

MiR-548ac Inhibits Wnt5a Expression and Promotes Proliferation of Glioma Cells Through the AKT/ERK Pathway

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Abstract

Background: Glioma one of the most frequently occurring and lethal primary malignant tumors in adults. Micro (mi)RNAs are a newly identified modulator involved in the occurrence and progression of glioblastoma multiforme (GBM). Previous studies found that miR-548ac had a role in suppressing laryngeal and breast cancer. This study investigated the role of mir-548ac in glioma progression.

Methods: The function of miR-548ac was studied by stable knockdown or overexpression of miR-548ac in GBM cells. Luciferase reporter constructs were used to investigate the correlation between Wnt5a and miR-548ac. Cell Counting Kit-8, Transwell, and colony formation assays were used to investigate Wnt5a and miR-548ac activity in glioma cells. Flow cytometry was used to describe the cell cycle. Xenograft experiments were used to evaluate tumor growth and metastasis *in vivo*. Western blotting was used to assess the function of the Wnt/ β -catenin pathway.

Results: Downregulation of miR-548ac promoted cell proliferation, migration, and invasion during GBM progression. Silencing of miR-548ac promoted expression of Wnt5a, a direct target of miR-548ac, which plays an oncogenic role in glioblastoma stem cells. Inhibiting the expression of Wnt5a partially rescued the consequences of increased cell proliferation, migration, and invasion caused by miR-548ac. Tumor growth was suppressed by Wnt5a knockdown *in vivo*.

Conclusions: miR-548ac suppressed GBM progression by targeting Wnt5a. The miR-548ac/Wnt5a axis may be a new potential strategy for glioma therapy.

Introduction

Glioblastoma multiforme (GBM) is one of the most frequently occurring and lethal primary malignant tumors [1, 2]. The highly invasive and metastatic growth pattern of glioma is responsible for the lack of successful treatments [3], including, for example, surgical intervention (tumor resection), radiotherapy and chemotherapy, which results in a dismal patient prognosis [4-7]. Therefore, understanding the mechanisms underlying the progression of this complicated disease is important to enable the development of new treatment methods.

MicroRNAs (miRNAs) are a class of highly conserved small noncoding RNAs containing 18 to 23 nucleotides [8]. They function as inhibitors of target mRNA degradation or silencing by binding the 3'-untranslated regions (UTRs) [6, 9-12]. They play key roles in the regulation of gene expression in numerous biological process [12, 13]. MiR-548ac expression has been described in various tumors. For example, miR-548ac inhibits cell proliferation in lung cancer via the PI3K/AKT signaling pathway and in breast cancer cells by targeting the *ECHS1* gene [14, 15]. There are few reports of the function of miR-548ac in glioma.

Studies have shown the mir-548 family is a large, poorly conserved primate-specific miRNA family. The enrichment pathway analysis of the miR-548 family shows that they play an important role in various

human diseases[16]. Mir-548ac is one member of mir-548 family, and plays a regulatory role in a variety of tumors, such as breast cancer[15], laryngeal cancer[14], etc. However, the mechanism of mir-548ac in glioma has not been elucidated. Based on the above findings, we put forward the hypothesis that mir-548ac may play a role in glioma, then designed related experiments. Elucidating the role of mir-548ac will be critical for understanding the tumor progression and identifying potential new biomarkers or therapeutic targets for gliomas.

The purpose of this study was to explore the effect of mir-548ac underlying the development of glioma and to identify biomarkers for diagnosis and treatment of glioma. Real time quantitative polymerase chain reaction (RT-qPCR) was used to demonstrate that mir-548ac is overexpressed in glioma. The anticancer effect of miR-548ac was then investigated by Cell Counting Kit-8 (CCK-8), colony formation, flow cytometry, western blotting, and tumor xenograft assays. MiR-548ac was found to bind the 3'-UTR of Wnt5a, inhibit the malignant behavior of GBM cells, and suppress the growth of GBM xenografts.

Methods

Animals and the xenograft tumor model

We used pregnant BALB/c nude mice purchased from the experimental animal center of Nantong University (Certificate No: SYXK (SU) 2012-0031). All animal experiments were conducted according to protocols approved by the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number and suffering of animals used in this study.

Lentivirus encoding Wnt5a and a nontargeting sequence NC were transfected into U87 cells to generate stably transfected cells. For assessing tumor growth in vivo, BALB/c nude mice were randomly assigned to three groups of six each. LV-NC U87 cells (1×10^6), LV-siWnt5a U87 cells (1×10^6) and LV-miR-548ac+sh-Wnt5a cells (5×10^6) were separately resuspended in 150 μ l PBS and then subcutaneously inoculated into the flanks of BALB/c nude mice. The tumors were monitored regularly at the indicated time points.

Cell culture

U87 and U251 human glioma cells were purchased from the Chinese Academy of Medical Sciences (Beijing, China) and cultured in Dulbecco's modified Eagle's medium/high glucose with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) at 37°C in 5% CO₂.

Cell transfection

An miR-548ac mimic, miR-548ac inhibitor, related negative control (NC) mimics, and an NC inhibitor were transfected into cells (RiboBio Co. Ltd, Guangzhou, China) using Lipofectamine 3000 reagents (Invitrogen, CA, USA). Stably transfected cells were collected after 48 h of culture. Wnt5a small interfering

(si)RNA, and an siRNA NC were purchased from GenePharma (Shanghai, China). The target sequence was 5'-GTTTTGGCCACTGACTGA-3'. A plasmid overexpressing Wnt5a (NM_001377271.1) was inserted in a pcDNA3.1 vector (Shanghai, China), and the empty vector was used as its NC. To explore the association between miRNA-548ac and Wnt5a in GBM, U87 and U251 cells were transfected with miR-548ac mimics or the mimic NC and pcDNA3.1-NC or pcDNA3.1-Wnt5a using Lipofectamine 3000 (Invitrogen, CA, USA). Cells were analyzed 48 h after transfection.

Flow cytometry

Cells were collected in 0.25% trypsin 48 h after transfection. For cell cycle analysis, the cells were fixed with ice cold 75% ethanol for 24 h and resuspended in phosphate buffered saline (PBS). After adding 500 µl/ml Pharmingen PI/RNase Staining Buffer (BD Biosciences, CA, USA) with incubation for another 30 min at room temperature, the labeled cells were assayed by flow cytometry (BD Biosciences, CA, USA). The distribution of cells in the G0/G1, S, and G2/M phases was analyzed with ModFit LT software (Verity Software House, <http://www.vsh.com/products/mflt/>).

Cell proliferation assays

Cell proliferation was measured with CCK-8 assay kits in accordance with the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). In brief, GBM cells were seeded in 96-well plates at 3000 cells/well. CCK8 solution (10 µl) was added, followed by incubated for 2 h at 37°C. The absorbance was evaluated at 450 nm with a SpectraMax M5 microplate reader (Molecular Devices, CA, USA).

Colony formation assays

GBM cells in growth phase were seeded at 1000 cells per well in culture medium. The cells were cultured for 2 weeks at 37°C, then fixed in 4% paraformaldehyde for 10 min. Cell colonies were stained with 0.5% crystal violet for 30 min at room temperature and washed in PBS.

Transwell invasion assays

Transwell assays were performed to detect cell invasion. Cells were transfected for 48 h, and then 2×10^5 cells were plated in the upper chamber (with Matrigel for invasion assays) of a 24-well Transwell plate, and 600 µl of FBS was added to the lower chamber. After incubation for 24 h, cells on the upper surface were removed, and cells migrating to the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For each assay, cells were counted in six random fields.

Wound healing assays

Wound healing assays were performed to analyze cell migration. Briefly, GBM cells were seeded into six-well plates and cultured at 37°C for 24 h. A linear wound was scratched with a 1 ml pipette tip, and the wells were washed twice with PBS to remove suspended cells and debris. The cells were cultured in

medium containing 10% FBS, then the wounds were photographed and the size was measured at 0 h, 24 h and 48 h.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions and reverse transcribed to cDNA with the SuperScript III First-strand Synthesis System (Thermo Fisher Scientific, MA, USA). For qRT-PCR analysis, the relative quantification of Wnt5a expression was calculated by the comparative CT method ($\Delta\Delta CT$). The Wnt5a forward primer was 5'-TCGACTATGGCTACCGCTTT-3' and the reverse primer was 5'-CACTCTCGTAGGAGCCCTT-3'.

The miR-548ac forward primer was 5'-CAAAAACCGGCAATTACTTTTG-3' and the reverse primer was 5'-CTCAACTGGTGTCGTGGA-3'. The GAPDH forward primer was 5'-ATTCCATGGCACCGTCAAGGCTGA-3' and the reverse primer was 5'-TTCTCCATGGTGGTGAAGACGCCA-3'.

Luciferase reporter assays

Potential miR-548ac binding sites in the Wnt5a 3'-UTR were predicted with the bioinformatics tools miRwalk (<http://starbase.sysu.edu.cn/>), miRDB (<http://mirdb.org/>) and TargetScan (<http://www.targetscan.org/>). The sites of the predicted target gene and a mutant variant were synthesized and cloned in the pmirGLO Dual-Luciferase miRNA target expression vector (GenePharma, Shanghai, China). GBM cells were seeded in 96-well plates and co-transfected 12 h later with plasmids or miR-548ac mimics with Lipofectamine 3000. The cells were harvested 48 h after transfection, and firefly and *Renilla* luciferase activity was analyzed with a dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western blot analysis

Cells were lysed and proteins were isolated in ice cold RIPA buffer (Thermo, MA, USA). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk, the membranes were incubated with p-ERK1/2, total ERK1/2, total RAF, p-RAF, Wnt5a, and β -actin primary antibodies purchased from Abcam.

Statistical analysis

Prism 6 statistical software was used for statistical analysis. All data are presented as the mean \pm standard error of mean (SEM) from at least three independent replicates. Statistical analysis of data was performed with Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

The effect of miR-548ac on the proliferation of GBM cells

To examine the function of miR-548ac in GBM proliferation and migration, miR-548ac mimics or miR-548ac NC were transfected into GBM cells. RT-qPCR was used to certify the transfection efficiency of miR-548ac. The results revealed that the expression of miR-548ac significantly increased after cells were transfected with miR-548ac mimics, respectively, as compared with NC mimics (Figure 1A). Ki67 assays showed that miR-548ac significantly decreased GBM cell proliferation (Figure 1B, C). We then analyzed the cell cycle distribution and evaluated the proliferation of U87 and U251 cells by fluorescence-activated cell sorting (FACS). MiR-548ac blocked the progression of the cell cycle in GBM cells, and the number of cells in G1 phase increased, whereas the number of cells in S and G2 phases decreased (Figure 1D). In agreement with this finding, CCK8 assays showed that cell proliferation was greatly reduced when miR-548ac was overexpressed in GBM cell (Figure 1E). The effect of miR-548ac on cell proliferation was also shown in colony formation assays (Figure 1F-G). These results indicated that miR-548ac inhibits GBM progression and consequently might function as a tumor suppressor of GBM.

The ability of migration and invasion be affected by knockdown or overexpression of miR-548ac

To assess the effects of miR-548ac on cell migration and invasion, we performed wound healing and Transwell assays. The wound healing assays showed that miR-548ac significantly decreased migratory capacity in the U87 and U251 cells (Figure 2A–B). Transwell assays used to analyze the invasion of cells after miR-548ac overexpression indicated that increased miR-548ac expression decreased invasion by U87 and U251 cells (Figure 2C, D). The results indicated that miR-548ac affected the invasion and migration of GBM cells.

miR-548ac regulates Wnt5a expression

The above results indicated that miR-548ac has remarkable effects on the biological behaviors of GSCs, but the underlying molecular mechanisms remain unclear. The bioinformatics databases TargetScan, miRwalk and miRBD suggested that several genes might be downstream targets of miR-548ac (Figure 3A). A total of 11 downstream molecules associated with tumorigenesis were screened with KEGG analysis, including Wnt5a (Figure 3B). Wnt5a facilitates tumor cell proliferation and migration and recruits macrophages for infiltration, and it is also highly expressed in tumor tissues [17] (Figure 3C). We found that miR-548ac directly binds the 3'UTR of Wnt5a mRNA (Figure 3D). We cloned the full-length 3'-UTR of Wnt5a mRNA containing wild-type (wt) or mutant (mut) sequences and inserted it into a luciferase reporter vector downstream of the firefly luciferase gene. When the possible binding sites were mutated, the inhibitory effect of Wnt5a was completely blocked (Figure 3E). In addition, qRT-PCR indicated that transfection with miR-548ac mimics inhibited Wnt5a expression, whereas transfection of miR-548ac inhibitor promoted Wnt5a expression (Figure 3F). Western blotting further confirmed that miR-548ac overexpression inhibited Wnt5a protein expression (Figure 3G). Therefore, these results demonstrated that miR-548ac binds the 3'-UTR of Wnt5a and inhibits its expression, and also confirmed a negative correlation between miR-548ac and Wnt5a expression.

miR-548ac suppresses GBM proliferation through Wnt5a

Given that miR-548ac can negatively regulate the expression of Wnt5a, we hypothesized that miR-548ac suppression of GBM progression might be mediated by Wnt5a. To validate this hypothesis, we restored Wnt5a after overexpressing miR-548ac in GBM cells and analyzed the phenotypes (Figure 4A, B). KI67 assays showed that Wnt5a restoration rescued the effect of miR-548ac on GBM growth, whereas Wnt5a knockout decreased proliferation (Figure 4C, D). The results of CCK8 assays further confirmed this conclusion (Figure 4E). FACS was then conducted to analyze the cell cycle, and the results revealed that Wnt5a rescue increased the number of cells in S phase and restored the number of cells in G1 phase to a normal level (Figure 4F). Colony formation assays indicated that when Wnt5a was knocked out, the number of colonies formed decreased, whereas colony expansion was restored when Wnt5a was rescued (Figure 4G). Thus, these data indicated that miR-548ac suppresses GBM progression through Wnt5a, inhibiting the proliferation of tumor cells.

miR-548ac suppresses migration and invasion of GBM through Wnt5a

We restored Wnt5a expression before overexpressing miR-548ac in GBM cells to analyze the effects on migration and invasion. Wound healing assays showed that the migration of the GBM cells was significantly reduced after Wnt5a knockout, whereas Wnt5a rescued the migratory behavior (Figure 5A, B). In addition, Transwell assays revealed that a decrease in the expression of Wnt5a impaired the invasive ability of GSCs, whereas Wnt5a rescued the impaired invasion of GSCs induced by miR-548ac overexpression (Figure 5C, D). These results revealed that Wnt5a mediates the tumor-suppressive effects of miR-548ac when overexpressed in GSCs.

MiR-548ac activates the ERK/AKT pathway through Wnt5a

Wnt5a is known to be a key factor in the development and progression of various cancers [18]. It has been shown to activate nuclear factor kappa B (NF- κ B) pathway-dependent signaling to maintain tumor cell survival [19] and to promote the formation of blood vessels in tumor tissues [17]. Therefore, we determined the status of the ERK and AKT oncogenic signaling pathways in GBM cells. Cellular levels of p-ERK1/2 and p-AKT markedly decreased in GBM cells stably overexpressing miR-548ac compared with miR-NC, whereas no statistically significant decrease in total ERK1/2 and AKT was observed (Fig 6A). Wnt5a overexpression restored the ERK and AKT signaling pathways that were inhibited by miR-548ac overexpression. Deletion of Wnt5a abrogated ERK and AKT signaling activation in GBM/anti-548ac GBM cells (Fig 6A). Together, these results indicate that inhibition of Wnt5a in GBM cells with low miR-548ac expression reversed malignant phenotypes in GBM.

Wnt5a restoration with overexpression of miR-548ac suppresses tumor growth

To further confirm the above findings, we used an *in vivo* tumor model. Implantation of cells with restored Wnt5a levels and miR-548ac overexpression resulted in tumors of the same size as those in the miR-NC group. In addition, tumors in the siWnt5a group were larger and had greater volumes than those in the other groups (Figure 6B, C). RT-qPCR confirmed that Wnt5a expression was significantly reduced in the siWnt5a group (Fig. 6D), indicating that Wnt5a knockout might decrease tumorigenesis *in vivo*.

Discussion

Studies have shown that miRNA play the key role in the procession of tumor, studies have yet shown that miRNA can inhibit the translation or degradation of target mRNA, thereby negatively regulating the expression of target genes after transcription [20]. Much attention has been focused on the function of miRNAs in cell regulation, differentiation, apoptosis, and angiogenesis [21, 22]. It has been reported that miR-548ac was express abnormally in tumor and inhibit the growth of several tumor types. For example, miR-548ac participates in proliferation, migration and invasion of lung cancer, breast cancer and laryngeal cancer [14, 15, 23]. It has been described an oncogene, but the function and underlying mechanisms of miR-548ac in glioma remain poorly understood.

The present study investigated the role of miR-548ac during glioma development. Through CCK8, FACS, Clonogenicity and transwell assays, we demonstrated that miR-548ac overexpression suppressed GBM cell proliferation, migration and invasion, indicating a tumor suppressor role of miR-548ac in GBM. Through binding partner predication, we found the interaction between wnt5a and miR-548ac and demonstrated that miR-548ac is a direct target of Wnt5a in dual-luciferase assays. At the same time, increasing the expression of Wnt5a rescued cell viability caused by the increase in miR-548ac. MiR-548ac had a negative expression relationship with wnt5a, and miR-548ac overexpression will led the expression of wnt5a decrease. Thus, wnt5a might be a functional target of miR-548ac in GBM. Our results suggest that miR-548ac suppresses the progression of glioma by controlling expression of Wnt5a.

Wnt5a is an evolutionarily conserved non-canonical Wnt ligand that activates several non-canonical WNT signaling pathways [24]. Wnt5a plays an important role in tumor progression, and Wnt5a overexpression is thought to be involved in some cancers; for example, the tumor-promoting effect of Wnt5a is to activate the CaMKII-ERK pathway in colorectal cancer [25]. Wnt5a is highly expressed in breast cancer, and also enhances the metastatic ability of ovarian cancer [26-29]. In our study, miR-548ac was overexpressed in GBM cells, and wnt5a knockdown suppressed GBM progression. This finding suggested that wnt5a served as a tumor progression. Further analysis showed that miR-548ac suppressed GBM progression through wnt5a. These results indicated that miR-548ac and wnt5a axis participated in GBM progression.

In summary, we shed new light on the role of miR-548ac and its target gene, Wnt5a, in controlling the cell cycle and their potential implications in pathological processes in GBM cells. miR-548ac overexpression down-regulated the expression of Wnt5 and inhibited malignant biological behavior in glioma. These findings highlight the function of miR-548ac in cells and offers novel insights into the regulatory network of the cell cycle that may prove to be a new target and a new strategy for the treatment of glioma.

Declarations

Author contributions

WS and HNL designed the study; WS, HNL, XL, and XTW performed the experiments and prepared the figures; WS contributed to drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethical approval and consent to participate

The study was approved by the Ethics Review Committee of Affiliated Hospital of Nantong University (Nantong, China). The animal study followed the Guidelines for the Animal Care and Use approved by Affiliated Hospital of Nantong University.

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Figures

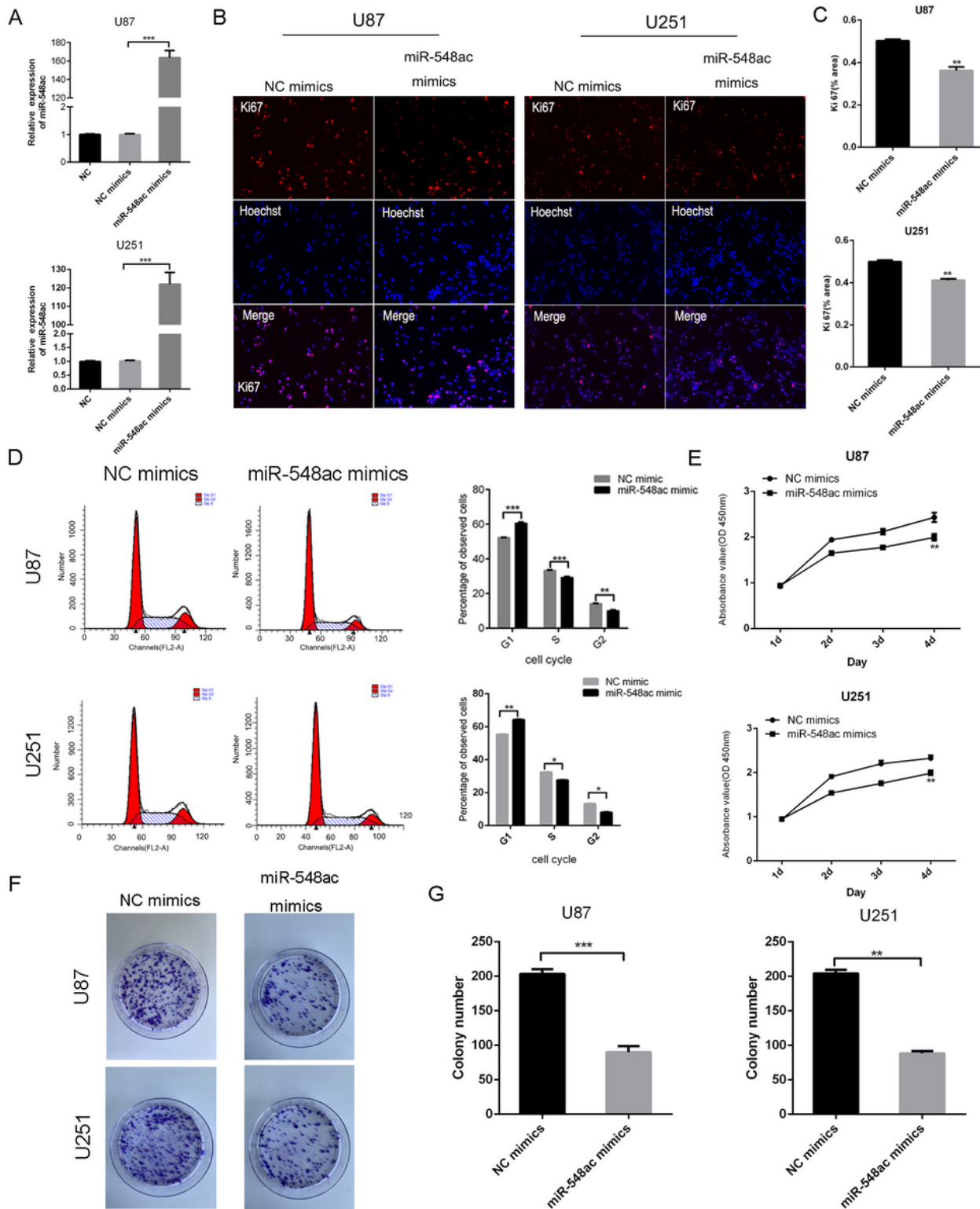


Figure 1

miR-548ac is indispensable for promoting GBM cell proliferation (A) qRT-PCR analysis of miR-548ac expression in U87 and U251 cells transfected with miR-548ac mimics, mimic controls, and miR-548ac inhibitor, or inhibitor control. (B) Ki67 assay of cellular proliferation in GBM cells transfected with miR-548ac mimics or miR-548ac inhibitor. Bar = 200 μ m. (C) Quantification of the percentage of Ki67-positive cells. (D) FACS measurement of cell cycle distribution and quantification of the percentage of cells in G1,

S or G2 stages. (E) CCK8 assay of cellular proliferation in cells transfected with miR-548ac mimics, or mimic controls, miR-548ac inhibitor or inhibitor control. (F, G) Colony formation assay of cellular proliferation with cells transfected with miR-548ac mimics, or mimic controls, miR-548ac inhibitor or inhibitor control. *P < 0.05, **P < 0.01, ***P < 0.001.

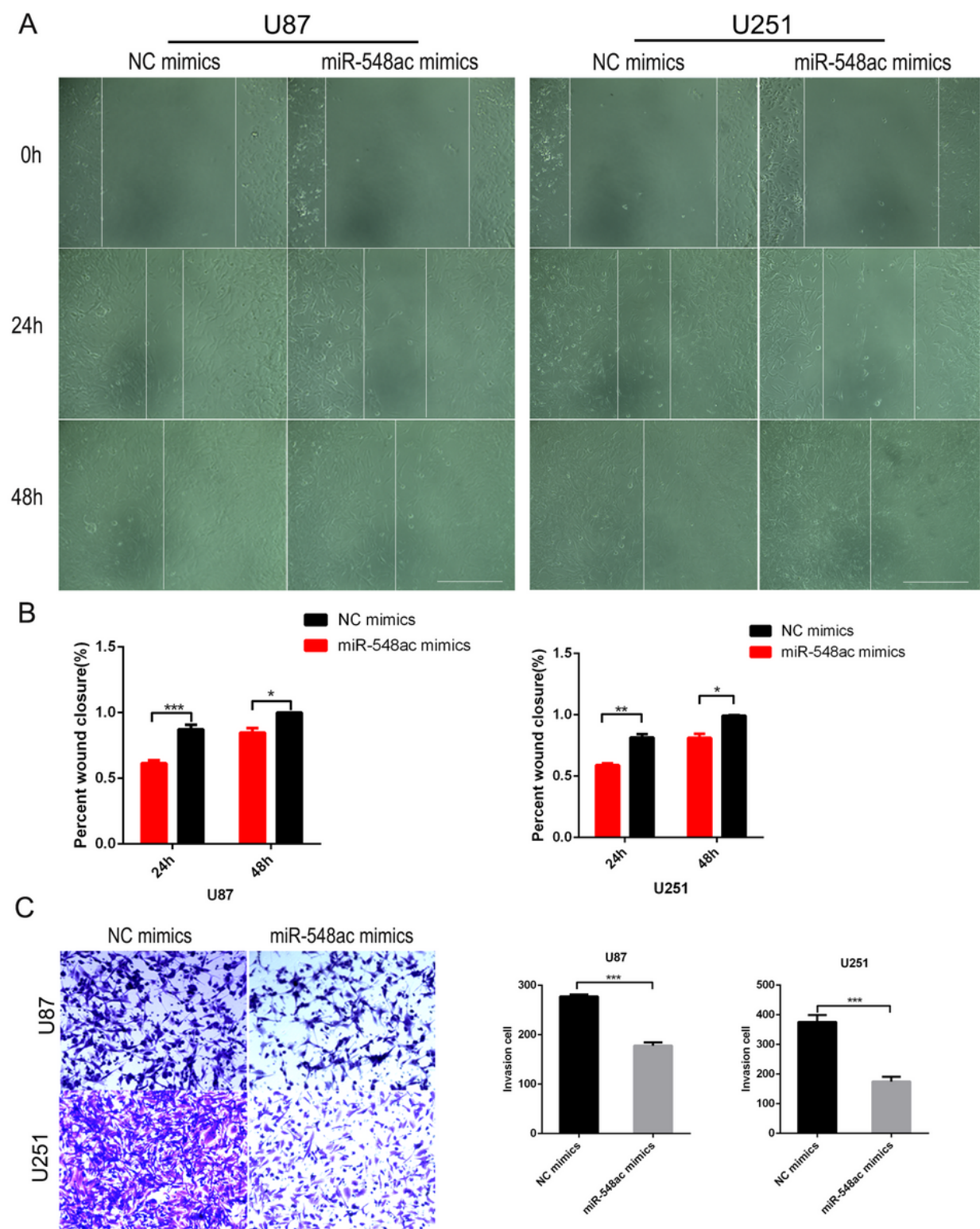


Figure 2

miR-548ac overexpression inhibited the migration and invasion of GBM cells in vitro. (A, B) Cellular migration in GBM cells was determined with wound healing assays. Wound healing analysis 24 h and 48 h after transfection and quantification of the percentage of wound recovery. Bar = 400 μ m. (C, D) miR-548ac overexpression reduced the rate of invasion by GBM cells, Bar = 400 μ m. *P < 0.05, **P < 0.01, ***P < 0.001.

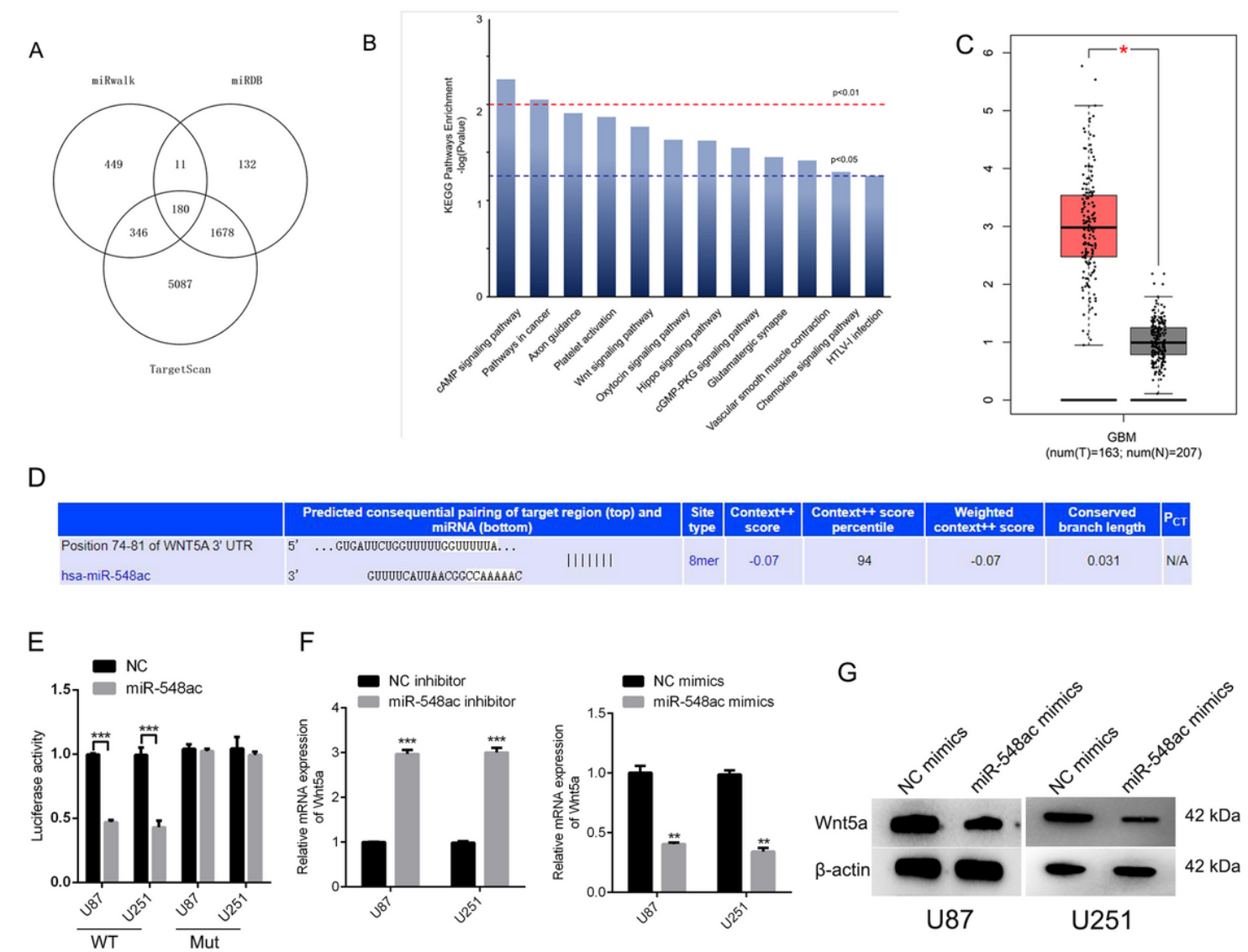


Figure 3

MiR-548ac targets Wnt5a and negatively regulates its expression in GBM cells. (A) Identification of the downstream target gene by online database analysis. (B) Use of KEGG analysis to identify factors associated with tumorigenesis in Wnt5a downstream molecules. (C) Wnt5a overexpression in GBM tissues compared with normal tissues according to GEPIA. (D) The predicted binding site of miR-548ac in the Wnt5a 3'UTR. (E) Luciferase assay with co-transfection of wt or mut Wnt5a 3'UTR constructs together

with miR-548ac or NC plasmids. (F) RT-qPCR analysis of Wnt5a expression. (G) Western blot analysis and quantification of Wnt5a expression. *P < 0.05, **P < 0.01, ***P < 0.001.

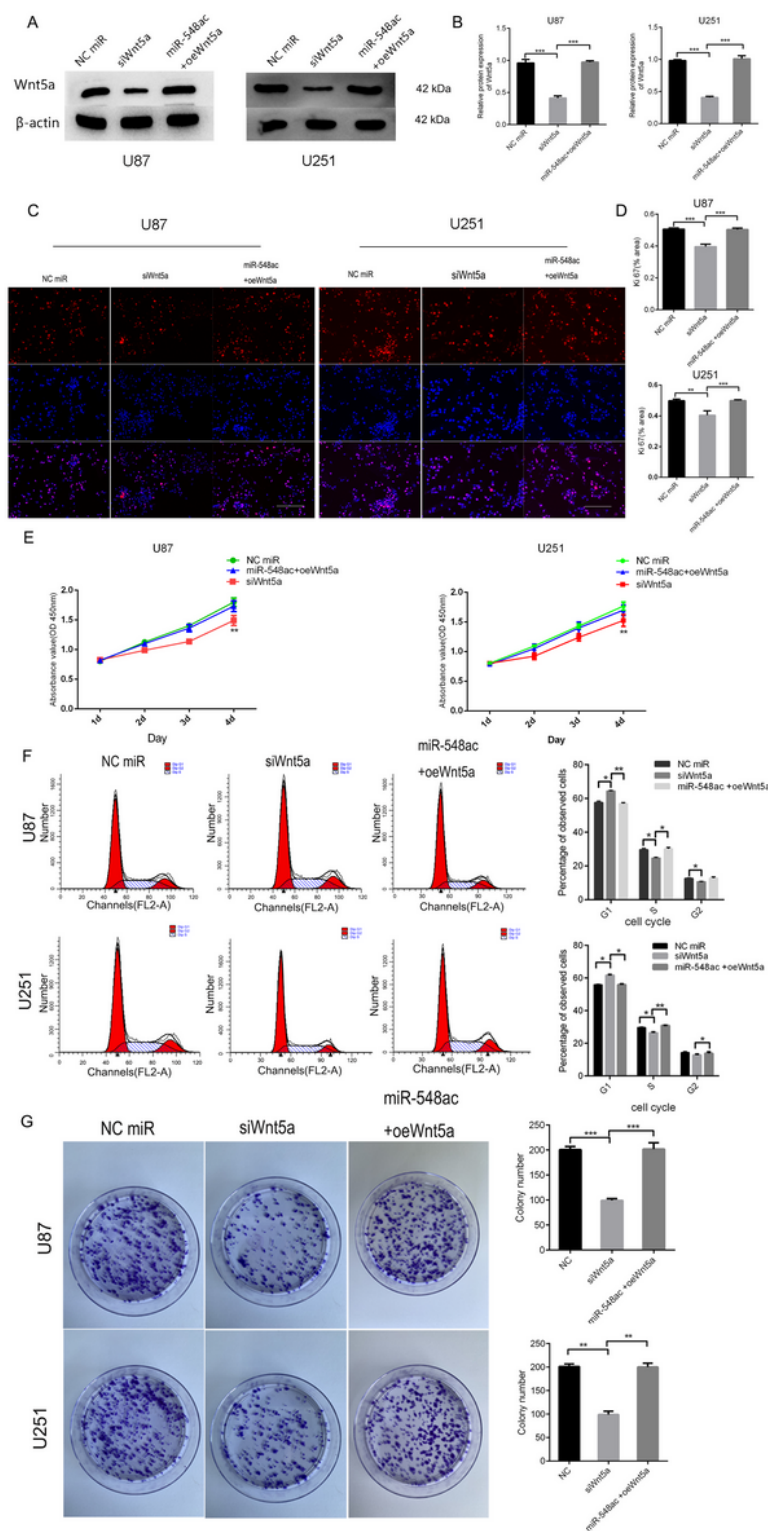


Figure 4

Knockdown of Wnt5a inhibits GBM cell proliferation, whereas Wnt5a rescues the effect of the miR-548ac. (A and B) Overexpression of Wnt5a increased its protein level, while miR-548ac restored the protein level of Wnt5a in GBM cells. (C and D) Ki67 assays performed 48h after transfection (bar = 200 μm) and

Knockdown of Wnt5a inhibits GBM cell migration, whereas Wnt5a rescues the effect of the miR-548ac mimics. (A, B) Wound healing analysis 24 h and 48 h after transfection (bar = 400 μ m) and quantification of the percentage wound recovery. (C, D) Transwell assays demonstrated the migration of GBM cells transfected with siWnt5a or miR-548ac mimics plus Wnt5a. Bar = 400 μ m. Quantification of migrated cells is shown. *P < 0.05, ***P < 0.001.

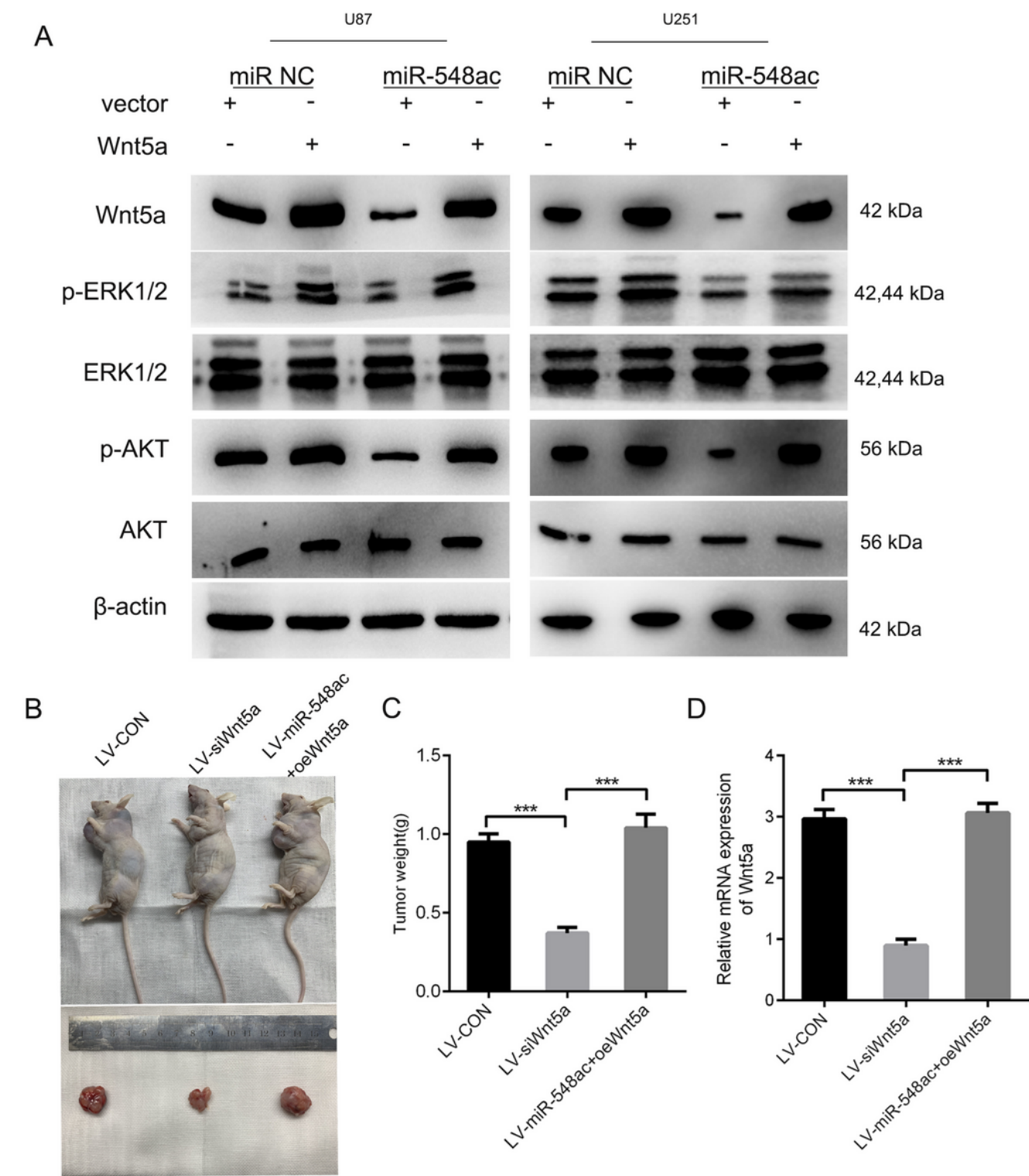


Figure 6

MiR-548ac inhibits the activation of AKT/ERK pathways by hampering the expression of Wnt5a, and miR-548ac suppresses glioma cell tumor growth in vivo. (A) Western blot assays of p-AKT, total AKT, p-ERK1/2, and total ERK1/2 expression in GBM/miR-NC and GBM/miR-548ac cells co-transfected with empty vector or Wnt5a expression vector (three replicates per group, three independent assays per group). GAPDH was used as the loading control. (B) Representative tumors obtained from mice transplanted with Lv-con, Lv-siWnt5a, or Lv-miR-548ac+oeWnt5a. (C) Tumor weight. Error bars, SEM. (D) Relative mRNA expression of Wnt5a in tumors from the study groups. Error bars, SEM.