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Circular RNA TTBK2 regulates cell proliferation, invasion and ferroptosis *via* miR-761/ITGB8 axis in glioma

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Abstract. – OBJECTIVE: Glioma is a primary intracranial tumor with an unfavorable prognosis. Evolving evidence indicates that circular RNA Tau tubulin kinase 2 (circ-TTBK2) is a cancer-associated gene. Therefore, this study was to explore the potential role of circ-TTBK2.

MATERIALS AND METHODS: Levels of circ-TTBK2, microRNA (miR)-761, and integrin subunit beta 8 (ITGB8) were determined by adopting quantitative real-time polymerase chain reaction (qRT-PCR) or Western blot. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to detect cell viability, and the invaded cells were distinguished utilizing transwell assay. Iron and lipid reactive oxygen species (ROS) assays were implemented to examine the iron (total iron and ferrous iron) and lipid-based ROS in glioma cells, respectively. Besides, dual-luciferase reporter assay was administrated to illustrate the interaction between miR-761 and circ-TTBK2 or ITGB8. The role of circ-TTBK2 was identified via xenograft tumor model.

RESULTS: Levels of circ-TTBK2 and ITGB8 were upregulated, whereas miR-761 level was low-expressed in glioma tissues and cells. Circ-TTBK2 was a sponge of miR-761 to modulate ITGB8. Additionally, circ-TTBK2 knockdown or miR-761 increase could retard cell proliferation, invasion, and promote ferroptosis in glioma cells. Interestingly, miR-761 inhibitor could abolish the repressive impact of circ-TTBK2 silencing on cell growth in vitro. Also, the influence of miR-761 mimic on cell phenotypes was regained after ITGB8 upregulation. Meanwhile, circ-TTBK2 deficiency caused the decrease of tumor growth.

CONCLUSIONS: Circ-TTBK2 regulated cell proliferation, invasion and ferroptosis via targeting ITGB8 by sponging miR-761 in glioma, providing a promising biomarker for the clinical therapy of human glioma.

Key Words:
Circ-TTBK2, MiR-761, ITGB8, Glioma, Proliferation, Invasion, Ferroptosis.

Introduction

Glioma is one of the general types of the intracranial tumor throughout the world and occurs from the normal glial tissues with the unfavorable prognosis[1]. The unlimited proliferation of tumor cells causes the characteristics of uncontrollable growth. Currently, the mortality of glioma is decreased, whereas its incidence rate is consistently improved[2]. Mountainous patients are diagnosed at the advanced or metastatic stages. Besides, surgery is implemented with curative intent for glioma patients with a surgically resectable feature. Although the development of the therapeutic strategies, incipient markers of diagnosis and prognosis, as well as the responsiveness to therapy, are still controversial, only a few validated markers were applied for the routine clinical practice[3]. More effective biomarkers needed to be found.

Despite the discoveries of circular RNAs (circRNAs) over 30 years ago, they attract more attention recently[4,5], and have been manifested to be involved in tumorigenesis[6-8]. The dysfunction of circRNAs result in tumor suppression or oncogenesis via regulating cell proliferation, migration, and invasion[9]. Importantly, circRNAs signatures may serve as the diagnosed or prognostic targets for human cancers, acting as a promising tool for assessing disease state[10]. The evidence highlights the significance of circRNAs in modifying the oncogenic progression[11]. Of note, circ_0034642 functions as an oncogenic mediator for the development of glioma by sponging microRNA (miRNA/miR)-1205[12]. Tau tubulin kinase 2 (TTBK2) is deemed as a kinase, and its upregulation can facilitate the process of amyotrophic lateral sclerosis[13]. TTBK2 leads to sunitinib-resistance kidney carcinoma[14]. Apart from that, circRNA TTBK2 (circ-TTBK2) functions as a master regulator in glioma malignancy by sponging miR-217[15]. How-

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ever, the underlying network of circ-TTBK2 in glioma needed to be further explored.

Until now, circRNAs are identified as the sponges of miRNAs to participate in tumorigenesis[16]. In regard to miRNAs, the strict relationship between miRNAs dysregulation and the biological or pathological process has been confirmed[17]. MiR-761 is an ordinary miRNA, located at chromosome 1p2. Earlier works suggest that miR-761 was connected with the onset and development of diverse cancers, such as hepatocellular carcinoma[18] and glioma[19]. Whether miR-761 could interact with circ-TTBK2 was the purpose of this present purpose. Furthermore, miR-761 goes in for the pathogenesis and progression of tumors by targeting tumor-related proteins[20,21]. Integrin subunit beta 8 (ITGB8) has been revealed to be related to the regulation of metastasis in human cancer[22]. We further investigated whether ITGB8 was a potential target of miR-761.

In this study, we clarified the expression statuses of circ-TTBK2, miR-761, and ITGB8 in glioma tissues and cells (LN299 and U251). The network of circ-TTBK2 was partially exposed by the functional assays.

Materials and Methods

Clinical Specimens and Cell Culture

Glioma patients (n=30) and intracranial injury patients (n=30) were recruited from Tianjin Medical University General Hospital for the glioma tissues and matched specimens. The experimental tissues were donated by the enrolled participators who signed the written informed consent. This research was authorized by the Ethics Committee of Tianjin Medical University General Hospital.

Glioma cells (LN229 and U251) and normal cells (NHA) were purchased from Be Na Culture Collection (Beijing, China) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South-Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin (HyClone, South-Logan, UT, USA) and 100 μg/mL streptomycin (HyClone, South-Logan, UT, USA). The cells continuously grew at 37°C in an incubator containing 5% CO_2.

Cell Transfection

Short hairpin (shRNA) targeting the junction region of circ-TTBK2 (sh-circ-TTBK2) and its negative control (sh-NC) was obtained from Sangon Biotechnology (Shanghai, China). MiR-761 mimic (miR-761), miR-761 inhibitor (anti-miR-761), and their controls (miR-NC and anti-miR-NC) were purchased from Hanbio (Shanghai, China). The overexpression vectors of circ-TTBK2 (circ-TTBK2) and ITGB8 (ITGB8), as well as their blank control (pcDNA), were designed and constructed in Sangon Biotechnology (Shanghai, China). Consistent with the previous paper, circ-TTBK2 contained two frames: a front circular frame and a back circular frame[5,23]. Cell transfection was conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the producer's protocol. Expect that, sh-circ-TTBK2 and sh-NC were adopted to establish the stably transfected cells *via* lentivirus-mediation.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was purified and extracted from the tissues (glioma and its matched control) and cells (LN229, U251, and NHA) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For circ-TTBK2, RNase-R (Epicentre, Madison, WI, USA) was added to eliminate the liner RNAs and verify the existent of circ-TTBK2. The measurement of circ-TTBK2 was performed using One Step PrimeScript™ RT-PCR Kits (TaKaRa, Dalian, China). In addition, total RNA acted as the template to synthesize the complementary DNA (cDNA) with the help of High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Fast SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) was employed to mix the reacted solution as per the manufacturer's directions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for circ-TTBK2 and ITGB8) or U6 (for miR-761) served as the endogenous control. The levels of circ-TTBK2, miR-761, and ITGB8 were normalized *via* the $2^{-\Delta\Delta Ct}$ method. The primer sequences were shown: circ-TTBK2 (Forward: 5'-AGTGCAACATTTTCCCTGGTG-3', Reverse: 5'-GCTTGATTTTGGCTTGGCTC-3'); probe: FAM+CCCCAATCTTTCTCAATGGTCTGACG+BHQ1; miR-761 (Forward: 5'-ACAGCAGGCACAGAC-3', Reverse: 5'-GAGCAGGCTGGAGAA-3'); ITGB8 (Forward: 5'-CTGAAGAAATACCCCGTGGA-3', Reverse: 5'-ATGGGGAGGCATACAGTCT-3'); GAPDH (Forward: 5'-ACTCCTCCACCTTTGACGC-3', Reverse: 5'-GCTGTAGCCAAATTCGTTGTC-3'); U6 (Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3').

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The glioma cells (LN229 and U251) were plated into the wells of a 96-well plate at 24 h post-transfection and cultured at 37°C. After incubation for indicated time, the cells were added with MTT solution (Sigma-Aldrich, St. Louis, MO, USA), and continuously cultured for 4 h. Subsequently, the reaction was terminated following the addition of 200 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The absorbance of the lysates was assessed *via* a microplate reader at 490 nm.

Matrigel Invasion Assay

Cell invasion assay was performed using transwell plates (8 mm pores; Corning, Costar, NY, USA) with pre-coated Matrigel (Corning, Costar, NY, USA). The transfected cells with serum-free media were seeded onto the upper chamber, and relative medium containing 10% FBS as the chemoattractant of cells filled with the low chamber. After culture for 48 h, the migrated cells that passed through the filter were stained by 0.5% crystal violet (Corning, Costar, NY, USA). Then, the average invasion rate was calculated to represent the ability of the invasion of the tested cells.

Iron Assay

The measurements of the total iron and ferrous iron were performed using the Iron Analysis Kit (Sigma-Aldrich, St. Louis, MO, USA). Firstly, 2×10^6 cells were immediately homogenized with 5 volumes of Iron Assay buffer. The insoluble material was wiped off *via* the centrifugation at 13,000×g under 4°C. For estimation of Fe^{2+} iron, 5 μl of Iron Reducer was supplemented per well. For the assay of total iron, sample wells were added Iron reducer to reduce the switch from Fe^{3+} to Fe^{2+}. The mixture was gently mixed *via* pipetting, and then, reacted for 30 min in the dark. Subsequently, 100 μL Iron Probe was added into the standard and test samples, and the thoroughly mixed sample was incubated for 1 h at room temperature darkly. In the end point, the absorbance was determined with the help of microplate reader at 593 nm.

Lipid Reactive Oxygen Species (ROS) Assay

Lipid ROS level was identified with the use of BODIPYC11 dye (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with previous studies[24,25]. Briefly, the cells were induced using ferrostatin (2.0 μM; MedChemExpress, Monmouth Junction, NJ, USA) or Erastin (10.0 μM; MedChemExpress, Monmouth Junction, NJ, USA). The cells treated accordingly were cultured in the well of a 6-well plate. After incubation for 48 h, the supernatant was removed and then added with fresh medium with 5 μM of BODIPY-C11. Next, the cells were continuously cultured for 20 min at 37°C. After that, the above cells were harvested and washed using phosphate buffered solution (PBS; HyClone, South Logan, UT, USA), and then shifted into 15 ml tubes. Subsequently, the sedimentary cells were re-suspended with 500 μL PBS followed by the filtration with the cell strainer (0.4 μm nylon mesh; BD Biosciences, Franklin Lakes, NJ, USA). The cell suspension was subjected to the analysis of flow cytometry to assess the abundance of the ROS within cells.

Western Blot Assay

The protocol was operated in accordance with the descriptions in a previous study[26]. Briefly, the segregated proteins were blotted on the polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). Then, the membranes were incubated with unique primary antibodies overnight at 4°C after blocking for 1 h. On the next day, the corresponding secondary antibody was used for the combination with the primary antibody, and the combined signals were appeared *via* adding the reagents of an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA). The primary antibodies were purchased from Abcam (Cambridge, MA, USA), and the special information was listed: ITGB8 (1:1000, ab80673) and GAPDH (1:20000, ab8245).

Dual-Luciferase Reporter Assay

The fragments of circ-TTBK2 and ITGB8 containing miR-761 complementary sequences were synthesized from Sangon Biotechnology (Shanghai, Cina). These sequences were inserted into psi-CHECK-2 (Promega, Madison, WI, USA), which was the basic vector of the Luciferase, named as WT-circ-TTBK2 and ITGB8 3'UTR-WT. Simultaneously, the mutated binding sites were used for the mutant reporters (MUT-circ-TTBK2 and ITGB8 3'UTR-MUT) in the same way. Next, the above wildtype or mutant reporter was transfected into LN229 and U251 cells with the presence of miR-761 mimic or its control. Luciferase activity was examined with the use of Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as per the manufacturer's manuals.

Xenograft Tumor Model

The stably transfected LN229 cells were set up *via* the lentivirus-mediation of sh-circ-TTBK2 or sh-NC. Then, the stably transfected LN229 cells (2×10^6) were injected into the Balb/c nude mice (4 weeks old, n=6/group) subcutaneously, which were purchased from Vital River Laboratory Animal Technology (Beijing, China). The *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University General Hospital. The tumor length (L) and width (W) were measured every 3 days for a total of 6 times, and the volume was calculated: volume=$L\times W^2/2$. Twenty-four days after injection, mice were sacrificed, and the tumor samples were collected, and these isolated tumors were weighed and applied for the subsequent assays.

Statistical Analysis

Statistical analyses were carried out utilizing SPSS 18.0 (SPSS Inc., Chicago, IL, USA), and the data were expressed as mean ± Standard Deviation (SD). Comparison between two groups was assayed using Student's *t*-test, and one-way analysis of variance with Tukey test was implemented to compare the difference among the multiple groups (three or more groups). For all analyses, $p<0.05$ was deemed as the indicator of statistical difference.

Results

Circ-TTBK2 with a High Level in Glioma Tissues and Cells was Associated with MiR-761

To investigate the biological function of circ-TTBK2 in glioma, we implemented qRT-PCR to evaluate the relative expression of circ-TTBK2 in glioma and selected cell lines. The results demonstrated that circ-TTBK2 was clearly upregulated in glioma tissues and cells in comparison with relative controls (Figure 1A and 1B). Besides, we also found that miR-761 was particularly decreased in glioma tissues and cells (Figure 1C and 1D). Subsequently, statistical analysis exposed that miR-761 was inversely correlated with circ-TTBK2 in glioma tissues (Figure 1E). These data showed that the aberrant expression of circ-TTBK2 and miR-761 might go in for the progression of glioma.

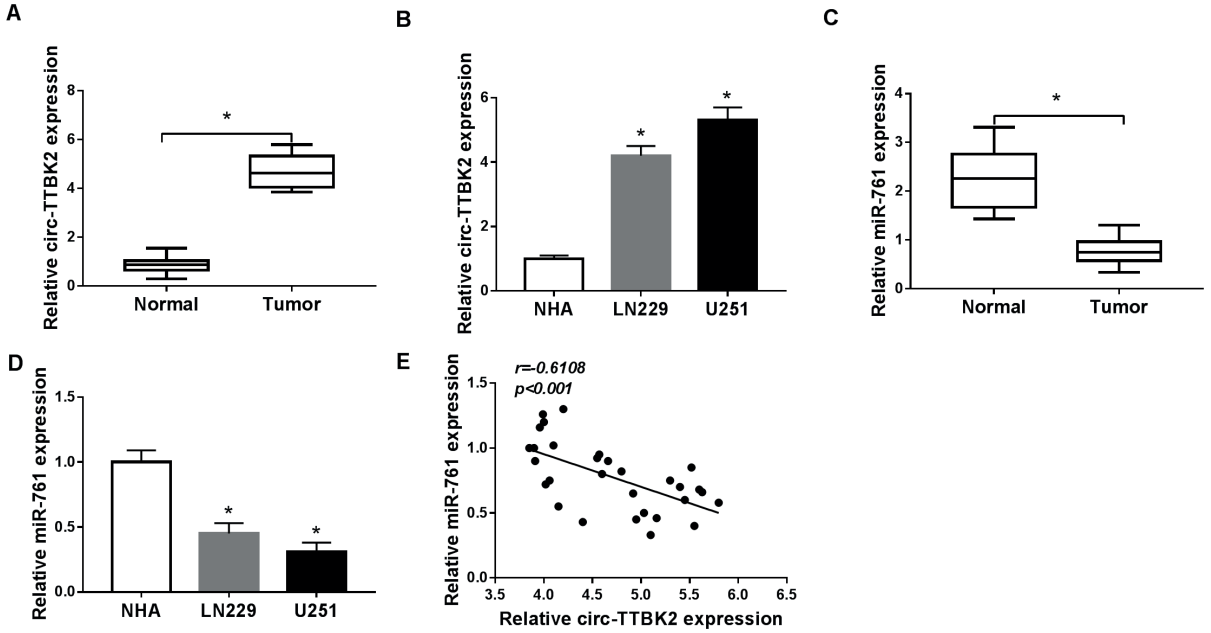

Figure 1. Circ-TTBK2 with a high level in glioma tissues and cells was associated with miR-761. **A-D,** The levels of (**A** and **B**) circ-TTBK2 and (**C** and **D**) miR-761 were assessed using qRT-PCR in glioma tissues and cells. **E,** The correlation between miR-761 and circ-TTBK2 was verified by Pearson's correlation analysis. *$p<0.05$.

Knockdown of circ-TTBK2 suppressed cell proliferation, invasion, and promoted ferroptosis in glioma cells

Considering the ectopic expression of circ-TTBK2, we next attracted the characteristics of circ-TTBK2 in glioma. Firstly, sh-circ-TTBK2 or sh-NC was introduced into LN229 and U251 cells, and the level of circ-TTBK2 was evidently reduced by sh-circ-TTBK2 transfection (Figure 2A). Then, MTT analysis confirmed that circ-TTBK2 deficiency could strikingly hamper cell proliferation in the two glioma cells (Figure 2B and 2C). In addition, the capacity of cell invasion was determined by transwell assay, and the results exhibited that the invasion of LN229 and U251 cells was distinctly impeded after circ-TTBK2 detection (Figure 2D). In addition, the level of ferroptosis was measured. The ferroptotic mode of programmed necrosis was identified as an apoptosis-independent form of cell death[27]. We supplemented ferrostatin-1 (2 μM) or Erastin (10 μM) into circ-TTBK2-silenced glioma cells for 48 h. The results from MTT manifested that circ-TTBK2 deficiency could enhance the Erastin-induced growth reduction in LN229 and U251 cells (Figure 2E and 2F). After treatment with Erastin (5 μM) for 48 h, the decrease of circ-TTBK2 caused the acceleration of the intracellular concentrations of the iron and ferrous iron (Figure 2G-2J). Similarly, circ-TTBK2 silencing also triggered the concentrations of intracellular lipid ROS *in vitro* (Figure 2K and 2L). In brief, the knockdown of circ-TTBK2 hindered cell proliferation, invasion, and reinforced ferroptosis in LN229 and U251 cells.

MiR-761 Inhibition Could Restore the Impact of Circ-TTBK2 Silencing on Cell Phenotypes In Vitro

Due to the negative correlation between miR-761 and circ-TTBK2, we predicted the common fragments between them using starBase v2.0, and the results were shown in Figure 3A. Next, Dual-Luciferase reporter analysis elucidated that miR-761 sharply decreased the Luciferase activity in presence of WT-circ-TTBK2 ($p<0.05$), but that with the mutant reporter was not remarkably affected (Figure 3B and 3C). The level of circ-TTBK2 was significantly upregulated after circ-TTBK2 transfection in LN229 and U251 cells (Figure 3D). Later, we found that circ-TTBK2 silencing resulted in the forceful augment of miR-761 level in the two glioma cells, while circ-TTBK2 transfection inversely modified the level of miR-761 (Figure 3E). Afterwards, sh-circ-TTBK2 or sh-circ-TTBK2 plus anti-miR-761 was introduced into LN229 and U251 cells, and the reintroduction of miR-761 inhibitor could relieve the acceleratory effect of circ-TTBK2 detection on the level of miR-761 (Figure 3F). Simultaneously, MTT analysis presented that the inhibitory impact of circ-TTBK2 reduction on cell proliferation was partially restored *via* co-transfection with anti-miR-761 (Figure 3G and 3H). As depicted by the analysis of transwell, cell invasion was specially suppressed by circ-TTBK2 detection, whereas synchronous introduction with miR-761 inhibitor could reverse this effect in the two glioma cells (Figure 3I). Apart from that, we also measured the alteration of ferroptosis. As described in Figure 3J and 3K, circ-TTBK2 silencing strengthened the role of Erastin in cell growth repression, but co-transfection with anti-miR-761 could rescue this effect *in vitro*. In addition, the boost of the intracellular iron and ferrous iron concentrations caused by circ-TTBK2 deficiency was diminished after transfection with miR-761 inhibitor in LN229 and U251 cells (Figure 3L-3O). Besides, the intracellular concentration of ROS was induced as a result of circ-TTBK2 silencing, and such facilitation effect could be abrogated after simultaneous introduction with miR-761 inhibitor in the two glioma cells (Figure 3P and 3Q). In general, miR-761 was directly targeted by circ-TTBK2 and miR-761 inhibition overturned the influence of circ-TTBK2 deficiency on cell proliferation, invasion and ferroptosis in glioma cells.

ITGB8 Was a Target of MiR-761

In view of the foregoing introductions, we attempted to seek the protein targets of miR-761. As predicted by starBase v2.0, there were complementary sequences between miR-761 and ITGB8 (Figure 4A). Dual-Luciferase reporter analysis indicated that miR-761 mimic declined the Luciferase intensity nearly 60% in wildtype group, but the mutant of the seed region abrogated the reduction effect of miR-761 mimic in both LN229 and U251 cells (Figure 4B and 4C). Next, efficient augments of the mRNA and protein levels of ITGB8 were observed in glioma tissues and cells (Figure 4D-4G). Subsequently, Pearson's correlation analysis exhibited that the level of miR-761 was negatively related to ITGB8 level in glioma tissues (Figure 4H). Furthermore, the opposite regulation between miR-761 and ITGB8 was confirmed by qRT-PCR and

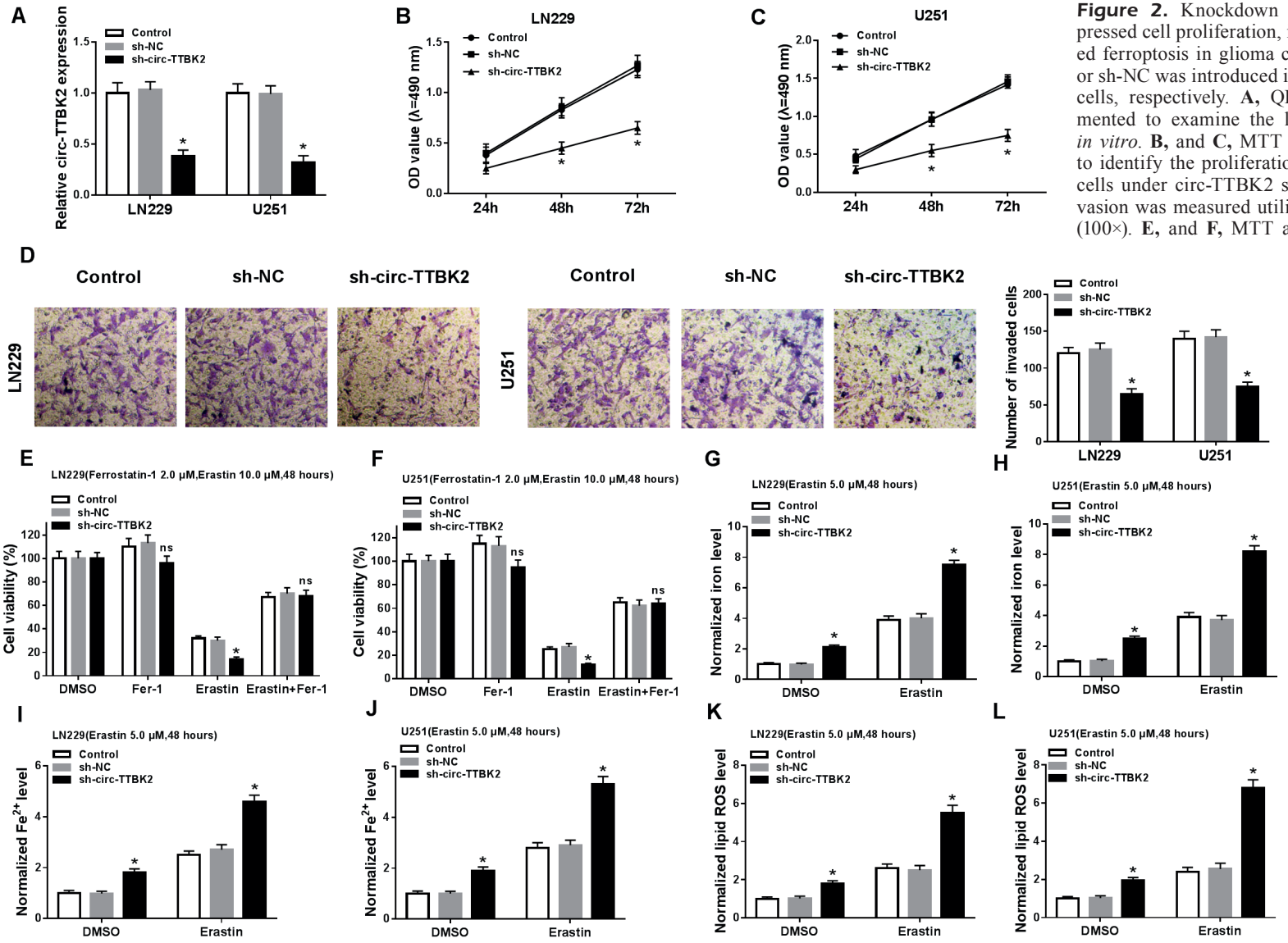

Figure 2. Knockdown of circ-TTBK2 suppressed cell proliferation, invasion and promoted ferroptosis in glioma cells. Sh-circ-TTBK2 or sh-NC was introduced into LN229 and U251 cells, respectively. **A,** QRT-PCR was implemented to examine the level of circ-TTBK2 *in vitro*. **B,** and **C,** MTT assay was employed to identify the proliferation of the two glioma cells under circ-TTBK2 silencing. **D,** Cell invasion was measured utilizing transwell assay (100×). **E,** and **F,** MTT assay was conducted to reckon the responses of LN229 and U251 cells stably low-expressing circ-TTBK2 to ferrostatin-1 (2 μM) or Erastin (10 μM). (G-L) Levels of (**G** and **H**) total iron, (**I** and **J**) ferrous iron, and (**K** and **L**) lipid ROS in the two glioma cells stably low-expressing circ-TTBK2 was evaluated. *p<0.05.

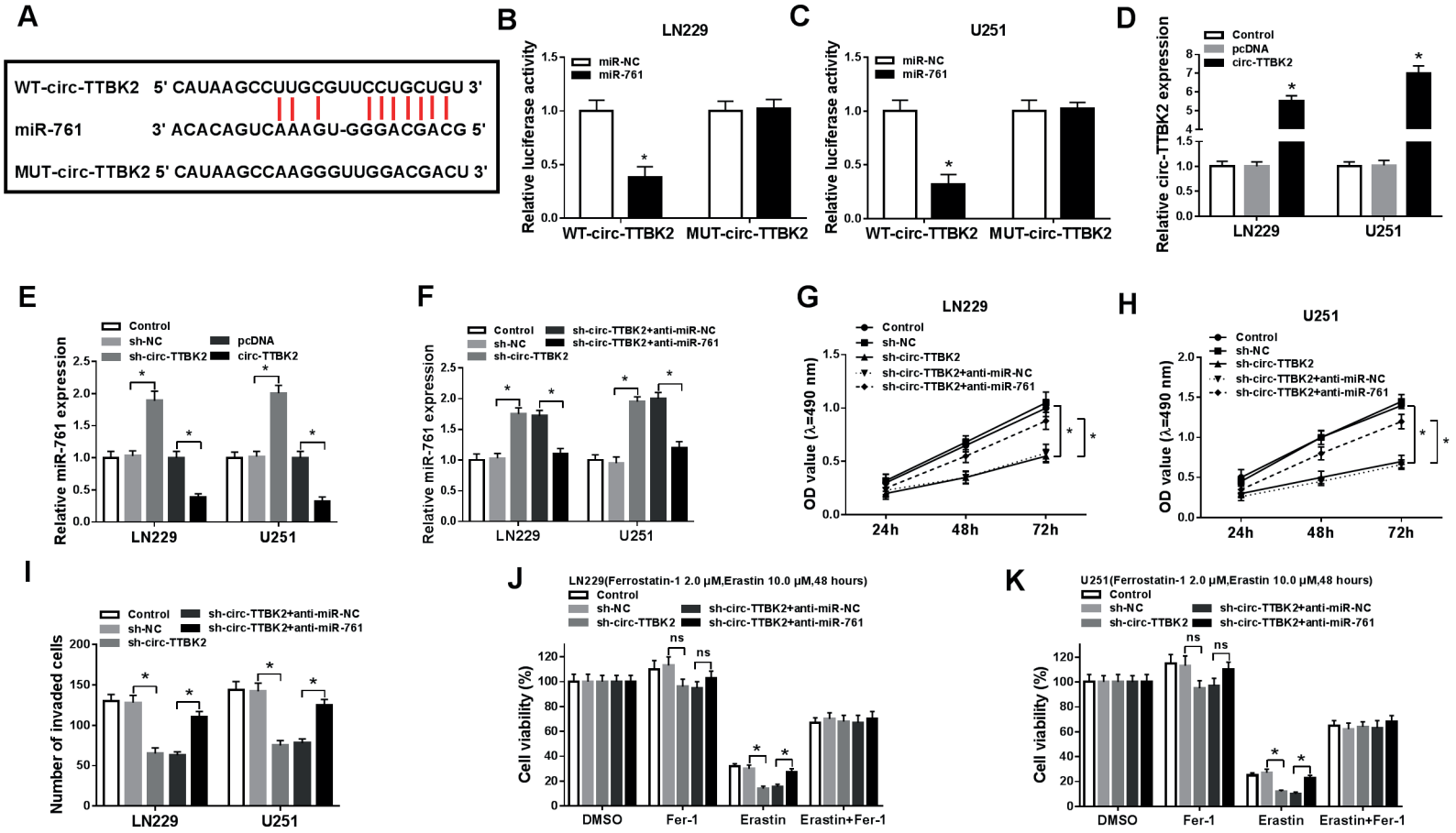

Figure 3. MiR-761 inhibition could restore the impact of circ-TTBK2 silencing on cell phenotypes and ferroptosis *in vitro*. **A,** StarBase v2.0 software was applied to predict the complementary sequence between circ-TTBK2 and miR-761. **B,** and **C,** The interrelation between miR-761 and circ-TTBK2 was confirmed using dual-luciferase reporter assay. **D,** The level of circ-TTBK2 was tested in LN229 and U251 cells with circ-TTBK2 transfection. **E,** The effect of circ-TTBK2 or sh-circ-TTBK2 transfection on the level of miR-761 in glioma cells. **F-Q,** LN229 and U251 cells were transfected with sh-NC, sh-circ-TTBK2, sh-circ-TTBK2+anti-miR-NC, or sh-circ-TTBK2+anti-miR-761, respectively. **F,** QRT-PCR was performed to evaluate the level of miR-761 *in vitro*. **G,** and **H,** The effects of sh-circ-TTBK2 and anti-miR-761 transfection on cell proliferation were detected by MTT in LN229 and U251 cells. **I,** Transwell assay was used to determine the ability of cell invasion *in vitro*. **J,** and **K,** MTT assay was administrated to assess the responses of glioma cells to ferrostatin (2.0 μM) and Erastin (10.0 μM).

Figure continued

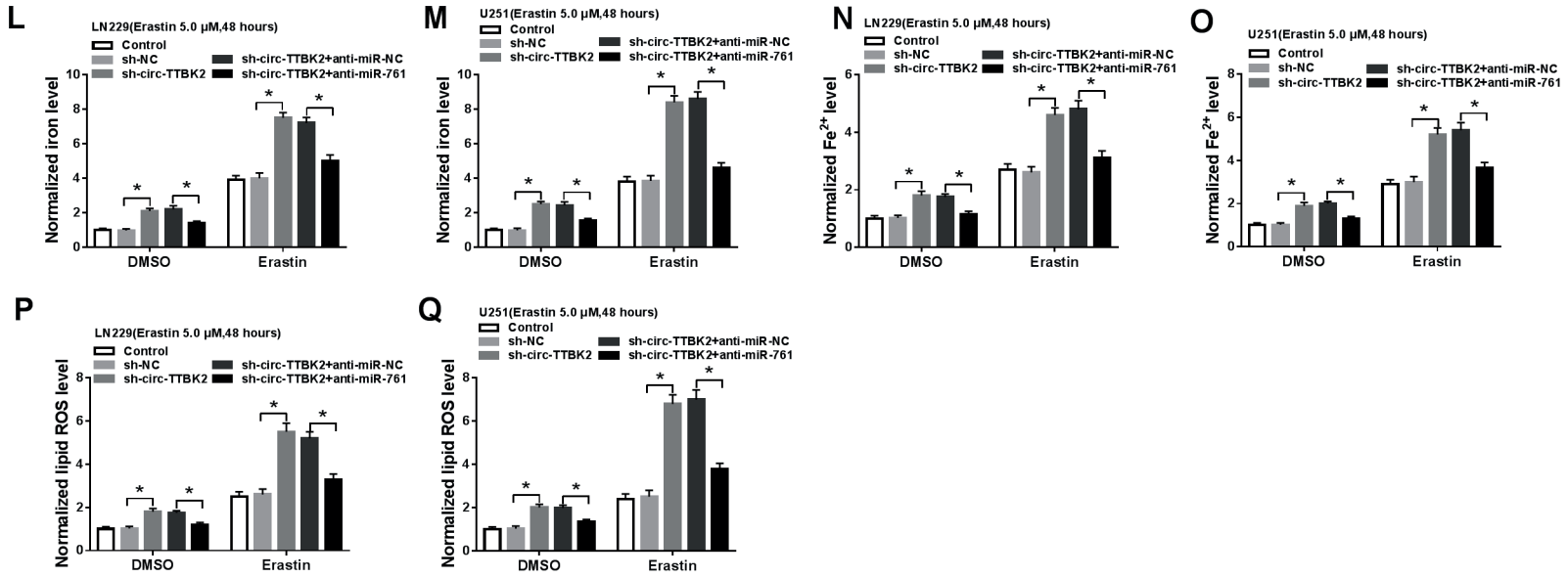

Figure 3. ***(Continued).*** **L-Q,** Levels of (**L** and **M**) total iron, (**N** and **O**) ferrous iron, and (**P** and **Q**) lipid ROS in LN229 and U251 cells were analyzed using iron and lipid ROS assays, respectively. *p<0.05.

Western blot assays, and the results implied that miR-761 inhibitor caused the enhancement of ITGB8 level at the mRNA and protein expression, but the role of miR-761 mimic exerted the contrary role *in vitro* (Figure 4I and 4J). All the data demonstrated that miR-761 targeted ITGB8 in glioma cells.

Influence of MiR-761 Mimic on Cell Behaviors was Relieved by ITGB8 Upregulation in Glioma Cells

Next, we systemically investigated the functional mechanism between miR-761 and ITGB8. Firstly, miR-761 alone or along with ITGB8 was introduced into LN229 and U251 cells, the level of ITGB8 was retarded by miR-761 mimic at mRNA and protein expression, but supplement with ITGB8 regained this effect *in vitro* (Figure 5A). Then, MTT analysis disclosed that the reintroduction of ITGB8 could recovered the inhibitory impact of miR-761 increase on cell proliferation in LN229 and U251 cells (Figure 5B and 5C). Besides, cell invasion was impeded as a result of miR-761 upregulation, and such repression influence could be recuperated after co-transfection with ITGB8 in the two glioma cells (Figure 5D). Moreover, miR-761 mimic could expedite Erastin-stimulated cell growth reduction in both LN229 and U251 cells, while this role was reverted *via* reintroduction with ITGB8 (Figure 5E and 5F). Meanwhile, the elevations of intracellular concentrations of iron, Fe^{2+}, and ROS caused by miR-761 mimic were greatly eliminated *via* supplement with ITGB8 in both LN229 and U251 cells (Figure 5G-5L). As a whole, the influence of miR-761 upregulation on cell phenotypes was abolished by ITGB8 overexpression in glioma cells.

ITGB8 Was Co-Regulated by Circ-TTBK2 and ITGB8

Based on the above information, we attempted to systemically clarify the regulatory mechanism between ITGB8 and circ-TTBK2 or miR-761. At first, sh-circ-TTBK2 alone or plus anti-miR-761 was introduced into LN229 and U251 cells. The mature ITGB8 level was evaluated *via* Western blot assay, and the results suggested that circ-TTBK2 silencing led to a significant curb of the level of protein ITGB8, but co-transfection with miR-761 inhibitor could partially regain this effect in the two glioma cells (Figure 6A and 6B). Namely, the level of ITGB8 was co-modulated by circ-TTBK2 and miR-761 in glioma cells.

Circ-TTBK2 Silencing Diminished Tumor Growth in Nude Mice

As mentioned above, the function of circ-TTBK2 was demonstrated by *in vitro* experiments. We further exposed how circ-TTBK2 regulated glioma growth utilizing xenograft tumor model. As shown in Figure 7A and 7B, the knockdown of circ-TTBK2 resulted in the repressions of tumor volume and weight at the same time point. Then, effective decrease of circ-TTBK2 and ITGB8, and a distinct augment of miR-761 were observed in sh-circ-TTBK2-mediated xenograft tumors (Figure 7C). Meanwhile, Western blot analysis implied that the protein level of ITGB8 was indeed restrained in excised specimens with sh-circ-TTBK2-mediation (Figure 7D). The evidence meant that circ-TTBK2 might act as an oncogene in the progression of glioma *via* miR-761/ITGB8 axis.

Discussion

Glioma is a leading cause of cancer-related deaths with refractory malignancy. Recurrence and distal metastasis are the major barriers for overcoming this refractory malignant tumor[3]. To date, molecular-associated treatment is extremely imperative in glioma cure. CircRNAs consist of non-coding RNAs, and emerging records imply that circRNAs play the critical functions in cancerous progression *via* transcriptional or post-transcriptional regulation of targeted gene expression[28,29]. The ectopic expression of circRNAs is tightly involved in the cell behaviors and drug-resistance of several cancer cells[30,31]. Hence, circRNAs may serve as the probable biomarkers for the diagnosis, prognosis, and therapy of human carcinomas.

Zheng et al[15] clarified that circ-TTBK2 was overexpressed in glioma tissues and cells. In this paper, we further illustrated this elevation in glioma samples and cells compared with the corresponding controls (noncancerous tissues and NHA cells). Additionally, the reprogramming of cellular balance is necessary for tumorigenesis, such as the modification of apoptotic and necrotic cell death[32,33]. Notably, an oxidative iron-dependent form of regulated necrosis, ferroptosis, has emerged as a new type of programmed death distinct from apoptosis and autophagy. Ferroptosis refers to metabolic dysfunction that causes the glutaminolysis and the production of iron-dependent reactive oxygen species (ROS) in intracel-

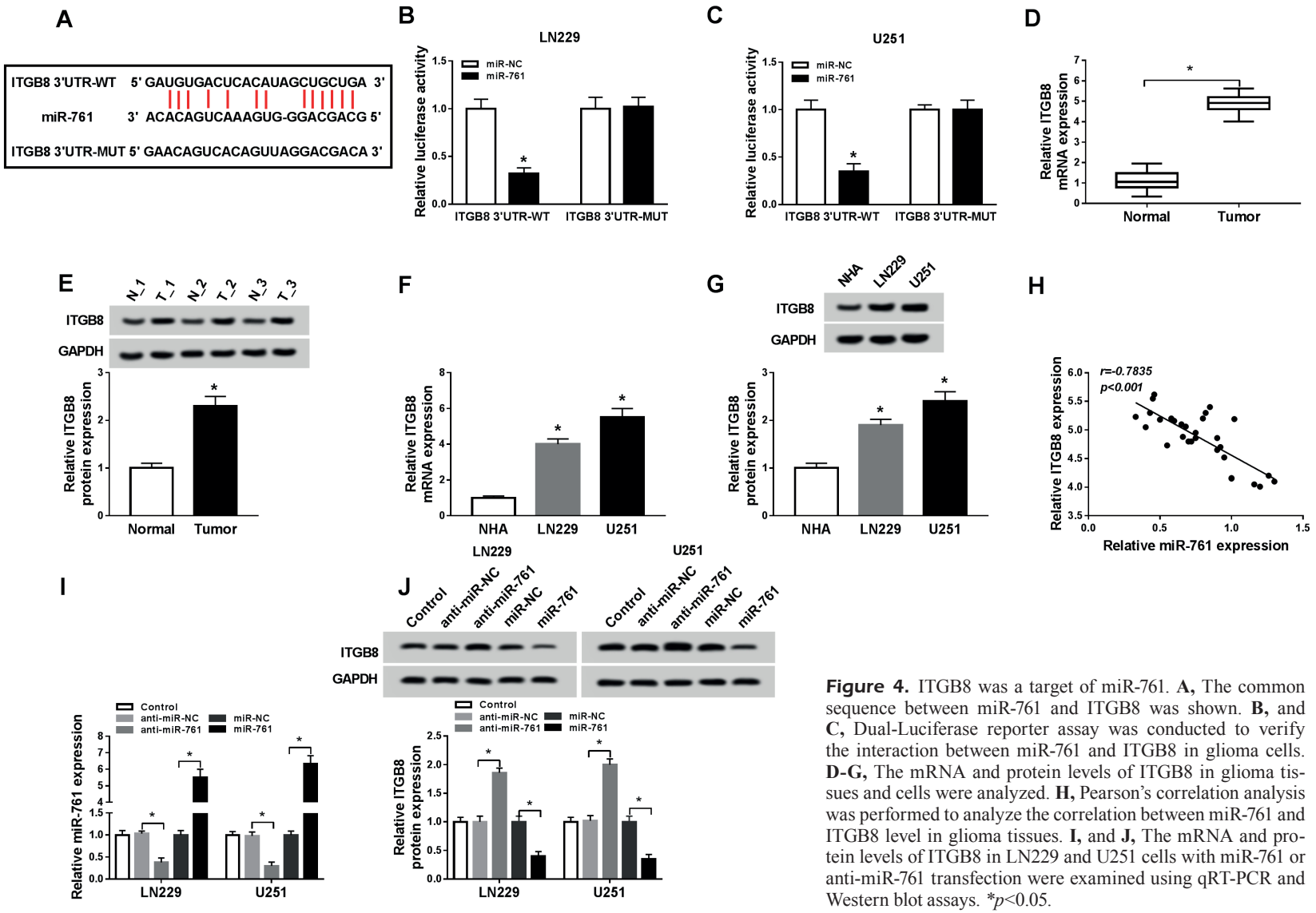

Figure 4. ITGB8 was a target of miR-761. **A,** The common sequence between miR-761 and ITGB8 was shown. **B,** and **C,** Dual-Luciferase reporter assay was conducted to verify the interaction between miR-761 and ITGB8 in glioma cells. **D-G,** The mRNA and protein levels of ITGB8 in glioma tissues and cells were analyzed. **H,** Pearson's correlation analysis was performed to analyze the correlation between miR-761 and ITGB8 level in glioma tissues. **I,** and **J,** The mRNA and protein levels of ITGB8 in LN229 and U251 cells with miR-761 or anti-miR-761 transfection were examined using qRT-PCR and Western blot assays. *$p<0.05$.

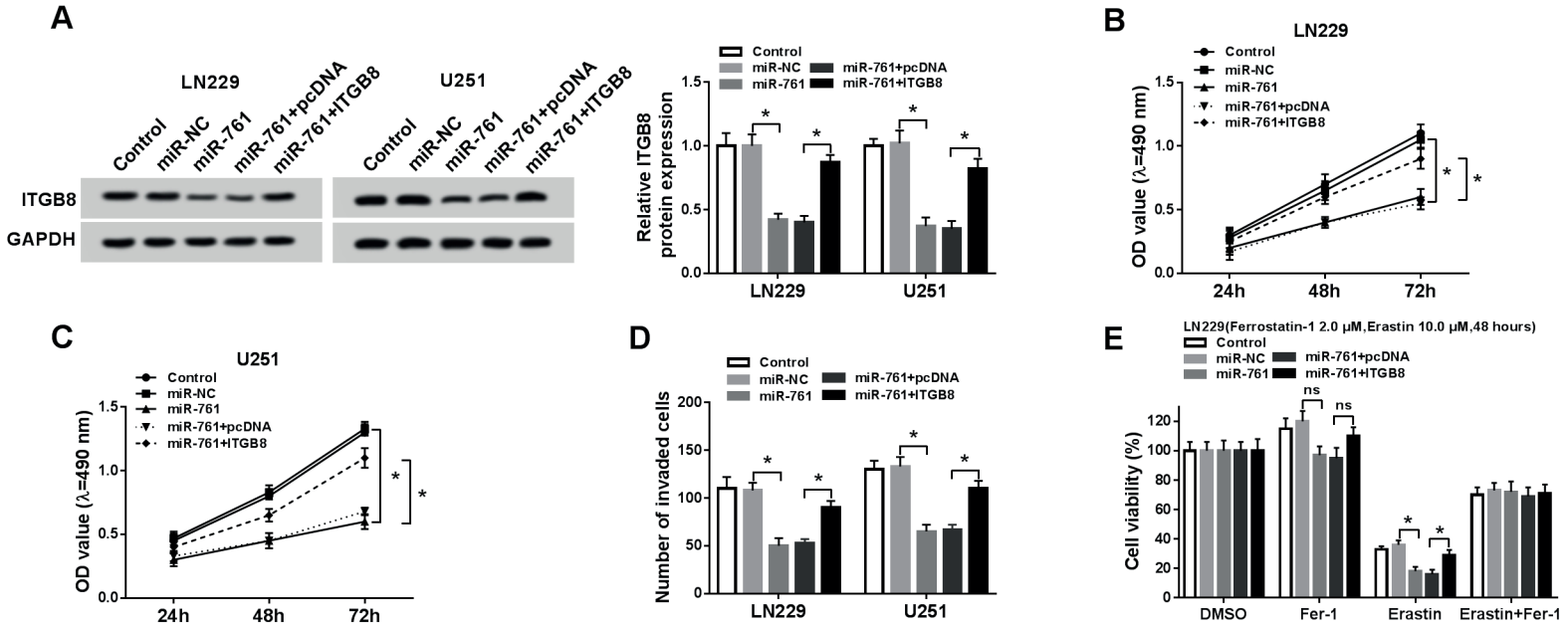

Figure 5. Influence of miR-761 mimic on cell behaviors and ferroptosis was relieved by ITGB8 upregulation in glioma cells. MiR-NC, miR-761, miR-761+pcDNA, or miR-761+ITGB8 was introduced into LN229 and U251 cells, respectively. **A,** QRT-PCR was administrated to assay the level of ITGB8 *in vitro*. **B,** and **C,** Cell proliferation was measured utilizing MTT assay. **D,** The capacity of cell invasion was evaluated by transwell assay in the two glioma cells. **E,** and **F,** MTT assay was carried out to identify the responses of glioma cells with stably overexpression of miR-761 or ITGB8 to ferrostatin (2.0 μM) and Erastin (10.0 μM). **G-L,** Levels of (**G** and **H**) total iron, (**I** and **J**) ferrous iron, and (**K** and **L**) lipid ROS in the two glioma cells with miR-761 or ITGB8 transfection were reckoned via corresponding detection kits. *p<0.05.

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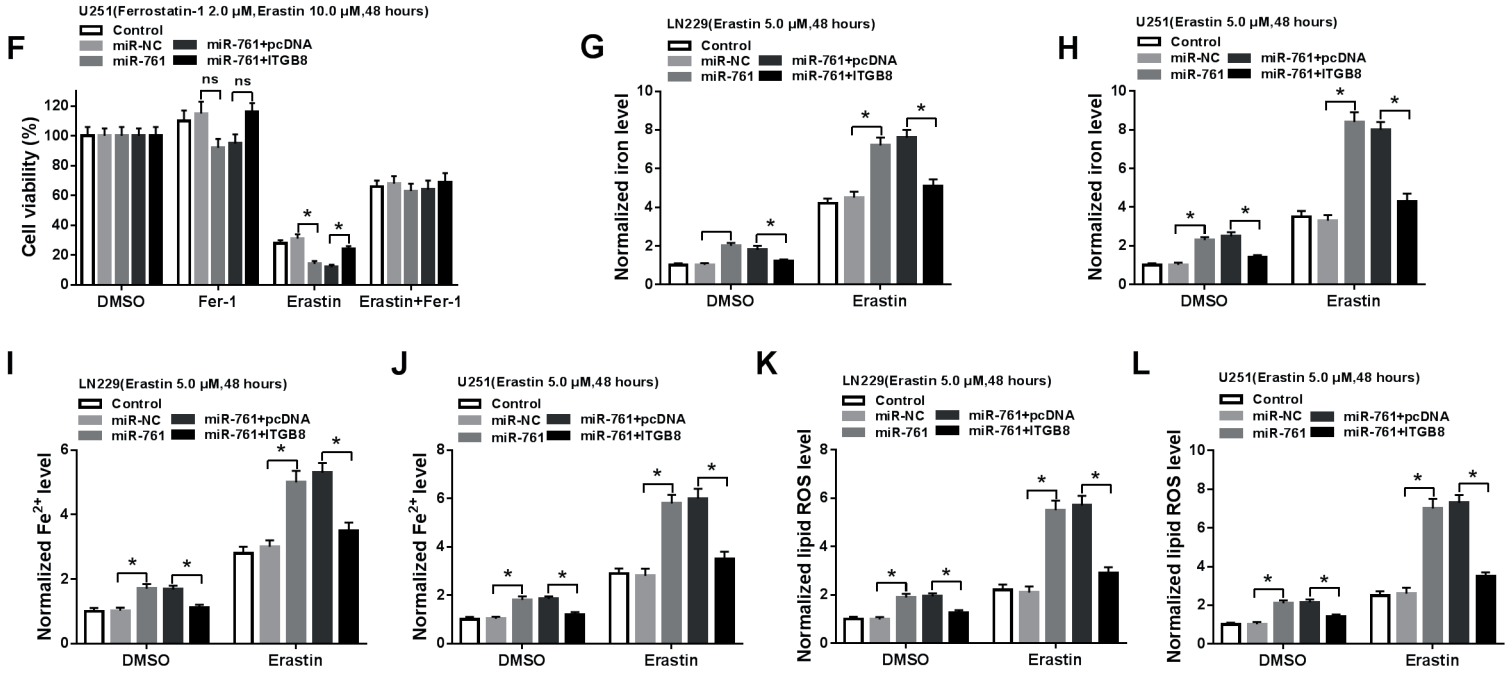

Figure 5 ***(Continued).*** Influence of miR-761 mimic on cell behaviors and ferroptosis was relieved by ITGB8 upregulation in glioma cells. MiR-NC, miR-761, miR-761+pcDNA, or miR-761+ITGB8 was introduced into LN229 and U251 cells, respectively. **A,** QRT-PCR was administrated to assay the level of ITGB8 *in vitro*. **B,** and **C,** Cell proliferation was measured utilizing MTT assay. **D,** The capacity of cell invasion was evaluated by transwell assay in the two glioma cells. **E,** and **F,** MTT assay was carried out to identify the responses of glioma cells with stably overexpression of miR-761 or ITGB8 to ferrostatin (2.0 μM) and Erastin (10.0 μM). **G-L,** Levels of (**G** and **H**) total iron, (**I** and **J**) ferrous iron, and (**K** and **L**) lipid ROS in the two glioma cells with miR-761 or ITGB8 transfection were reckoned via corresponding detection kits. **p*<0.05.

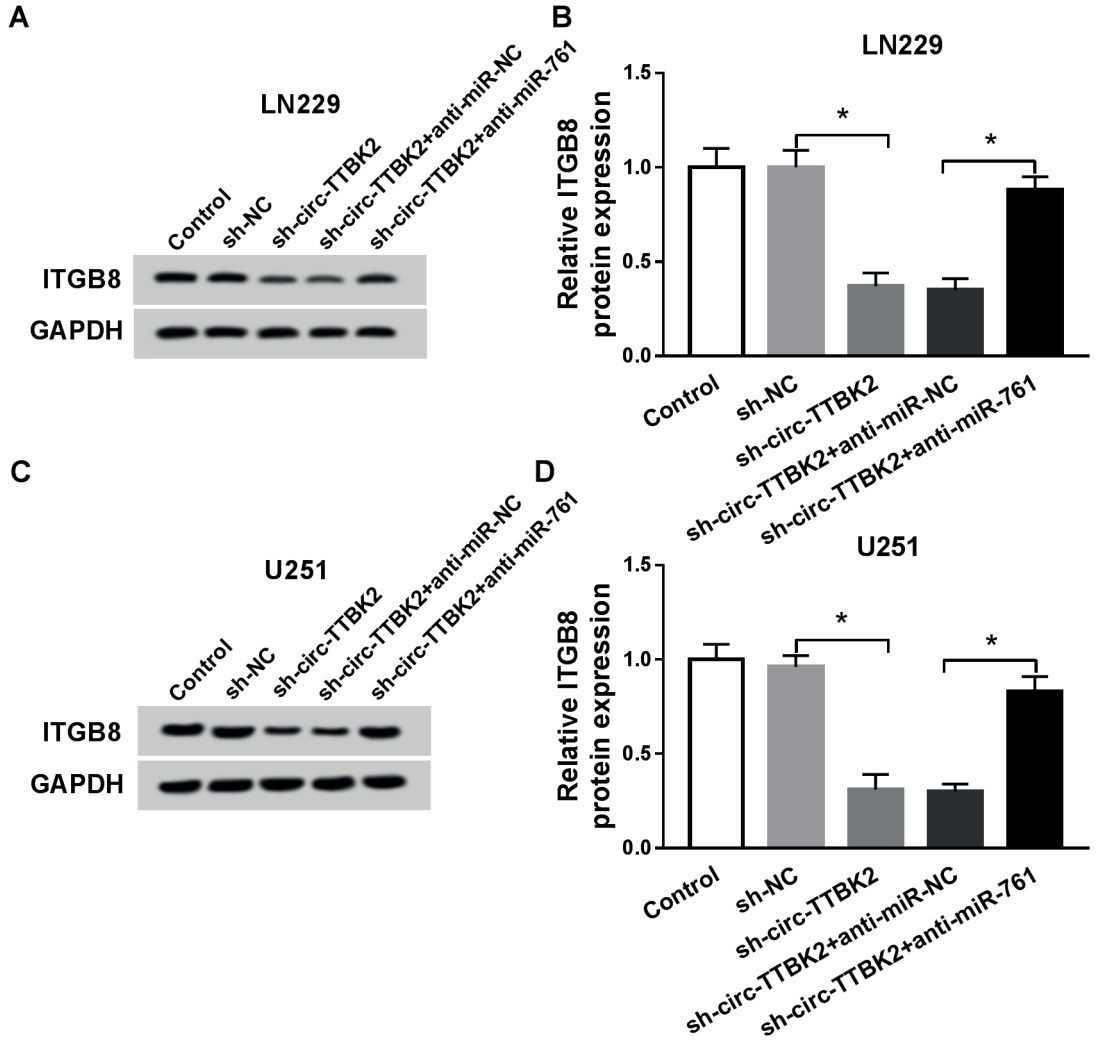

Figure 6. ITGB8 was co-regulated by circ-TTBK2 and ITGB8. **A,** and **B,** The protein level of mature ITGB8 was tested by adopting Western blot in LN229 and U251 cells under sh-circ-TTBK2 or anti-miR-761 supplement. *$p<0.05$.

lular metabolism[24,25]. Meanwhile, ferroptosis is a major mediator of p53 activity when tumor suppression occurs[34]. Intriguingly, ferroptosis could be induced by drugs, such artemisinin, siramesine, and lapatinib in some cancer cells with strong resistance to apoptosis, thereby inhibiting the growth of these cancer cells[35,36]. In this project, the knockdown of circ-TTBK2 could diminish cell proliferation, metastasis, and promote ferroptosis in LN229 and U251 cells. The results documented that circ-TTBK2 acted as an oncogene in glioma initiation and progression.

CircRNAs attract more attention and are discovered to function as competing endogenous RNAs (ceRNAs)[37]. Salzman[38] shows that the aberrant expression of circRNAs ubiquitously existed in heterogeneous tumors. Together with results from earlier missions, we further searched for the potential targets of circ-TTBK2. As predicted by the starBase v2.0, miR-761 was one of the possible targets of circ-TTBK2. Subsequent Dual-Luciferase reporter and qRT-PCR assays confirmed that circ-TTBK2 was a sponge of miR-761 and hindered the level of miR-761 in glioma cells. About miR-761, it acts as an opposite function in different tumors. MiR-761 could constrain the development of ovarian carcinoma[39] and enhance the sensitivity of 5-Fluorouracil in colorectal cancer cells[40]. On the contrary, miR-761 contributed to the aggressive phenotypes of non-small cell lung cancer cells, thereby promoting the progression of cancer[21]. This controversial gene was al-

so expounded in glioma, and the low expression of miR-761 hinted that it was a tumor suppressor in glioma. Further functional assays verified this conjecture which agreed with Li et al[19]. Besides, the influence of circ-TTBK2 deficiency on cell proliferation, invasion, and ferroptosis was reverted *via* co-transfection with miR-761 inhibitor. Collectively, miR-761, targeted by circ-TTBK2, suppressed the aggressive behaviors and boosted ferroptosis in glioma cells.

Next, we also researched the downstream targets of miR-761. Firstly, the prediction was performed using bioinformatics analysis, and the results exhibited that ITGB8 was one of the probable proteins of miR-761. The rescue assay indicated that miR-761 could directly target ITGB8, which was a member of the integrin (ITG) family. Based on the previous studies, ITGB8, a target of miR-19b-3p, could regulate tumor growth and metastasis in colorectal cancer[41]. In this research, we illustrated that ITGB8 was expressed at a high level in glioma tissues and cells. Also, the ectopic expression of ITGB8 functioned as an oncogene in glioma, which was in line with a previous report[42]. Finally, we also unraveled that ITGB8 was co-modified by circ-TTBK2 and miR-761 in glioma.

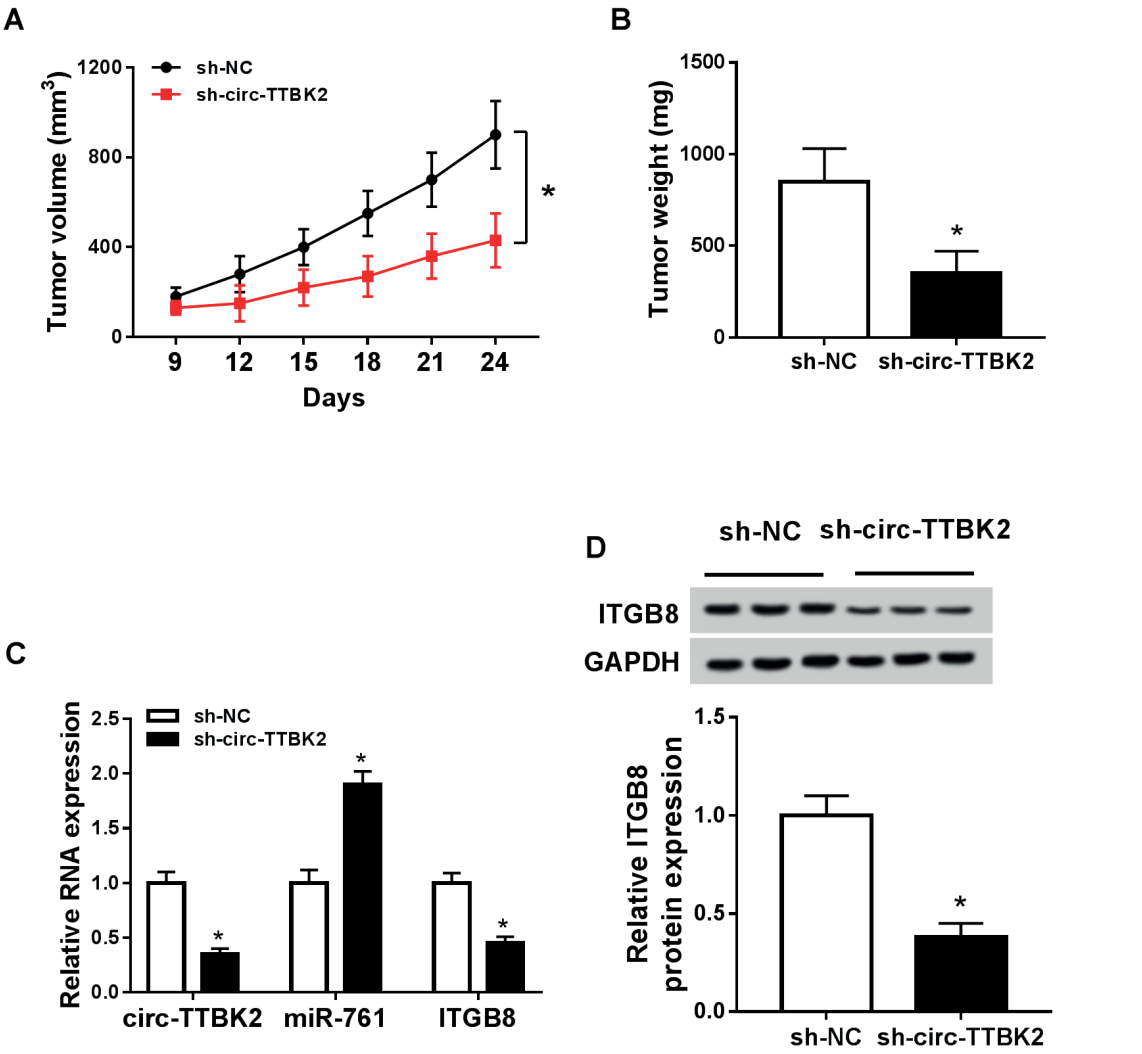

Figure 7. Circ-TTBK2 silencing diminished tumor growth in nude mice. Nude mice assay was shown, (**A**) the formation of tumor was monitored at indicated days after injection. **B,** Excised tumor weight was recorded and analyzed (n=6). **C,** Levels of circ-TTBK2, miR-761, and ITGB8 were determined by adopting qRT-PCR in xenograft tumors. **D,** Western blot assay was implemented to detect the protein level of ITGB8. *$p<0.05$.

Conclusions

Circ-TTBK2 and ITGB8 were expressed at high levels, whereas miR-761 was downregulated in glioma tissues and cells. The knockdown of circ-TTBK2 could weaken the aggressive phenotypes, including proliferation and invasion, and simultaneously improve ferroptosis in LN229 and U251 cells. More importantly, circ-TTBK2 exerted its oncogenic role *via* miR-761/ITGB8 axis *in vitro* and *in vivo* assays. However, other evidence needs to be discovered for providing circ-TTBK2 as a novel target in clinical therapy of human glioma.

Conflict of Interests

The authors declare that they have no conflict of interests.

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