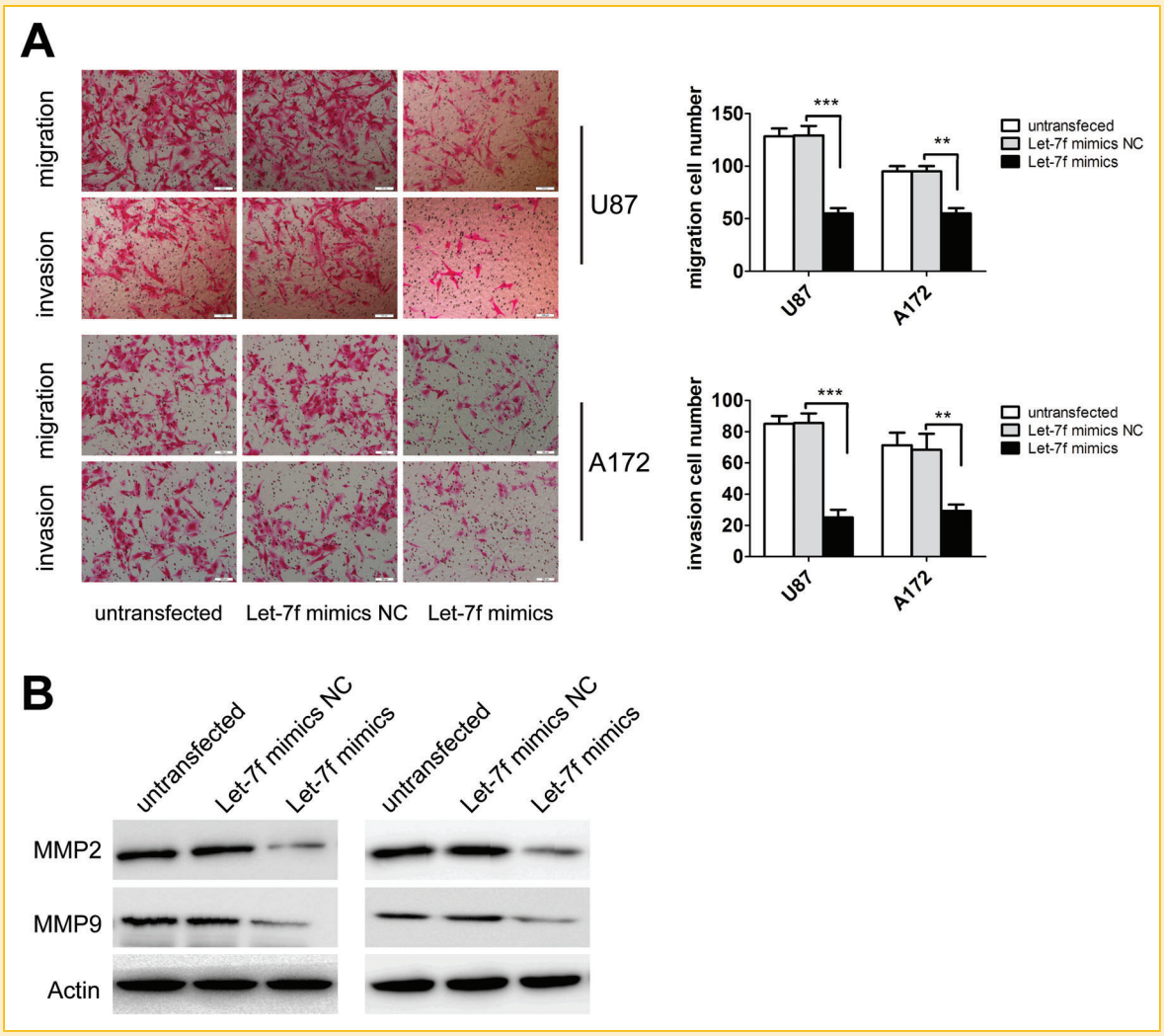

Fig. 4. Let-7f inhibits glioma cell migration and invasion. A: Effect of Let-7f on cell movement ability was assessed by transwell migration and Matrigel invasion assays. Compared with the control cells, Let-7f over-expression decreased the migration and invasion abilities of U87 and A172 at 24 h after transfection. B: Expression levels of MMP2 and MMP9 proteins were detected by Western blot analysis. Actin was used as the loading control. Scale bar, 100 μm. All experiments were performed in triplicate. The data are shown as the mean ± SD ($^{**}P < 0.01$, $^{***}P < 0.001$).

revealed that Let-7f over-expression significantly suppressed the activity of the pGL3-WT-periostin-3′ UTR plasmid in glioma cells without changing the luciferase activity of the pGL3-MUT-periostin-3′ UTR plasmid (Fig. 5C). These results demonstrated that Let-7f directly regulated periostin expression by binding to the 3′ UTR of periostin in glioma cells.

PERIOSTIN IS INVOLVED IN THE REGULATION OF CELL PROLIFERATION, MIGRATION, AND INVASION BY LET-7F

Periostin expression was knocked down using siRNA to further investigate whether the function of Let-7f in glioma cells is related to the modulation of periostin, and the role of periostin in glioma cell growth, migration, and invasion was investigated. The transfection of glioma cells with siRNA for periostin effectively inhibited periostin protein expression (Fig. 6A). The down-regulation of periostin expression suppressed cell growth (EdU assay, Fig. 6B), migration (transwell migration assay, Fig. 6C), and invasion (Matrigel transwell invasion assay, Fig. 6D) in both cell lines. The results suggested that the knockdown of periostin expression partly phenocopied the effects of Let-7f in glioma cells. In addition, whether the up-regulation of periostin expression reversed the suppression of cell phenotypes caused by Let-7f over-expression in glioma cells was examined. The periostin cDNA plasmid with the MUT 3′ UTR region and Let-7f mimics or NC were co-transfected into glioma cells. As shown in Figure 6E, the decreased level of periostin that was caused by Let-7f over-expression was overcome via the introduction of periostin cDNA. More importantly, the data clearly showed that the ectopic expression of periostin effectively restored the suppression of glioma cell growth (Fig. 6F), migration (Fig. 6G), and invasion (Fig. 6H) caused by Let-7f over-expression. These results suggested that Let-7f inhibited glioma cell growth, migration, and invasion by regulating periostin expression.

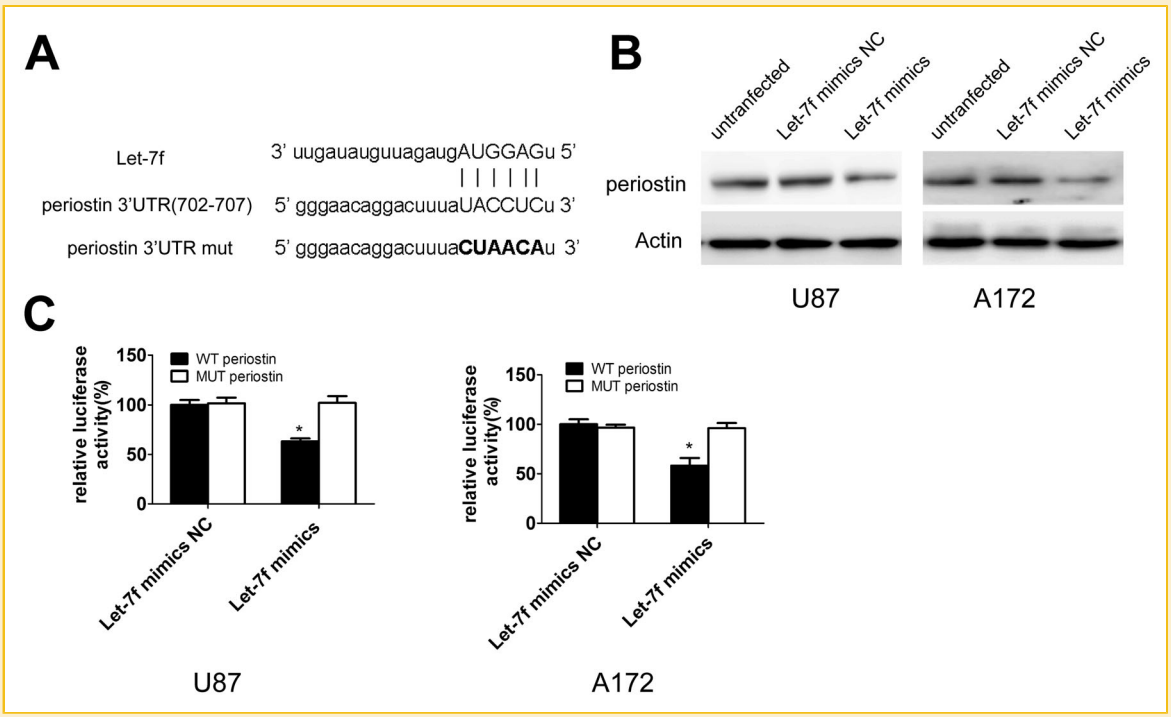

Fig. 5. Periostin is a direct target gene of Let-7f. A: Bioinformatic analysis of the predicted interactions between Let-7f and its binding sites within the 3′ UTR region of periostin. The corresponding mutant nucleotides of the periostin 3′ UTR are labeled in bold type. B: Periostin protein expression was assayed by Western blot analysis at 48 h after U87 and A172 treatment in the Let-7f mimics-transfected group and in the NC-transfected and blank groups. Actin was detected on the same blot as the loading control. C: Luciferase activity was determined after 48 h in glioma cell lines co-transfected with the luciferase reporter construct containing 3′ UTR together with Let-7f mimics or NC. The results show the mean of the normalized luciferase intensity. All experiments were performed in three independent experiments. The data are shown as the mean ± SD $^{*}p < 0.05$.

LET-7F SUPPRESSES THE AGGRESSIVE PHENOTYPE OF GLIOMAS IN VIVO

The in vitro results suggested that Let-7f over-expression might be sufficient to inhibit tumor progression and prompted the investigation of the effect of Let-7f on the malignant properties of gliomas using a glioma xenograft model in vivo.

When the mean tumor volume of the mice was approximately 200 mm^3, Let-7f mimics, NC, or PBS administration by intratumoral injections began and was continued every 3 days for 4 weeks. As shown in Figure 7B, no difference in tumor size was observed in the three groups during the first 4 days of observation. From day 10 on, the tumor volume was significantly smaller in the Let-7f mimics-treated mice at the same time points compared with the controls. At the end of the study, the difference in tumor size reached the maximum between the Let-7f mimics-treated group and the control group ($P < 0.01$). Moreover, the mean tumor weight at the end of the experiment was markedly lower in the Let-7f mimics-treated group compared with the NC-treated group. However, no difference in tumor volume or weight was observed between the NC- and PBS-treated groups (Fig. 7C). The level of Let-7f in the Let-7f mimics-treated group was up-regulated within the tumor injection (Fig. 7E). Immunohistochemical analysis showed marked decreases in periostin, Ki67, and MMP2/MMP9 protein levels in the Let-7f mimics-treated group (Fig. 7D and F). In addition, a TUNEL assay showed increased levels of apoptosis in tumors administered Let-7f mimic compared with the control group (Fig. 7D and G).

In the intracranial tumor model, the borders of control and NC-treated tumors were blurred, and tumor cells infiltrated into surrounding normal tissue. However, the border between the tumor and the surrounding tissues was clear in the Let-7f mimics treatment, and few tumor cells infiltrated into normal tissue (Fig. 7H). More importantly, Kaplan–Meier analysis showed that mice treated with Let-7f mimics had a significantly longer survival time (median survival time, 35 days) compared with control or NC-treated mice (median survival time, 23 days) (Fig. 7I). In contrast, no significant difference in survival was observed between control mice or NC-treated mice (Fig. 7I).

These results further suggested that Let-7f could inhibit glioma growth, migration, and invasion in vivo and that Let-7f might be a novel new therapeutic target for malignant glioma therapy.

DISCUSSION

Uncontrolled cell proliferation and invasion are considered important features of glioma [Deng et al., 2010]. Because of the poor understanding of the molecular mechanisms governing glioma development and progression, the therapeutic efficacy for glioma patients is not satisfactory, despite multiple advanced treatments [Shen et al., 2010]. In the present study, the data showed that the expression level of Let-7f is down-regulated in glioma samples and cell lines and that Let-7f serves as a tumor suppressor that directly

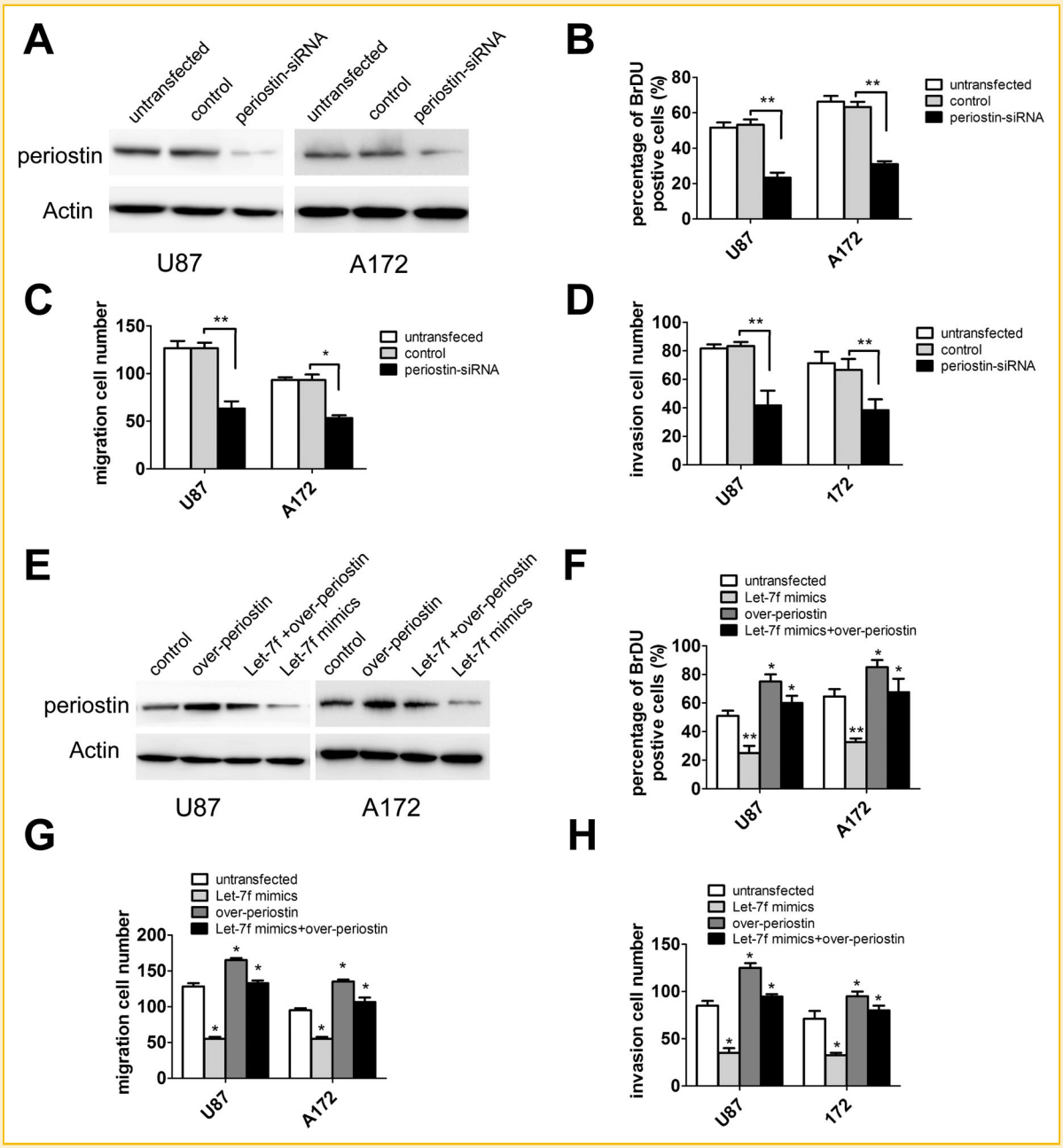

Fig. 6. Targeting periostin expression mediates the inhibitory function of Let-7f. U87 and A172 cells were transfected with siRNA or siRNA-negative control. A plasmid containing periostin with MUT 3′ UTR and Let-7f mimics or NC was co-transfected into cells. (A) and (E) Effect of periostin siRNA or co-transfection at 48 h after transfection as determined by Western blot analysis. (B) and (F) Cell proliferation (EdU incorporation assay). (C) and (G) Cell migration (transwell migration assay). (D) and (H) Cell invasion (transwell invasion assay). The data are represented as the mean ± SD from three independent experiments. The results are the mean ± SD of three different experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

targets periostin. Let-7f over-expression inhibits glioma cell growth, migration, and invasion.

miRNAs, which are a class of small non-coding RNAs that primarily regulate gene expression at the posttranscriptional level, are involved in various physiological and pathological events [Shi et al., 2012]. Aberrant miRNA expression is thought to play key roles in cancer development, enhancing tumor suppressive or oncogenic roles by regulating their target genes [Nohata et al., 2013]. Thus, understanding the functions and mechanisms of miRNAs is an important research area.

miRNA expression is abnormal in most cancers. Recently, several reports demonstrated that miRNA expression is down- or up-regulated in gliomas compared with normal brain tissues. miR-221, miR-21, and miR-155 were up-regulated [Quintavalle et al., 2013;

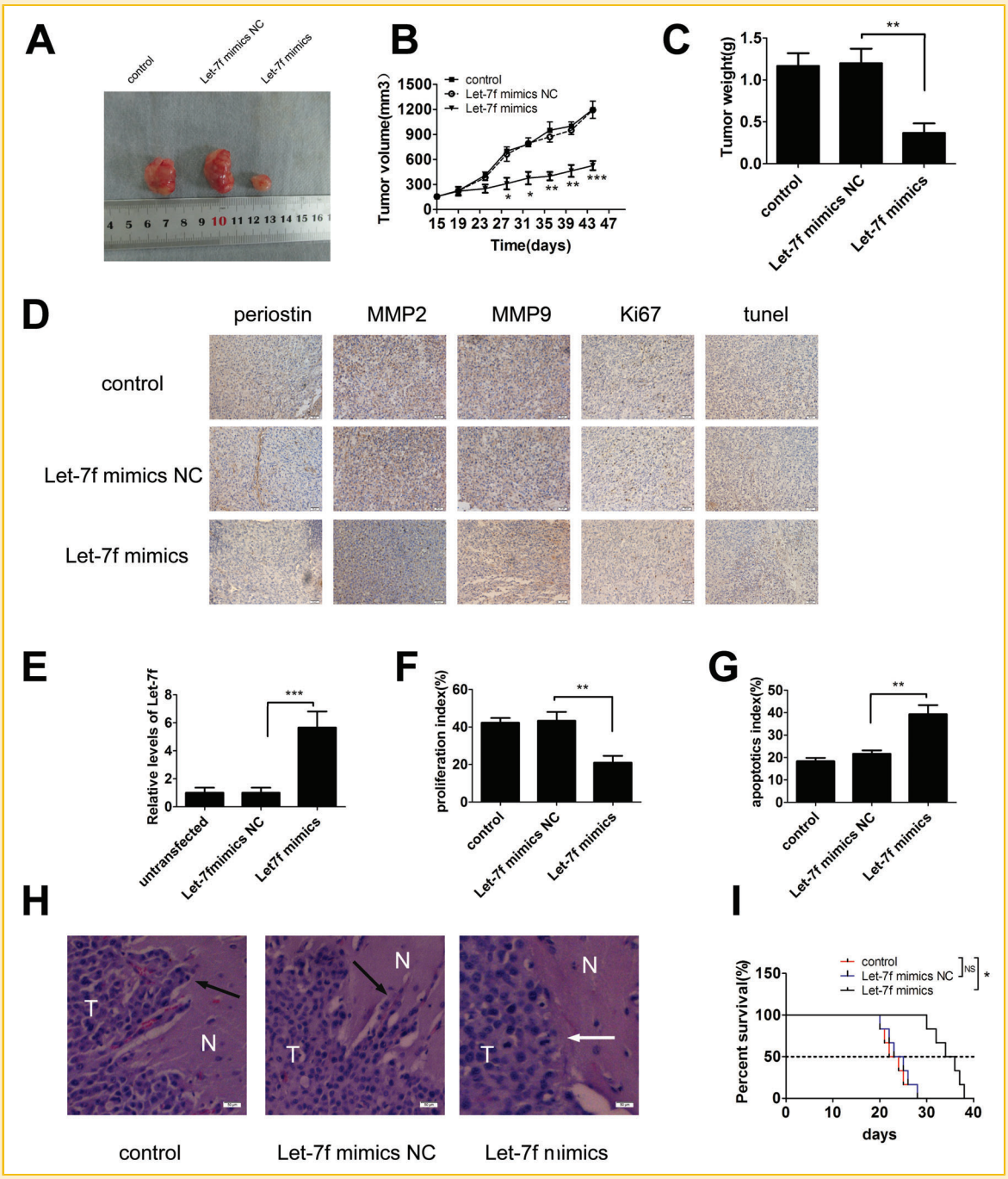

Fig. 7. Let-7f inhibits the progression of glioma cells in a mouse xenograft model. A: The image shows representative features of tumor xenografts in this experiment. B: Tumor volumes following Let-7f mimics treatment significantly were decreased compared with the control mice and NC-treated mice. C: Tumor weights were significantly decreased after Let-7f mimics administration compared with the control and NC-treated mice. E: The expression level of Let-7f within the tumor injection was evaluated by qPCR in a tumor model. (D, F, and G) The effect of Let-7f expression on cell aggressiveness in the above implanted tumors was determined by immunohistochemical assays against periostin, MMP2/9, and Ki67 and TUNEL assay. The proliferation and apoptosis indices of the cancer cells were the positively stained cells/total cells. Scale bar, 50 μm. H: H&E staining shows that the invasion phenotype of Let-7f mimics-treated glioma cells differed from that of control or NC-treated cells in vivo. Scale bar, 50 μm (T, the tumor tissues; N, the normal brain tissues; the black arrows, the tumor cells infiltrated the surrounding normal brain tissues in the control and NC-treated groups; the white arrows, the border between the tumor and the surrounding normal brain tissues in the Let-7f mimics treatment group). I: Kaplan–Meier survival analysis reveals significantly prolonged survival in the Let-7f mimics-treated group compared with the control group or the NC-treated group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Sun et al., 2014; Yang et al., 2014], whereas miR-128, miR-181a, and miR-320a were down-regulated in glioma specimens [Chen et al., 2010; Shi et al., 2012; Guo et al., 2014]. Let-7f is a member of the Let-7 family, which is down-regulated in several malignancies, including hepatocellular carcinoma, ovarian carcinoma, and head and neck cancer [Vaksman et al., 2011; Zheng et al., 2013; Ge et al., 2014]; however, Let-7f is up-regulated and has oncogenic roles in primary breast cancer [Kuehbacher et al., 2007]. These results highlight conflicting data regarding the role of Let-7f in different types of cancers, and whether Let-7f actually participates in gliomagenesis requires further investigation. Our results showed that Let-7f expression was significantly down-regulated in glioma tissues and cell lines compared with human brain tissues and NHAs. Furthermore, Let-7f expression decreased with increasing pathological glioblastoma grade. Our result is consistent with recent reports of miRNA expression profiles in gliomas [Loftus et al., 2012; Wang et al., 2013c]. Taken together, these results indicated that Let-7f might function as a tumor suppressor in glioma.

Let-7f has shown various effects on physiological and pathological processes [Liang et al., 2011]. Let-7f is a novel regulator of the innate immune response in human endocervical cells and is involved in the induction of immune tolerance [Sathe et al., 2014]. Let-7f also regulates secreted protein acidic and rich in cysteine (SPARC)-mediated cisplatin resistance in medulloblastoma cells [Sathe et al., 2014] and is involved in the differentiation capacity of human mesenchymal stem cells (hMSCs)[Egea et al., 2012]. Anti-Let7f mediates neuro-protection [Selvamani et al., 2012]. Let-7f was shown to play an important role in cell growth, migration, invasion, and angiogenesis in tumors [Kuehbacher et al., 2007; White et al., 2010; Liang et al., 2011].

A series of experiments was performed to confirm the functions of Let-7f in glioma cells. We observed that cell proliferation and colony formation were inhibited, and G1 cell cycle arrest was induced by transfected exogenous Let-7f mimics, which are involved in the down-regulation of cell cycle regulators (cyclin D1 and cyclin E) and in the up-regulation of cyclin-dependent kinases inhibitors (P27 and P21). In addition, the study showed that apoptosis was promoted via up-regulating Bax expression, down-regulating Bcl-2 expression, and activating the caspase-3 signal in glioma cells treated with Let-7f mimics. Glioma cells are able to not only proliferate but also invade the surrounding brain tissue, leading to extremely poor prognoses for patients suffering from glioma [Xie et al., 2014]. In this study, the data showed that Let-7f over-expression dramatically repressed the migration and invasion of glioma cells by regulating MMP2 and MMP9 expression in vitro. Moreover, intratumoral delivery of Let-7f mimics significantly suppressed glioma tumor cell growth in nude mice. More importantly, Let-7f mimics decreased the protein levels of Ki67, MMP2, and MMP9, which are markers for cell proliferation and invasion. A TUNEL assay showed that Let-7f induced apoptosis in vivo. These data are consistent with the in vitro studies and strongly suggested that Let-7f plays an important role in glioma development and progression.

To date, few targets of Let-7f have been reported. Let-7f over-expression in gastric cancer inhibits the invasion and migration of gastric cancer cells through directly targeting the tumor metastasis-associated gene MYH9 [Liang et al., 2011]. Let-7f is also involved in the induction of immune tolerance by targeting Blimp-1 [Sathe et al., 2014], and a tumor suppressor miRNA that is expressed in breast cancer directly targets the aromatase gene [Selvamani et al., 2012]. Aromatase inhibitor treatment of breast cancer cells increases the expression of Let-7f, which targets CYP19A1 [Selvamani et al., 2012]. Let-7f can affect cell proliferation by targeting KLKs [Liang et al., 2011]. The underlying molecular mechanism of the effects of Let-7f on glioma remains unclear. We attempted to determine the potential target genes of Let-7f in glioma by bioinformatic analysis (microRNAs, TargetScan, and miRBase). Bioinformatic prediction and experimental demonstration identified periostin as a direct target of Let-7f in glioma cells.

Periostin, which was originally designated osteoblast-specific factor 2, is up-regulated in many types of cancer, including breast cancer, prostate cancer, cholangiocarcinoma, and glioma [Wang et al., 2013a]. Periostin has an important role in cancer development and progression and may be involved in the progression and invasion of pancreatic cancer [Ben et al., 2011]. The re-expression of periostin in colon cancer cells or in head and neck cancer cells promotes tumorigenicity in nude mice [Kudo et al., 2012; Xiao et al., 2013]. Periostin has been reported to be a ligand for vß3 integrins, inducing integrin-dependent cell adhesion and motility [Watanabe et al., 2012]. More importantly, the previous reports showed that periostin expression correlates with glioma patient survival and with glioma grade and is an independent prognostic factor in high-grade glioma patients because periostin promotes cellular invasion and proliferation [Wang et al., 2013a; Tian et al., 2014]. Thus, periostin functions as an oncogene in gliomas.

In the present study, periostin expression could be significantly down-regulated after transfecting exogenous Let-7f mimics. A luciferase reporter assay also confirmed that Let-7f targeted the 3′ UTR of periostin. The results of the present study are the first to reveal the negative regulatory effects of Let-7f on periostin expression in glioma cells. Periostin was knocked down by siRNA in glioma cells to further elucidate the mechanisms of the tumor-suppressive effect of Let-7f, and the proliferation, migration, and invasion of these cells decreased similar to the phenotype observed upon Let-7f over-expression in glioma cells. Consistent with these results, periostin over-expression overcame the inhibitory effect of Let-7f on the growth, migration, and invasion of glioma cells. To our knowledge, our study is the first to show that Let-7f inhibits the proliferation, migration, and invasion of glioma cells through a possible mechanism of negatively regulating periostin expression.

Although we examined only two cell lines (U87 and A172) in these experiments, we speculate that other glioma cells will show similar results.

In summary, this study showed that Let-7f is down-regulated in glioma tissue and cell lines, and the results demonstrated that Let-7f plays an important role in glioma pathogenesis. Let-7f has a suppressive role in tumor progression by suppressing cell proliferation, migration, and invasion, inducing cell cycle arrest in the G1 phase, and promoting apoptosis in glioma. More importantly, the mechanism by which Let-7f mediates the inhibition of glioma cell aggressiveness is related to the direct regulation of its target periostin. Although knowledge regarding the role of Let-7f in glioma remains

incomplete, the role of Let-7f as a tumor suppressor indicates that Let-7f may be a novel therapeutic target for glioma.

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