MicroRNA-197 inhibits cell proliferation by targeting GAB2 in glioblastoma

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Abstract. Glioblastoma is the most common type of primary brain tumor in adults, and is usually fatal in a short duration. Acquiring a better understanding of the pathogenic mechanisms of glioblastoma is essential to the design of effective therapeutic strategies. Grb2-associated binding protein 2 (GAB2) is a member of the daughter of sevenless/Gab family of scaffolding adapters, and has been reported to be important in the development and progression of human cancer. Previously, it has been reported that GAB2 is expressed at high levels in glioma, and may serve as a useful prognostic marker for glioma and a novel therapeutic target for glioma invasion intervention. Elucidating why GAB2 is overexpressed in glioma, and investigating how to downregulate it will assist in further understanding the pathogenesis and progression of the disease, and to offer novel targets for therapy. The present study used *in situ* hybridization to detect microRNA (miR)-197 expression levels and Targetscan to predict that the 3'-UTR of GAB2 was targeted by miR-197. Northern blotting and reverse transcription-quantitative polymerase chain reaction were also conducted in the current study. miR-197 is downregulated in glioblastoma tissues, compared with adjacent normal tissues, however it involvement continues to be detected in the disease. The results of the present study demonstrated that miR-197, as a tumor suppressor gene, inhibited proliferation by regulating GAB2 in glioblastoma cells. Furthermore, GAB2 was not only upregulated in glioma, but its expression levels were also associated with the grades of glioma severity. In addition, overexpression of GAB2 suppressed the expression of miR-197 in glioblastoma cells. Therefore, restoration of miR-197 and targeting GAB2 may be used, in conjunction with other therapies, to prevent the progression of glioblastoma.

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Introduction

MicroRNAs (miRNAs; miRs) are short, non-coding RNAs, which regulate target mRNA by binding predominantly to the 3'-untranslated region (3'-UTR), inducing either translational repression or the degradation of the mRNA target (1-3). Previously, the involvement of miRNAs in the phenotypic modulation of human glioma has been reported. For example, miRNA-21 knockdown disrupts glioma growth *in vivo* and exhibits synergistic cytotoxicity with neural precursor cell-delivered secretable tumor necrosis factor-related apoptosis-inducing ligand in human glioma (4); miRNA-34a acts as a tumor suppressor in brain tumors and glioma stem cells (5); and miRNA-181a sensitizes human U87MG malignant glioma cells to radiation by targeting B cell lymphoma 2 (6). Although miR-197 is downregulated in glioblastoma, its roles in malignant tumor progression remain to be elucidated (7).

Grb2-associated binding protein (GAB)2 belongs to a family of evolutionarily conserved proteins consisting of three mammalian paralogues: GAB1, GAB2 and GAB3; *Drosophila melanogaster* homolog daughter of sevenless; and *Caenorhabditis elegans* homolog suppressor of clear. Family members exhibit 40-50% sequence homology, however they are associated with unique cellular functions (8). GAB1 and GAB2 are expressed ubiquitously, but are expressed at the highest levels in the brain, kidney, lung, heart, testis and ovary (9). GAB3 also has a widespread expression pattern, although its expression is highest in lymphoid tissues (9). DNA amplification is a common mechanism underlying oncogenic activation in human cancer. GAB2 is located on chromosomal band 11q14.1, and amplification of 11q13-14.1 is frequently observed in human malignancies (10). The identification of GAB2 as a potential oncogene has been reported in studies investigating breast (11,12) and ovarian cancer (13), leukemia (14), melanoma (15) and gastric cancer (16). Previously, GAB2 was found to be expressed at high levels in glioma and a subset of cancer cell lines. Statistical analysis suggested that the upregulation of GAB2 was correlated with the World Health Organization (WHO) grade of glioma, and that patients with high expression levels of GAB2 exhibited reduced survival rates (17). In a previous animal experiment, knockdown of GAB2 inhibited the invasive ability of glioma cells in the brains of mice with severe combined immunodeficiency (17). As demonstrated by

cellular investigations, the downregulation of GAB2 inhibits the migration and invasion of glioma cells (17). Elucidating why the GAB2 gene is overexpressed and determining how it may be downregulated will assist in further understand the pathogenesis and progression of glioma, and offer novel therapeutic targets.

Elucidation of the overexpression of GAB2 and methods to downregulate the expression levels may aid in understanding the pathogenesis and progression of the disease and aid in development of novel targets for therapeutic strategies.

Materials and methods

Glioma tissue samples, cells and GAB2-expressing plasmids. Human glioma tissue samples were obtained from the Department of Neurosurgery, Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China). The patients included 38 World Health Organization (WHO) grade I; 39 WHO grade II; 44 WHO grade III and 40 WHO grade IV cases) (18). The patients comprised 60 women and 111 men, with a mean age of 58 years (range, 31-78 years). The human tissue samples were used according to internationally recognized guidelines, as well as local and national regulations. Investigations involving humans followed international and national regulations. The medical ethics committee of Shandong Provincial Hospital Affiliated to Shandong University (Shandong, China) approved the experiments of the present study. Informed consent was obtained from each individual. Human A172 glioblastoma cell lines were donated by Dr Rosalind Segal (University of Heidelberg, Heidelberg, Germany). The A172 cells were maintained at 37˚C in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). GAB2-expressing plasmids were donated by Dr Xiao Yao (University of Heidelberg).

miRNA precursors. The miR-197 miRNA precursor (pre-miR-197) and a control precursor (control miR) were purchased from Ambion (Thermo Fisher Scientific, Inc.).

Transfection. For the transfection experiments, $5x10^4$ A172 cells were cultured in serum-free medium without antibiotics to 60% confluence for 24 h at 37˚C, prior to being transfected with Invitrogen Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following incubation at 37˚C for 6 h, the medium was removed and replaced with normal culture medium for 48 h at 37˚C, unless otherwise specified.

Detection of miRNAs by in situ hybridization in formalin-fixed paraffin-embedded (FFPE) sections. Paraffin-embedded specimens of gliomas and adjacent normal tissue were obtained from the Pathology Department of the Shandong Provincial Hospital affiliated to Shandong University. Sections (5 μm) were deparaffinized in 70% xylene (Tiangen Biotech Co., Ltd., Beijing, China) and then rehydrated through a series of graded ethanol dilutions (100-25%). The sections on slides and submerged in diethyl pyrocarbonate-treated water (Tiangen Biotech Co., Ltd.) and subjected to proteinase K (Tiangen Biotech Co., Ltd.) (10 μg/ml) digestion and 0.2% glycine treatment, refixed in 4% paraformaldehyde (Tiangen Biotech Co., Ltd.), and treated with acetylation solution (Tiangen Biotech Co., Ltd.) containing 66 mmol/l HCl, 0.66% (v/v) acetic anhydride and 1.5% (v/v) triethanolamine. The slides were rinsed three times with 1X phosphate-buffered saline (PBS) between treatments. The slides were then prehybridized in hybridization solution (Tiangen Biotech Co., Ltd.), containing 50% formamide, 5X saline sodium citrate (SSC), 500 μg/ml yeast tRNA and 1X Denhardt's solution, at 50˚C for 30 min. Subsequently, 5-10 pmol fluorescein isothiocyanate-labeled, LNA-modified DNA probe (Exiqon A/S, Vedbeak, Denmark) complementary to miR-197 was added to the 180 μl hybridization solution and hybridized for 2 h at a temperature 20-25˚C below the calculated melting temperature of the LNA probe (37˚C). Following washes in SSC at increasing stringency (2-0.2X) at the same temperature as hybridization, a tyramide signal amplification reaction was performed using a GenPoint Fluorescein kit (Dako, Glostrup, Denmark), according to the manufacturer's protocol. Finally, the slides were mounted with Prolong Gold solution (Invitrogen; Thermo Fisher Scientific, Inc.). The signals were visually quantified by Dr Li-Qiang Tian and Dr En-Qin Liu using a quick score system (scores 0-5), which combines the intensity of the signal and the percentage of positive cells (signal: 0, no signal; 1, weak signal; 2, intermediate signal; 3, strong signal; percentage: 0, 0%; 1, <30%; 2, >30%). The tissue sections were then examined by Jian Li and Guang-Ming Xu in a blinded-manner, who reported results in agreement with the initial quantifications.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miRNA. Total RNA from the cultured A172 cells, with efficient recovery of small RNAs, was isolated using an mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.). In addition, detection of the mature form of miRNAs was performed using an mirVana qRT-PCR miRNA Detection kit, according to the manufacturer's protocol. Reverse Transcription system (Promega Corporation, Madison, WI, USA) was used with the miR-197 primer as follows: 5'-TTCACCACCTTCTCCACCCAGC-3'. The U6 small nuclear RNA was used as an internal control. The RT-qPCR and quantification were conducted as described previously (19).

Cell counting assay. At 48 h post-transfection, A172 cells were seeded in 96-well plates in triplicate at a density of $5x10^3$ cells/well in 100 μl RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% antibiotics (Hyclone; GE Healthcare Life Sciences). Cell proliferation was evaluated using a Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol, and the absorbance value for each well was measured at 450 nm using a microplate reader (Spectra Max 180; Molecular Devices, LLC, Sunnyvale, CA, USA).

Colony formation assay. For the colony formation assay, $5x10^4$ A172 cells were transfected with pre-miR-197 or control miR for 24 h, prior to being seeded into a 6-well plate. A total of 0.2 ml FBS was added per well on day 5. Following 9-10 days incubation at 37˚C, the plates were washed with PBS

and stained with 0.1% crystal violet (Tiangen Biotech Co., Ltd.). Colonies with >50 cells were manually counted (IX-70 inverted microscope; Olympus Corporation, Tokyo, Japan). Plating efficiency was calculated by dividing the number of colonies formed in the treated group by that in the control.

Cell cycle analysis. The A172 cells ($8.0x10^5$ cells) were seeded into a 100 mm culture plate and allowed to attach overnight. The cells were transfected, as previously described, for 24 h at 37˚C, washed twice with NaCl/P_i (Tiangen Biotech Co., Ltd.), and then centrifuged at 200 x g at room temperature for 10 min. The pellet was resuspended in 1 ml cold NaCl/P_i and fixed in 70% ethanol for ≥12 h at 4˚C. The fixed cells were incubated with 100 μl DNase-free RNaseA (200 μg/ml; Tiangen Biotech Co., Ltd.) for 30 min at 37˚C, prior to the addition of 1 mg/ml propidium iodide (Tiangen Biotech Co., Ltd.). The stained cells were analyzed using a fluorescence-activated cell sorter (BD Accuri C6; BD Biosciences, San Jose, CA, USA). The percentages of cells in the G_1, S and G_2/M phases of the cell cycle were determined using Cell Quest Pro software (FlowJo; Tree Star, Inc., Ashland, OR, USA).

BrdU proliferation analysis. Cell proliferation was assessed using a colorimetric BrdU Proliferation kit, according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN, USA). Briefly, the transfected A172 cells were labeled with BrdU for 4 h at 37˚C. The genomic DNA was fixed and denatured by BrdU, and then incubated with a rat peroxidase-conjugated anti-BrdU antibody (Sigma-Aldrich, St. Louis, MO, USA; cat. no. 11202693001) for 90 min. A substrate for the conjugated peroxidase, a component included in the kit, was then added, and the reaction product was quantified by measuring the absorbance at a wavelength of 270 nm using a SpectraMax M5 (Molecular Devices, LLC). The results were then normalized to the number of total viable cells.

Western blot analysis. Confluent A172 cells were washed twice with cold PBS and denatured in a radioimmunoprecipitation assay lysis buffer (containing 20 mM Tris-HCl, 200 mM NaCl, 0.2% Nonidet P-40, 0.5% Triton X-100 and protease inhibitors; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) and boiled at 100˚C for 10 min. Protein was quantified by BCA Protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) and western blot analysis was performed, as described previously (20). Equal quantities of protein (5 μg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Beijing Solarbio Science and Technology Co., Ltd.) and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking in Tris-buffered saline (TBS; Beijing Solarbio Science and Technology Co., Ltd.) with 0.1% Triton X-100 and 5% milk, the membranes were incubated with the following primary antibodies: Rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA; 1:500; Abcam, Cambridge, MA, USA; cat. no. ab18197), rabbit polyclonal anti-retinoblastoma (Rb; 1:500; Abcam; ab1765), rabbit polyclonal anti-Ki67 (1:500; Abcam; ab15580), rabbit monoclonal anti-cyclin-dependent kinase (CDK)2 (1:500; Abcam; ab32147), rabbit monoclonal anti-CDK4 (1:500; Abcam; ab108357), rabbit monoclonal anti-E2F transcription factor 1 (E2F1; 1:500; Abcam; ab179445), rabbit monoclonal anti-GAB2 (1:500; Abcam; ab32365) and mouse monoclonal anti-β-actin (1:500; Abcam; ab129348) overnight at 4˚C. Following washing with TBS three times, the membranes were incubated with donkey anti-mouse Alexa Fluor 488 secondary antibodies (1:5,000; Abcam; cat. no. ab150105) and goat anti-rabbit immunoglobulin G biotin-conjugated secondary antibodies (1:5,000; Abcam; cat. no. ab6720) for 30 min at room temperature. Signal detection was conducted with an enhanced chemiluminescence system (GE Healthcare Life Sciences, Chalfont, UK). The specific proteins were visualized and analyzed using an Odyssey™ Infrared Imaging system (Gene Company, Ltd., Hong Kong, China).

Bioinformatics. Analysis of the potential miRNA target sites was performed using the TargetScan algorithm (http://www.targetscan.org).

RT-qPCR for GAB2. Total RNA was isolated from A172 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand cDNA was synthesized from the total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corporation), 2.5 μl 5X buffer and 2 μl random hexamer primers (Sangon Biotech, Co., Ltd., Shanghai, China). The concentration of the cDNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The reaction was performed with the following thermocycling conditions: Denaturation for 30 sec at 95˚C, annealing for 45 sec at 52-58˚C, depending on the primers used, and extension for 45 sec at 72˚C. The PCR products were visualized on 2% agarose gels (Tiangen Biotech Co., Ltd.) stained with 20% ethidium bromide (Tiangen Biotech Co., Ltd.) under ultra violet transillumination. RT-qPCR was performed using Power SYBR® Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The primer sequences for GAB2 were forward, 5'-CTGAGACTGATAACGAGGAT-3' and reverse, 5'-GAGGTGTTTCTGCTTGAC-3'.

Immunofluorescence analysis. For immunofluorescence analysis, $5x10^4$ A172 cells were plated on glass coverslips in six-well plates and transfected with 50 nM pre-miR-197 or control miR. At 36 h post-transfection, the coverslips were stained with the previously mentioned anti-GAB2 antibodies. Alexa Fluor 488 goat anti-rabbit IgG antibody was used as a secondary antibody (Invitrogen; Thermo Fisher Scientific, Inc.). The coverslips were counterstained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for visualization of the nuclei. Microscopic analysis was performed using a confocal laser-scanning microscope (FV300; Olympus Corporation). Fluorescence intensity was measured in a small number of viewing areas with 200-300 cells per coverslip, and analyzed using ImageJ 1.37 software (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter assay. Luciferase reporter plasmids were purchased from Tianjin Biotech Co., Ltd. (Tianjin, China). Briefly, the 3'-UTR of human GAB2 mRNA was cloned into pRL-TK (Promega Corporation) using three PCR-generated fragments, including positions of 3885-3891, 1151-1158,

2044-2050 in the 3'UTR of GAB2. Site-directed mutagenesis of the miR-197 target sites in the GAB2-3'-UTR was performed using a Quik Change Mutagenesis kit (Agilent Technologies GmbH, Waldbronn, Germany), with GAB2-WT-luc (containing position 1151-1158) a template. For reporter assays, the A172 cells were transiently transfected with wild-type or mutant reporter plasmid and miRNA using Lipofectamine® 2000. Reporter assays were performed 36 h post-transfection using a Dual Luciferase Assay system (Promega Corporation), normalized for transfection efficiency with co-transfected *Renilla* luciferase (Tiangen Biotech Co., Ltd.).

Immunohistochemistry. Immunohistochemistry was performed using standard laboratory techniques. Antigen retrieval was performed by autoclaving at 121˚C for 8 min (model, V8879l; Tiangen Biotech Co., Ltd.), following which the above-mentioned glioma tissue sample sections were incubated with 10% normal goat serum in PBS for 15 min at 37˚C to eliminate non-specific staining. Incubation with anti-GAB2 antibody was then performed. Finally, the sections were counterstained with 10% Mayer hematoxylin (Tiangen Biotech Co., Ltd.), dehydrated, mounted, and observed. Staining was evaluated by a neuropathologist and an investigator in a blinded-manner (H-7000 electron microscope; Hitachi, Ltd., Tokyo, Japan). The sections were classified as - (negative), + (focal and weak immunoreactivity), ++ (diffuse and weak or focal and intense immunoreactivity) or +++ (diffuse and intense immunoreactivity). The data were analyzed using SPSS 11.5 (SPSS, Inc., Chicago, IL, USA). Quantitative image analysis was performed, as previously described (21). The comparison between high expression levels was achieved using a χ^2 test.

Examination of cell proliferation using an MTT assay. The effect on cell proliferation was assessed using MTT (Sigma-Aldrich). A172 cells were suspended in 200 μl medium and seeded in 96-well plates ($1x10^4$/ml and grown to 50% confluence and synchronized with serum-free medium for 24 h prior to adding serum to a concentration of 10%. MTT reagent (20 μl; 5 mg/ml; Amresco, LLC, Solon, OH, USA) was added at 48 h. The cells were incubated at 37˚C for 4 h and following centrifugation at 500 x g for 5 min at room temperature, 150 μl dimethyl sulfoxide (Beijing Solarbio Science and Technology Co., Ltd.) was added to halt the reaction. The cell confluence was determined by absorbance at a wavelength of 570 nm on a spectrophotometer (UV-16; Shimadzu Corporation, Kyoto, Japan) and absorbance was directly proportional to the number of surviving cells.

miRNA microarray. Total RNA from the cultured A172 cells, with efficient recovery of small RNAs, was isolated using an mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.). The cRNA for each sample was synthesized using a 3' IVT Express kit (Affymetrix, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol. The purified cRNA was fragmented by incubation in fragmentation buffer (provided in the 3' IVT Express kit) at 95˚C for 35 min, and chilled on ice. The fragmented labeled cRNA was applied to a microRNA 2.0 array (Affymetrix, Inc.) and hybridized in a Genechip hybridization oven 640 (Affymetrix, Inc.) at 45˚C for 18 h. Following washing and staining in a Genechip fluidics station 450 (Affymetrix, Inc.), the arrays were scanned using a Genechip Scanner 3000 (Affymetrix, Inc.). The gene expressions levels of the samples were normalized and compared using Partek GS 6.5 (Partek, Inc, St. Louis, MO, USA). The average-linkage hierarchical clustering of the data was applied using Cluster (http://rana.lbl.gov) and the results were displayed using TreeView (http://rana.lbl.gov) (22).

Northern blotting analysis. Northern blot analysis of miRNAs was performed, as previously described (23). Total RNA was fractionated on a denaturing 12% polyacrylamide gel containing 8 M urea (Tiangen Biotech Co., Ltd.). The RNA was transferred to a Nytran N membrane (Schleicher & Schuell Bioscience GmbH, Dassel, Germany) by capillary method and fixed by ultraviolet cross-linking. The membranes were probed with ^{32}P-labelled standard DNA and LNA-modified oligonucleotides, complementary to the mature miRNA-197 and U6 snRNA, 10 pmol of each probe was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Prehybridization of the filters (Tiangen Biotech Co., Ltd.) was conducted in 50% formamide, 0.5% SDS, 5X SSPE, 5X Denhardt's solution (all from Tiangen Biotech Co., Ltd.) and 20 μg/ml sheared and denatured salmon sperm DNA (Beijing Solarbio Science and Technology Co., Ltd.). Hybridizations were performed in the same solution at 34-45˚C. The labeled probes were heated for 1 min at 95˚C prior to addition of the filters in the prehybridization solution. Following hybridization, the membranes were washed at low stringency in 2X SSC and 0.1% SDS at 34-45˚C twice for 5 min each time or at high stringency in 0.1 SSC and 0.1% SDS at 65˚C twice for 5 min. The results of the northern blot were visualized by Quantity One software (Bio-Rad Laboratories, Inc.)

Statistical analysis. Each experiment was repeated at least three times. All results are expressed as the mean ±standard deviation. The difference between the means was analyzed using the Student's t-test or the χ^2 test. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc.) and $P<0.05$ indicated a statistically significant difference.

Results

miR-197 functions as a tumor suppressor gene in glioblastoma. In order to determine the expression levels of miR-197 in glioblastoma, *in situ* hybridization was performed to analyze the expression levels of miR-197 between glioblastoma tissues and adjacent normal tissues. An LNA-modified DNA probe complementary to the indicated miRNA was used on consecutive 5 μm sections obtained from archived FFPE. The results of *in situ* hybridization demonstrated that miR-197 was markedly downregulated in the majority of the glioblastoma tissue samples, compared with adjacent normal tissue samples (Fig. 1A). These results suggested that miR-197 functioned as a tumor suppressor gene in glioblastoma. The roles of miR-197 in glioblastoma were then investigated in A172 cells. To examine whether the expression levels of miR-197 were upregulated by pre-miR-197, the A172 cells were transfected with pre-miR-197. Pre-miR-197 significantly upregulated the expression levels of miR-197

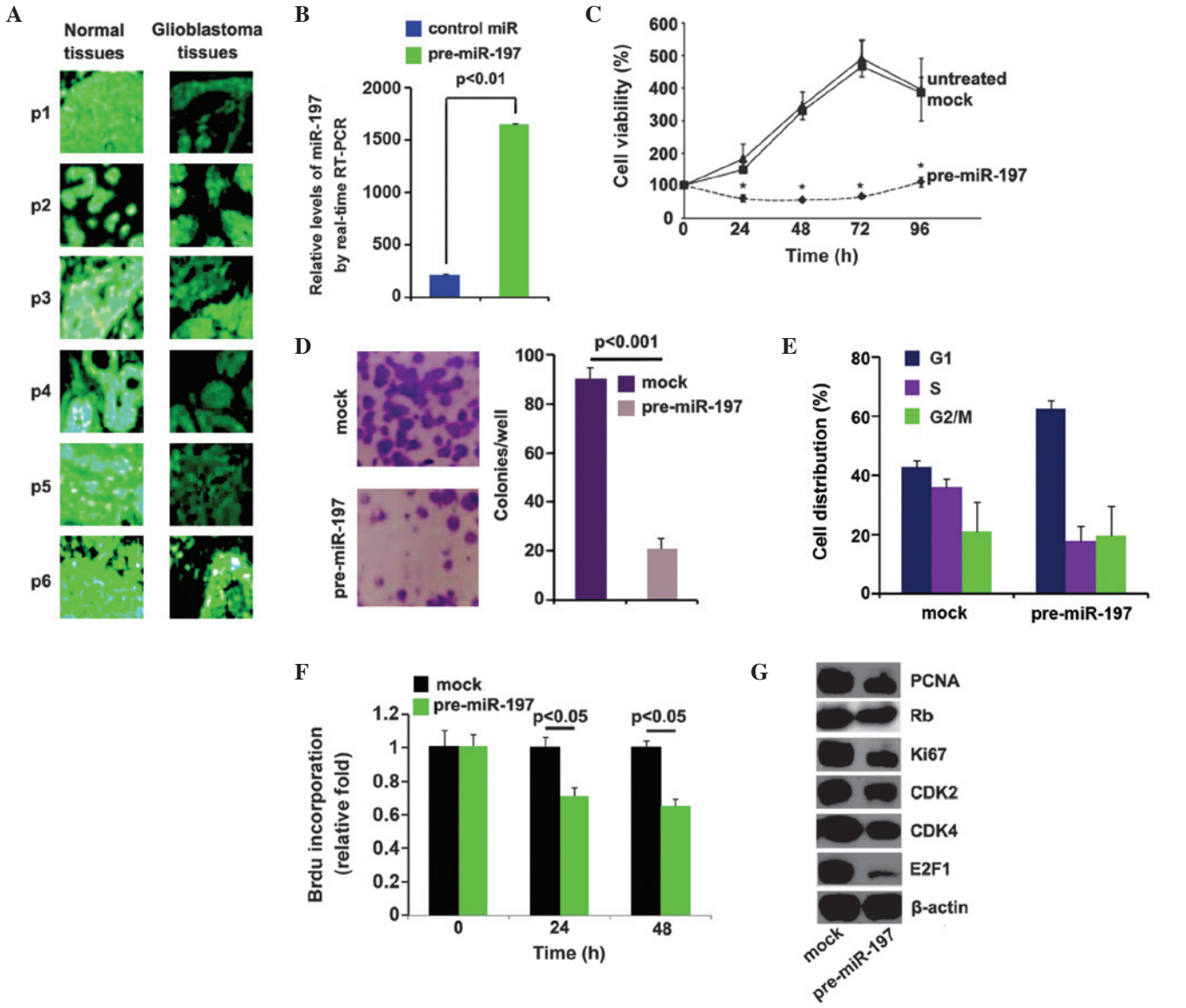


Figure 1. miR-197 is downregulated in glioblastoma tissues and promotes proliferation in glioblastoma cells. (A) *In situ* hybridization analysis of the expression of miR-197 in gliobastoma tissues and adjacent normal tissues in six patients with glioblastoma (p1-p6). (B) Reverse transcription-quantitative polymerase chain reaction for miR-197 in A172 cells transfected with pre-miR-197. Mock groups were transfected with control miR. U6 served as a loading control (n=3). (C) Cell counting assay for A172 cells transfected with pre-miR-197. Each time point is expressed as a relative value (fold change, Y axis) to time 0 (n=3). *P<0.05 vs. mock group. (D) Colony formation assay for A172 cells transfected with pre-miR-197. Colonies with >50 cells were counted. Mock groups were transfected with control miR (n=3). (E) Cell cycle analysis for A172 cells transfected with pre-miR-197. Mock groups were transfected with control miR (n=3). (F) BrdU analysis of A172 cells transfected with pre-miR-197. Mock groups were transfected with control miR. BrdU incorporation analysis was performed at the indicated time points (n=3). (G) Western blotting of proliferation-associated markers-PCNA, Rb, Ki67, CDK2, CDK4 and E2F1. Mock groups were transfected with control miR. β-actin served as a loading control (n=3). Bars indicate the standard error of the mean and magnification x200. miR, microRNA; PCNA; proliferating cell nuclear antigen; Rb, retinoblastoma; CDK, cyclin-dependent kinase; E2F1, EF2 transcription factor 1.

in the A172 cells (Fig. 1B). The effects of miR-197 on proliferation were also examined in the cells using a cell counting assay. miR-197 inhibited proliferation 24-96 h post-transfection in the A172 cells, compared with the untreated or control miR-treated groups (Fig. 1C). A colony formation assay was also performed for the A172 cells transfected with pre-miR-197 or control miR. Consistent with the results of the cell counting assay, the colony formation assay demonstrated that miR-197 significantly inhibited colony formation in the A172 cells (Fig. 1D). To further investigate the effects of miR-197 on proliferation, cell-cycle analysis was performed to analyze its effects on cell cycle. The A172 cells transfected with pre-miR-197 exhibited lower S phase fractions compared with the cells transfected with control miR (Fig. 1E). To examine whether the inhibition of DNA synthesis contributed to lower S phase fractions in the A172 cells transfected with pre-miR-197, a BrdU incorporation assay was performed to detect DNA synthesis within the cells. miR-197 significantly suppressed DNA synthesis in the cells (Fig. 1F). In addition, western blotting was performed to identify whether the protein expression levels of the proliferation markers were affected by miR-197 in the cells. The expression levels of PCNA, Rb, Ki67, CDK2, CDK4 and E2F1 were downregulated by miR-197 in the cells (Fig. 1G). These results suggested that miR-197 inhibited the proliferation of A172 cells.

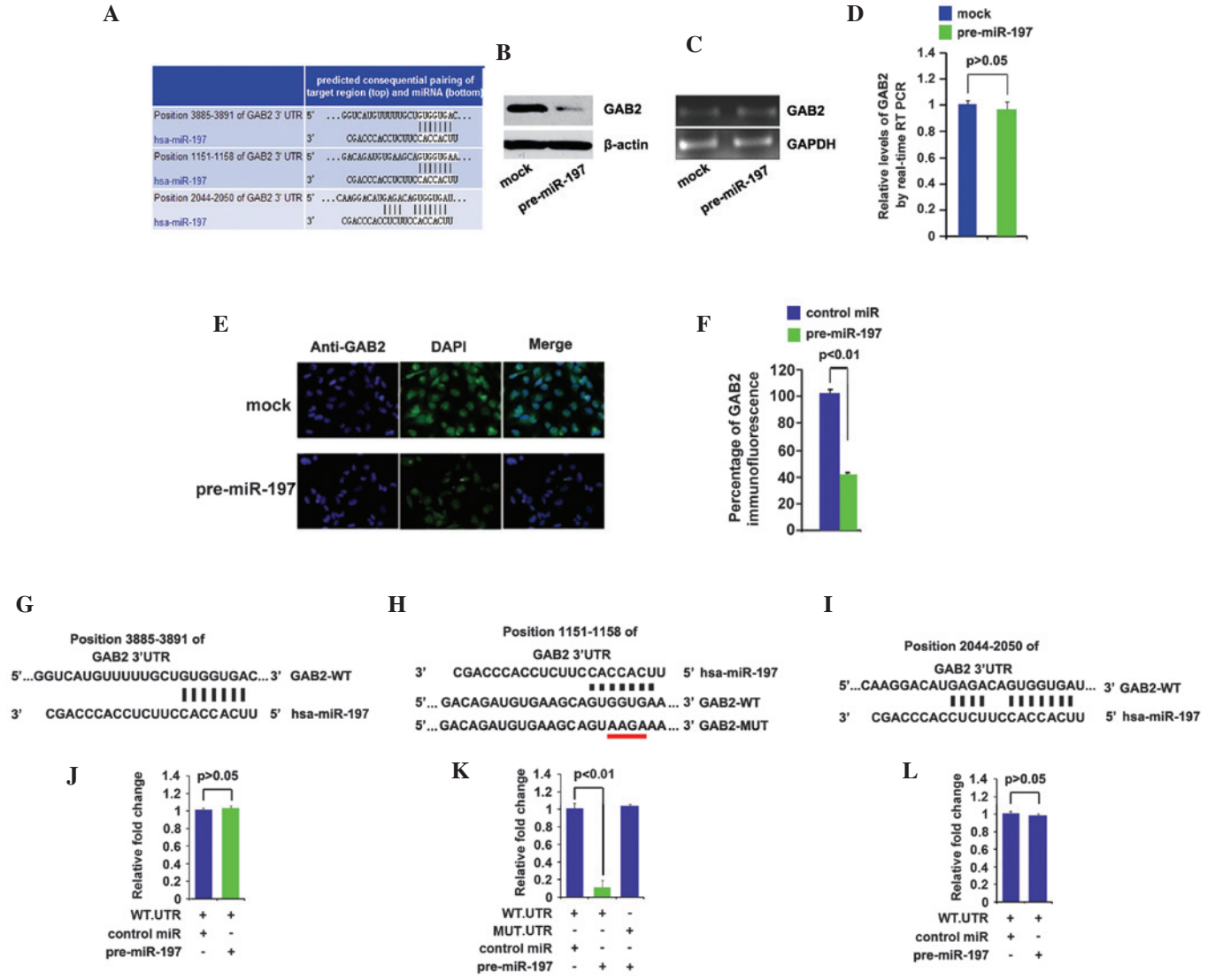


Figure 2. miR-197 downregulates the protein expression levels of GAB2 by targeting its 3'-UTR in glioblastoma cells. (A) Schematic of predicted miR-197 binding sites in the 3'-UTR of GAB2 mRNA. (B) Western blot analysis of the protein expression levels of GAB2 in A172 cells. Mock groups were transfected with control miR. β-actin was used as a loading control (n=3). (C) RT-qPCR of the mRNA expression levels of GAB2 in A172 cells transfected with pre-miR-197. Mock groups were transfected with control miR. U6 served as a loading control (n=3). (D) Quantitative analysis of the RT-qPCR for GAB2 in A172 cells transfected with pre-miR-197. Mock groups were transfected with control miR. U6 served as a loading control (n=3). (E) Immunofluorescence analyses of A172 cells transfected with pre-miR-197 or control miR. Images of immunofluorescence staining of a representative experiment (magnification, x200; n=3). (F) Quantitative presentation of mean immunofluorescence intensities of GAB2 (n=3). (G) GAB2 3'-UTR position 3885-3891. (H) GAB2 3'-UTR position 1151-1158. MUT contains a four base mutation (indicated by the red line) at the miR-197 target region, inhibiting its binding. (I) GAB2 3'-UTR position, 2044-2050. (J) Reporter assay conducted with co-transfection of 500 ng WT-reporter and 50 nM control-miR (mock), or pre-miR-197 (n=3). (K) Reporter assay following cotransfection of 500 ng WT-or MUT-reporter and 50 nM control-miR (mock), or pre-miR-197 (n=3). (L) Reporter assay following co-transfection of 500 ng WT-reporter and 50 nM control-miR (mock), or pre-miR-197 (n=3). Bars indicate the standard error of the mean. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAB2, Grb2-associated binding protein 2; 3'-UTR, 3' untranslated region; WT, wild-type; MUT, mutant.

miR-197 downregulates protein expression levels of GAB2 by targeting its 3'-UTR in A172 cells. miRNAs are short, non-coding RNAs, which regulate the target mRNA by binding predominantly to the 3'-UTR, inducing either translational repression or the degradation of the target (1-3). The target genes of miR-197 were screened using TargetScan (http://www.targetscan.org/vert_61/), which identified a large number of target genes, however, GAB2 was examined as several reports have demonstrated that GAB2 is an oncogene in malignant tumors (10-16). Target sites on the 3'-UTR of GAB2 are shown in Fig. 2A. It was hypothesized that miR-197 downregulates the expression of GAB2 in A172 cells. Western blot and RT-qPCR analyses were performed to detect the expression levels of GAB2 in the A172 cells transfected with pre-miR-197. The protein expression levels of GAB2 (Fig. 2B) were markedly downregulated in the cells transfected with pre-miR-197, however, mRNA expression levels remained unchanged (Fig. 2C and D). Concordant with the results of the western blot analysis, immunofluorescence analyses in the A172 cells transfected with pre-miR-197 or control miR revealed that the protein expression levels of GAB2 were significantly suppressed in the A172 cells transfected with pre-miR-197 (Fig. 2E and F). Fig. 2E shows microscopy images of immunofluorescence staining of a representative experiment (magnification, x100), and Fig. 2F shows the mean fluorescence intensities of three independent experiments.

To further demonstrate the direct regulation of GAB2 by miR-197, luciferase reporters were constructed with three targeting sequences: Wild-type-GAB2-WT-luc-1 (Fig. 2G), GAB2-WT-luc-2 (Fig. 2H) and GAB2-WT-luc-3 (Fig. 2I). Following GAB2-WT-luc-1 introduction into the A172 cells, miR-197 did not inhibit GAB2-WT-luc-1 plasmids (Fig. 2J). Therefore, GAB2-WT-luc-2 plasmids were investigated to determine whether they targeted by miR-197 in the A172 cells. The luciferase reporter assays demonstrated that the luciferase activities of the GAB2-WT-luc-2 plasmids were significantly suppressed in the cells (Fig. 2K). To further examine whether miR-197 targeted the 3'-UTR of GAB2 in the predicted sites, four bases were mutated (Fig. 2H). The mutant reporters were subsequently introduced into the A172 cells and demonstrated that the luciferase activity levels of GAB2-MUT-luc-2 were not suppressed in the cells (Fig. 2K). Having demonstrated that GAB2-WT-luc-2 plasmids were inhibited by miR-197, luciferase reporter assays were used to investigate whether miR-197 targeted GAB2-WT-luc-3. miR-197 did not affect the luciferase activity levels of GAB2-WT-luc-3 in the cells (Fig. 2L). These results suggested that miR-197 suppressed the protein expression levels of GAB2 by targeting its 3'-UTR at position 1151-1158.

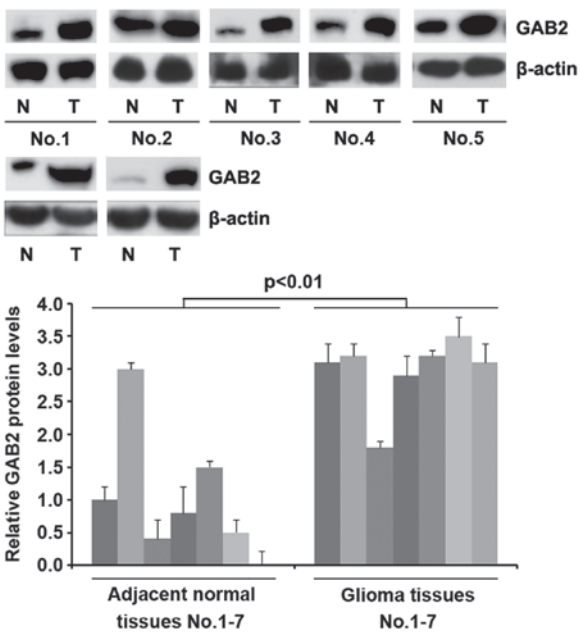
GAB2 acts as an oncogene in glioblastoma. To assess the expression levels of GAB2 in glioblastoma, western blot analysis was performed in seven pairs of glioblastoma tissue and matched adjacent normal tissue samples. The expression levels of GAB2 were higher in the majority of the glioblastoma tissue samples, compared with the normal tissue samples (Fig. 3A).

To demonstrate the importance of GAB2 in the pathogenesis and progression of glioblastoma, immunohistochemistry was performed to detect the expression of GAB2 in human glioma tissue specimens. The protein expression levels of GAB2 (++/+++) in glioma grades I, II, III and IV were 23.68, 38.46, 59.09 and 75%, respectively (Fig. 3B). Statistical analysis demonstrated that the expression levels of GAB2 were higher in the high-grade glioblastoma tissues, compared with the low grade glioma tissues: Grade I, vs. IV, P<0.001; grade II, vs. grade IV, P<0.001; grade I, vs. grade III, P<0.001.

In order to further confirm that high expression levels of GAB2 were associated with high-grade human glioblastoma, quantitative image analysis was performed to analyze the protein expression of GAB2 in the tissue sections. The expression levels of GAB2 were positively correlated with glioma grade (Fig. 3C).

GAB2 promotes proliferation in A172 cells. In order to identify the role of GAB2 in the regulation of A172 cell proliferation, the cells were transfected with GAB2-expressing plasmids. Following stable transfection, protein expression levels of GAB2 were quantified by western blot analysis. The GAB2-expressing plasmids increased the protein expression levels of GAB2 in the A172 cells (Fig. 4A). Furthermore, the proliferation of the A172 cells was determined using an MTT assay. Overexpression of GAB2 significantly increased the proliferation of A172 cells, in a dose-dependent manner (Fig. 4B). These data supported the hypothesis that GAB2 acts as a tumor suppressor in glioblastoma.

A



B

GAB2 expression in human glioma

Grade	n	GAB2	
		-/+	++/+++
I	38	29	9(23.68%)
II	39	24	15(38.46%)
III	44	18	26(59.09%)
IV	40	10	30(75.00%)

C

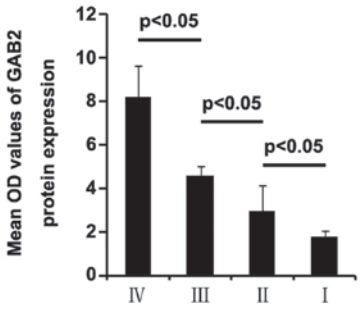


Figure 3. GAB2 is upregulated in glioblastoma and associated with grade of glioma. (A) Western blot analysis of the protein expression levels of GAB2 in seven pairs of glioblastoma tissue and adjacent normal tissue samples. β-actin served as a loading control (n=8). (B) Protein expression levels of GAB2 (++/+++) in glioma grades I, II, III and IV. Immunohistochemical analysis of GAB2 in glioma. Tissue sections of WHO grade I glioma (n=38), WHO grade II glioma (n=39), WHO grade III glioma (n=44) and WHO grade IV glioma (n=40). (C) Mean optical density values of immunohistochemical images for GAB2 in glioblastoma grades I, II, III and IV. Tissue sections of WHO grade I glioma (n=38), grade II glioma (n=39), WHO grade III glioma (n=44) and WHO grade IV glioma (n=40). Bars indicate the standard error of the mean. WHO, World Heath Organization; GAB2, Grb2-associated binding protein 2; OD, optical density; T, tumor tissue; N, normal tissue.

Introduction of GAB2 cDNA lacking predicted 3'-UTR sites inhibits the cellular function of miR-197. Due to the fact that miR-197 directly targets GAB2 through its 3'-UTR, the present study hypothesized that ectopic expression of GAB2 by transfection with cDNA lacking the predicted 3'-UTR target (in the present study, the GAB2 expression plasmids did not contain 3'-UTR position 1151-1158) can escape the regulation of miR-197, and thus attenuate or decrease miR-197 function. The GAB2-expressing plasmid or empty vector

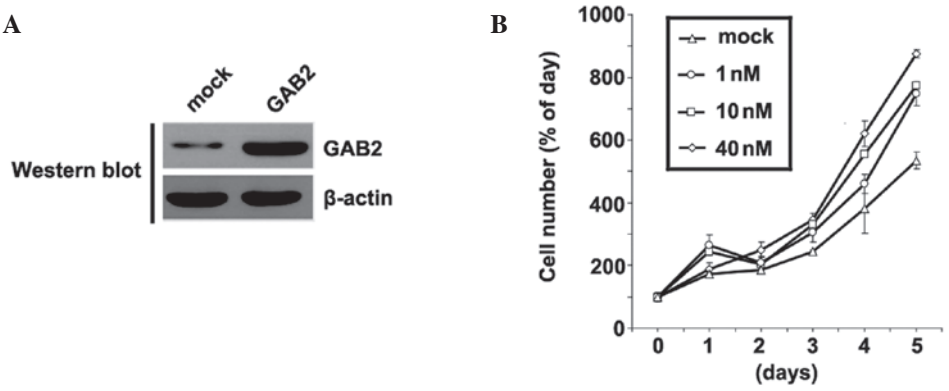


Figure 4. GAB2 promotes proliferation in glioblastoma A172 cells. (A) Western blot analysis of the protein expression levels of GAB2 in A172 cells transfected with GAB2-expressing plasmids. The mock group was transfected with empty vectors. β-actin served as a loading control (n=3). (B) Number of A172 cells transfected with various doses of GAB2-expressing plasmids. The doses of GAB2 transfection are shown in the inset of the histograms. The mock group was transfected with empty vectors (n=3). Bars indicate the standard error of the mean. GAB2, Grb2-associated binding protein 2.

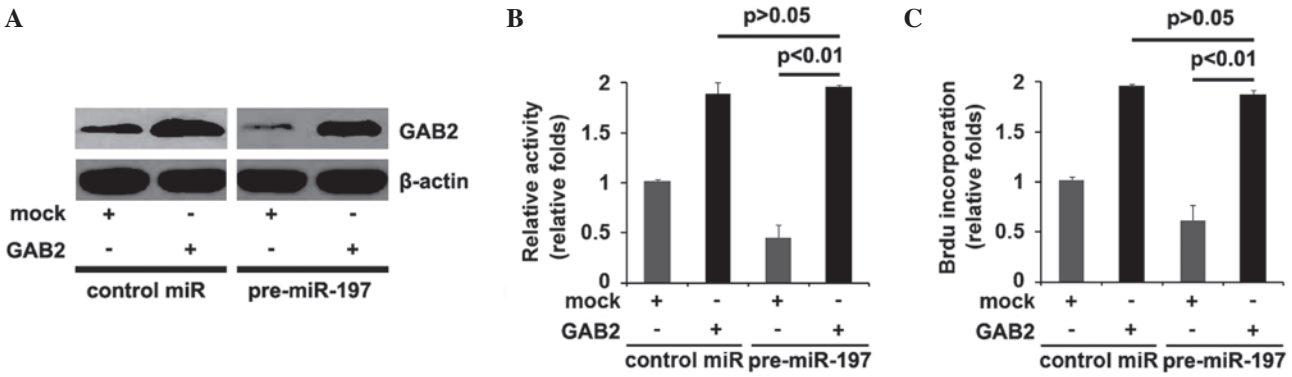


Figure 5. Introduction of GAB2 cDNA lacking predicted 3'-UTR sites inhibits miR-197 cellular function. (A) Western blot analysis of the expression levels of GAB2 in control miR- or pre-miR-197-treated A172 cells transfected with GAB2-expressing plasmids or empty vector (mock). β-actin served as a loading control (n=3). (B) MTT assay in A172 cells. Control miR- or pre-miR-197-treated A172 cells transfected with GAB2-expressing plasmids or empty vector (mock; n=3). (C) BrdU analysis of A172 cells. Control miR- or pre-miR-197-treated A172 cells transfected with GAB2-expressing plasmids or empty vector (mock; n=3). Bars indicate the standard error of the mean. miR, microRNA; GAB2, Grb2-associated binding protein 2.

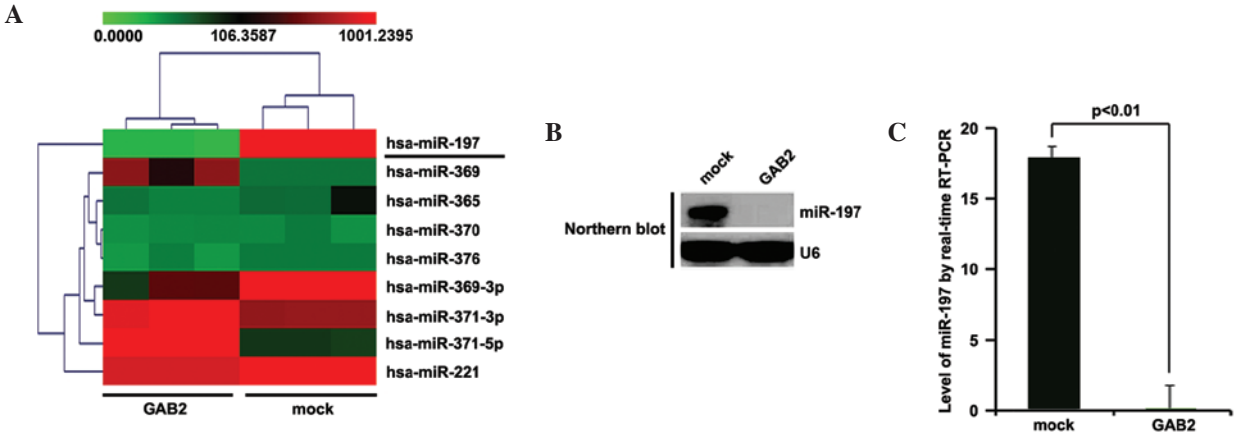


Figure 6. GAB2 downregulates miR-197 expression in glioblastoma A172 cells. (A) miRNA microarray analysis of A172 cells transfected with GAB2-expressing plasmids or empty vectors (mock; n=3). The colours indicate fold change as presented in the legend. (B) Northern blot analysis of miR-197 expression in A172 cells transfected with GAB2-expressing plasmids or empty vectors (mock) (n=3). (C) Reverse transcription-quantitative polymerase chain reaction of miR-197 expression levels in A172 cells transfected with GAB2-expressing plasmids or empty vectors (mock) (n=3). Bars indicate the standard error of the mean. miR, microRNA; GAB2, Grb2-associated binding protein 2.

plasmid (pcDNA3.1) were transfected into the control miR or pre-miR-197 treated-A172 cells. Immunoblotting revealed that transfection with the GAB2 plasmids attenuated the effects of miR-197 on GAB2 (Fig. 5A).

As the overexpression of miR-197 in glioblastoma A172 cells inhibited proliferation, the present study investigated whether GAB2 suppressed the function of miR-197 in proliferation by treating the control miR or pre-miR-197-transfected A172 cells with either GAB2-expressing plasmids or empty vector (pcDNA3.1). MTT and BrdU incorporation assays were performed, which demonstrated that the pre-miR-197-treated A172 cells exhibited a ~30-50% decrease in proliferation (Fig. 5B) and DNA synthesis (Fig. 5C), compared with the control miR-treated cells. Overexpression of GAB2 sufficed to reverse the loss of proliferation (Fig. 5B) and DNA synthesis (Fig. 5C) observed in the pre-miR-197-treated cells.

GAB2 significantly downregulates the expression levels of miR-197 in A172 cells. Tumor suppressor genes can exert their functions by regulating miRNA expression in glioma (24), several of which function as tumor suppressor genes or oncogenes (5,25,26). The present study hypothesized that GAB2 functions as an oncogene by regulating relevant miRNAs. An miRNA microarray was performed, in which RNAs isolated from A172 cells transfected with GAB2 or empty vectors were hybridized to a custom miRNA microarray platform. Following three cycles of hybridization, quantification and normalization, several miRNAs, specifically miR-197, were downregulated >1,000-fold in the cells (Fig. 6A). To further examine the regulation of GAB2, northern blotting was performed to detect the expression levels of miR-197. Concordant with the previous results, the results of the northern blot (Fig. 6B) demonstrated that GAB2 significantly downregulated the expression of miR-197. Furthermore, RT-qPCR was performed to quantify the expression levels of miR-197 in the A172 cells transfected with GAB2-expressing plasmids or empty vectors. As with the results of the northern blot analysis, the RT-qPCR analysis demonstrated that the expression levels of miR-197 were significantly suppressed by GAB2 (Fig. 6C).

Discussion

The expression levels of certain miRNAs are upregulated or downregulated, and function either as oncogenes or tumor suppressor genes by regulating target genes in malignant tumors (27-34). For example, miR-10b has been found to be markedly upregulated in breast cancer tissue samples, and initiated tumor invasion and metastasis (27). miR-214 is frequently downregulated in ovarian cancer, and induces cell survival and cisplatin resistance by targeting the 3'-UTR of the phosphatase and tensin homolog (PTEN), which leads to downregulation of PTEN protein and activation of the Akt signaling pathway (28). The polycistronic miRNA cluster, miR-17-92, is overexpressed in human lung cancer, enhancing cell proliferation (29). miRNA-101 is downregulated in gastric cancer and is involved in cell migration and invasion (30). miR-96 is significantly downregulated (>5-fold) in pancreatic cancer tissues, compared with normal tissues (31), suppresses Kirsten rat sarcoma viral oncogene homolog and functions as a tumor suppressor gene in the disease (32). miR-184 functions as a potential oncogenic miRNA in squamous cell carcinoma of the tongue (33). miR-125b exerts tumor-suppressive effects in hepatic carcinogenesis via suppression of the expression of the LIN28B oncogene, suggesting a therapeutic application for miR-125b in hepatocellular carcinoma (34). Although the expression of miR-197is downregulated in glioblastoma, its roles in the malignant tumor remain to be elucidated (7). The present study investigated why the gene is downregulated and whether it functions as a tumor suppressor gene to further understand the pathogenesis and progression of the disease, and offer novel therapeutic targets.

The results of the present study demonstrated that the expression of miR-197 was downregulated in glioblastoma tissue samples. The roles of miR-197 were examined in the disease, and demonstrated that it inhibited colony formation and proliferation in the glioblastoma cells. The mechanism underlying miR-197-induced suppression proliferation was also investigated. The results suggested that miR-197 downregulated the expression of GAB2 in glioblastoma cells by targeting its 3'-UTR, and the introduction of GAB2 cDNA lacking predicted 3'-UTR sites inhibited miR-197 cellular function. This suggested that miR-197 functions as a tumor suppressor by targeting GAB2. The results also showed that GAB2 acted as an oncogene in glioblastoma, was associated with grades of glioma and promoted proliferation in the A172 cells. Furthermore, the expression of miR-197 was significantly suppressed by GAB2. These results suggested that, due to the downregulated expression of miR-197 in glioblastoma, GAB2 was overexpressed, and this overexpression further promoted tumorigenesis and progression by suppressing the expression of miR-197. The miR-197-mediated GAB2 regulation in glioblastoma A172 cells, demonstrated in the present study, has potential basic and clinical implications. miR-197 may be used as a potent tumor suppressor by inhibiting proliferation and regulating various oncogenes in human glioblastoma, therefore, the pharmacological restoration of miR-197 may represent a promising therapeutic strategy. GAB2 acts as an oncogene, therefore, restoration of miR-197 may decrease its expression levels. Future investigations are required to further examine the role of miR-197 in glioblastoma.

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