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Sevoflurane impedes the progression of glioma through modulating the circular RNA has_circ_0012129/miR-761/TGIF2 axis

W. XU[1], R. XUE[2], R. XIA[1], W.-W. LIU[1], J.-W. ZHENG[1], L. TANG[1], L.-Y. KANG[1], W. WANG[1], W.-T. WEI[3]

[1]Department of Anesthesiology, The First People's Hospital of Jingzhou, The First Affiliated Hospital of Yangtze University, Jingzhou, Hubei, China
[2]Department of Anesthesiology, Renmin Hospital, Hubei University of Medicine, Shiyan, China
[3]Department of Clinical Laboratory, Jining No. 1 People's Hospital, Jining, China

Wei Xu and Rui Xue contributed equally to this work

Abstract. – OBJECTIVE: Glioma is a highly aggressive and lethal brain tumor. Anesthetics have been shown to have important effects on the biological characteristics of cancer cells. Nevertheless, the molecular mechanism of anesthetic-mediated glioma cells progression remains unclear.

MATERIALS AND METHODS: Sevoflurane (sev) was employed to treat glioma cells. The biological characteristics (viability, colony formation, apoptosis, cell cycle, migration, and invasion) of glioma cells were determined via Cell Counting Kit-8 (CCK-8), cell colony formation, flow cytometry, PI cytometry, or transwell assays. The protein levels of Cell Cycle Dependent Kinase (CDK) 2, CDK4, E-cadherin, N-cadherin, Vimentin, and Transforming Growth Factor Beta (TGFB) induced factor homeobox 2 (TGIF2) were assessed through Western blot analysis. Glucose consumption and lactate production were measured using special commercial kits. The expression of circular RNA has_circ_0012129 (circ_0012129) and miR-761 was detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between circ_0012129 or TGIF2 and miR-761 was verified with Dual-Luciferase reporter assay. Sevoflurane-mediated molecular mechanisms have been confirmed via xenograft assay.

RESULTS: Sevoflurane suppressed viability, colony formation, cell cycle, migration, and invasion and promoted apoptosis of glioma cells *in vitro*, and impeded tumor growth *in vivo*. Circ_0012129 and TGIF2 were downregulated and miR-761 was upregulated in sevoflurane-treated glioma cells. Circ_0012129 elevation abolished sevoflurane-mediated biological characteristics of glioma cells. MiR-761 served as target for circ_0012129 and miR-761 targeted TGIF2. Moreover, both miR-761 overexpression and TGIF2 suppression restored circ_0012129 enhancement-mediated biological characteristics of sevoflurane-treated glioma cells.

CONCLUSIONS: Sevoflurane mediated the progression of glioma *via* regulating the circ_0012129/miR-761/TGIF2 axis.

Key Words:
Sevoflurane, Glioma, Circ_0012129, MiR-761, TGIF2.

Introduction

Glioma is a highly invasive and fatal brain tumor that originates in glial cells[1]. At present, surgery combined with postoperative radiotherapy and thermotherapy is the most effective method for its treatment[2]. However, the prognosis of patients with glioma is extremely poor due to the high recurrence rate, and its 5-year survival rate is less than 5%[3]. Snyder and Greenberg[4] have shown that anesthetics could affect the invasion and migration of tumors cells, and even affect the long-term survival of patients. Sevoflurane is a volatile anesthetic commonly used in craniocerebral surgery. Also, sevoflurane has been revealed to affect cell proliferation and invasion in glioma[5,6]. Nevertheless, the mechanism through which sevoflurane affects the biological characteristics of glioma cells remains unclear.

Circular RNAs (circRNAs) are a type of non-coding RNA that forms a closed circular structure by covalent attachment[7]. They have been elucidated to be involved in the regu-

Corresponding Author: Wenting Wei, MD; e-mail: wwt198908@163.com

lation of transcription and alternative splicing, acting as microRNA (miRNA) sponges, and interacting with RNA-binging proteins[8,9]. Liu et al[10] manifested that circRNA was involved in the progression of many diseases, including glioma. Circular RNA NFIX accelerated the progression of glioma[11]. CircRNA PTN facilitated cell stemness and proliferation in glioma[12]. Circular RNA hsa_circ_0012129 (circ_0012129) originated from chromosomal region 1p34.1. Moreover, circ_0012129 was uncovered to accelerate the invasion and proliferation of glioma cells[13]. However, it is unclear whether circ_0012129 is connected with sevoflurane-mediated biological characteristics of glioma cells.

MicroRNAs (miRNAs) are a class of small endogenous RNAs that primarily regulate gene expression at the post-transcriptional level[14]. They are involved in many aspects of cancer biology, such as invasion, metastasis, apoptosis, proliferation, and angiogenesis[15]. MicroRNA-761 (miR-761) was revealed to play different roles in different tumors. Guo et al[16] demonstrated that miR-761 induced an invasive phenotype in triple negative breast cancer cells. Also, miR-761 could repress cell invasion and proliferation in colorectal cancer cells[17]. Furthermore, it was reported that miR-761 was associated with the migration and proliferation of glioma cells[18]. Currently, whether miR-761 is involved in sevoflurane-mediated biological characteristics of glioma cells has not been reported.

Transforming Growth Factor Beta (TGFB) induced factor homeobox 2 (TGIF2) is a transcriptional corepressor for TGFB receptor activated Smads[19]. It was reported that TGIF2 was implicated in the development of diverse tumors. In particular, TGIF2 repressed the progression of prostate cancer by controlling Smad pathway and epithelial-mesenchymal transition (EMT) process[20]. In addition, TGIF2 was revealed to promote the development of glioma[21]. To date, the molecular mechanisms associated with TGIF2 in sevoflurane-mediated biological characteristics of glioma cells have rarely been reported.

As consequence, the influence of sevoflurane on the biological characteristics of glioma cells was probed. Moreover, the role of circ_0012129 on sevoflurane-mediated biological characteristics of glioma cells was explored. Additionally, the mechanism of the has_circ_0012129/miR-761/TGIF2 axis in sevoflurane-treated glioma cells was investigated.

Materials and Methods

Subjects

The research was delegated by the Ethics Committee of The First People's Hospital of Jingzhou, The First Affiliated Hospital of Yangtze University. Brain tissue samples from 35 glioma patients and 35 normal brain tissues samples (from healthy individuals who died in traffic accidents) were acquired from The First People's Hospital of Jingzhou, The First Affiliated Hospital of Yangtze University. All glioma patients in this study did not receive radiation or chemotherapy prior to surgery. Informed consent was signed by the subject or the family of the subject.

Cell Culture and Treatment

Human glioma cells T98G and LN229 were acquired from the American Type Culture Collection (Manassas, VA, USA). Human astrocytes NHA were purchased from BeNa Culture collection (Jiangsu, Suzhou, China). Dulbecco's Modified Eagle's Medium (DMEM; Biochrom, Berlin, Germany) was applied to maintain T98G and NHA cells. Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, St. Louis, MO, USA) was employed to culture LN229 cells. Additionally, fetal bovine serum (FBS, 10%) (Sigma-Aldrich), streptomycin (100 microg/mL, Sigma-Aldrich, St. Louis, MO, USA), and penicillin (100 U/mL, Sigma-Aldrich, St. Louis, MO, USA) were supplemented into DMEM and EMEM to keep T98G, NHA, and LN229 cells. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. For sevoflurane treatment, T98G and LN229 cells at the exponential growth phase were seeded into 6-well plates and maintained for 24 h. Next, the cell plates were transferred to a sealed glass chamber which was connected to an anesthesia machine. An anesthesia vaporizer (Bisen, Guangzhou, China) was utilized to send sevoflurane to the glass chamber. The concentration of sevoflurane was monitored through a gas analyzer (Puritan-Bennett, Tewksbury, MA, USA). T98G and LN229 cells were treated in different concentrations (1.7%, 3.4%, and 5.1%) of sevoflurane for 6 h prior to incubation under normal conditions for 24 h before analysis.

Cell Transfection

The lentivirus-mediated circ_0012129 overexpression vector (circ_0012129) or negative control vector (vector) was amplified and cloned GenePharma (Shanghai, China). MiR-761 mimic and

inhibitor (miR-761 and anti-miR-761) and their negative control (miR-NC and anti-miR-NC) were purchased from GenePharma (Shanghai, China). Small interference RNA targeting circ_0012129 (si-circ_0012129) and si-TGIF2 and their negative control (si-NC) were procured from GenePharma (Shanghai, China). Oligonucleotides or vectors were transfected into T98G and LN229 cells through Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA).

Cell Viability Assay

The viability of treated T98G and LN229 cells was determined by Cell Counting Kit-8 (CCK-8) assay. In brief, the transfected or untransfected T98G and LN229 cells were treated with different concentrations (1.7%, 3.4%, and 5.1%) of sevoflurane for 6 h. After culturing for 24 h in cell medium, CCK-8 reagent (10 μL, Dojindo Molecular Technologies, Kumamoto, Japan) was added and incubated for 2 h. At last, a Microplate Absorbance Reader (Thermo Fisher Scientific, Waltham, MA, USA) was performed for the measurement of the color reaction at 450 nm.

Cell Colony Formation Assay

Treated T98G and LN229 cells were seeded in the cell culture dish and maintained at 37°C in an incubator with 5% CO_2 for 9 days, and the medium was changed every 3 days. As follows, ethanol (75%) was added to fix the cells for 2 h and stained with crystal violet (0.2%, KeyGen, Jiangsu, China) for 2 h. The colonies (> 50 cells/colony) were counted and photographed *via* the light microscope (Olympus, Tokyo, Japan).

Flow Cytometry Assay

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) was applied to evaluate the apoptosis rate of treated T98G and LN229 cells. Briefly, the transfected or untransfected T98G and LN229 cells were treated with different concentrations of sevoflurane for 6 h, and then, were maintained in cell medium for 48 h. After washing, treated T98G and LN229 cells (1×10^5) were resuspended in binding buffer. Following this, Annexin V-FITC (5 μL) and PI (10 μL) were replenished into the binding buffer and incubated for 15 min in the dark. The FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was executed to determine the apoptosis rate of treated T98G and LN229 cells.

PI Cytometry Assay

After treatment with sevoflurane for 6 h, the transfected or untransfected T98G and LN229 cells were cultured for 24 h. Then, ethanol (70%) was added and incubated at 4°C for overnight. Next, the PI/RNase Staining Buffer (BD Biosciences, Franklin Lakes, NJ, USA) was supplemented to stain the cells. The cell cycle was assessed with FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Analysis

Glioma tissues and normal brain tissues, as well as treated T98G and LN229 cells were lysed *via* radio-immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), and the Western blot analysis was executed as previously described[22]. The Immobilon TM Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was applied to visualize the protein bands. The primary antibodies were displayed as follows: rabbit-anti-Cell Cycle Dependent Kinase (CDK) 2 (ab32147, 1:1000, Abcam, Cambridge, MA, USA), rabbit-anti-CDK4 (ab199728, 1:2000, Abcam, Cambridge, MA, USA), rabbit-anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab181602, 1:10000, Abcam, Cambridge, MA, USA), rabbit-anti-E-cadherin (ab40772, 1:10000, Abcam, Cambridge, MA, USA), rabbit-anti-N-cadherin (ab76011, 1:5000, Abcam, Cambridge, MA, USA), rabbit-anti-Vimentin (ab92547, 1:1000, Abcam), and rabbit-anti-TGIF2 (ab155948, 1:1000, Abcam, Cambridge, MA, USA). GAPDH was regarded as a loading control.

Transwell Assay

The migration of treated T98G and LN229 cells was evaluated with the transwell chamber (8 μm, Corning Costar, Corning, NY, USA). In brief, DMEM or EMEM with FBS (10%) was replenished into the lower chamber and treated T98G and LN229 cells (5×10^4) were supplemented into the upper chamber with serum-free media. After culturing for 48 h, the cells on the upper layer of the membrane were removed with a cotton swab. Afterward, the cells on the lower layer of the membrane were fixed with methanol (100%) and stained with 0.1% crystal violet. A light microscope (Olympus, Tokyo, Japan) was applied to count the migrated cells. The invasion of treated T98G and LN229 cells was assessed *via* the same procedure with the migration assay, but the upper chamber coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA).

Measurement of Glucose Consumption and Lactate Production

After culture for 24 h, the supernatants of culture media of treated T98G and LN229 cells were collected. Glucose levels and lactate production were measured using either the Glucose Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) or the Lactic Acid Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' instruction.

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

Total RNA of glioma tissues and normal brain tissues as well as treated T98G and LN229 cells was extracted with the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). PrimeScript RT reagent Kit (TaKaRa, Dalian, China) or MiRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to the synthesis of the first-strand complementary DNA for circ_0012129, TGIF2, and miR-761, respectively. SYBR Premix Ex Taq (TaKaRa, Dalian, China) was applied to perform the qRT-PCR. The $2^{-\Delta\Delta Ct}$ method was used to figure the levels of circ_0012129, TGIF2, and miR-761, and GAPDH or U6 small nuclear RNA (snRNA) was used as an internal control. The primers were exhibited as follows: circ_0012129 (F: 5'-GATGACCGCAACCTATACCG-3', R: 5'-AAGTAGAGGATGACGGCCAC-3'), TGIF2 (F: 5'-TCTCTGTGTTGCCTCCCTCT-3', R: 5'-CCACCTCAGCCCAATACACT-3'), GAPDH (F: 5'-GACTCCACTCACGGCAAATTCA-3' and R: 5'-TCGCTCCTGGAAGATGGTGAT-3'), miR-761 (F: 5'-ACAGCAGGCACAGA-3', R: 5'-GAGCAGGCTGGAGAA-3'), U6 snRNA (F: 5'-GCTCGCTTCGGCAGCACA-3', R: 5'-GAGGTATTCGCACCAGAGGA-3').

Dual-Luciferase Reporter Assay

The starBase 2.0 was utilized to predict the possible binding sites between circ_0012129 or TGIF2 and miR-761. The fragments of wild type circ_0012129 or the 3' Untranslated Regions (UTR) of TGIF2 (with predicted miR-761 binding sites) and mutant circ_0012129 or 3'UTR of TGIF2 were amplified and cloned into the pGL3-control vector (Promega, Madison, WI, USA) to construct the Luciferase reporter vectors circ_0012129 WT and circ_0012129 MUT or TGIF2 3'UTR WT and TGIF2 3'UTR MUT. T98G and LN229 cells were co-transfected with the Luciferase reporter vectors and miR-761 or miR-NC by using Lipofectamine 3000 reagent. After transfection for 48 h, the Luciferase activities of Luciferase reporter vectors in T98G and LN229 cells were evaluated through the Luciferase reporter assay kit (Promega, Madison, WI, USA).

Xenograft Assay

The animal experiment of this study was approved by the Ethics Committee of The First People's Hospital of Jingzhou, The First Affiliated Hospital of Yangtze University. 8 BALB/c nude mice (4-6 weeks old) were purchased from Shanghai Experimental Animal Center (Shanghai, China) in order to establish the mouse xenograft model. Briefly, circ_0012129 or vector was transfected into T98G cells. After treatment with sevoflurane for 6 h, transfected T98G cells were cultivated for 24 h, and then, digested by trypsin (0.25%). Afterward, transfected T98G cells (4×10^5) were subcutaneously injected into the dorsal side of nude mice (4 mice/group). The tumor volume was measured using a digital caliper every 7 days and calculated through the equation: Volume = (length $\times$width^2)/2. After injection at 35 days, the mice were euthanized to take tumor tissue to assess the weight of the tumor tissue and conduct subsequent studies.

Statistical Analysis

The Statistical Product and Service Solution (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) were employed for statistical analysis. The experiments in this study were repeated 3 times. The data in this study were displayed as mean ± standard deviation (SD). The differences between the two groups were assessed through Student's *t* test and one-way variance analysis (ANOVA) was applied to compare the differences among more groups. $p < 0.05$ was considered statistically significant.

Results

Sevoflurane Impeded Viability, Colony Formation, Cell Cycle, Migration, and Invasion and Promoted Apoptosis of Glioma Cells

In the first place, we examined the viability of glioma cells treated with different concen-

trations of sevoflurane (1.7%, 3.4%, and 5.1%) through CCK-8 assay. Compared to the control group, the viability of T98G and LN229 cells was specially repressed with the elevation of sevoflurane (Figure 1A and 1B). After that, the influence of different concentrations of sevoflurane on colony formation capacity of glioma cells was probed. Colony formation assay manifested that sevoflurane evidently impeded cell colony formation ability in T98G and LN229 cells in a concentration-dependent manner in comparison to the control group (Figure 1C and 1D). Afterward, we probed into the impacts of different concentrations of sevoflurane on

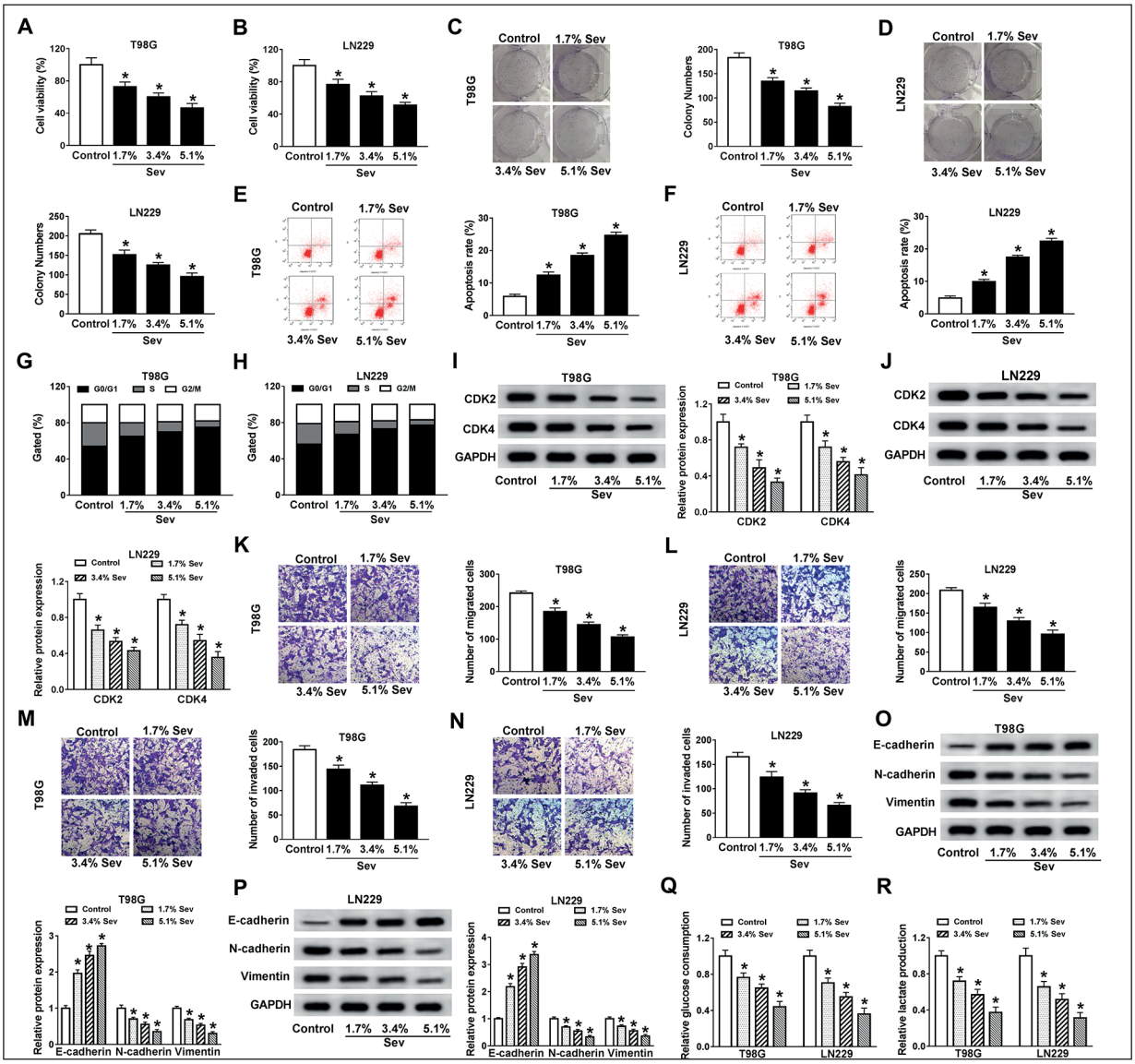

Figure 1. Effects of sevoflurane on cell biological characteristics in glioma cells. **A-R,** T98G and LN229 cells treated with different concentrations of sevoflurane (1.7%, 3.4%, and 5.1%). **A,** and **B,** Effect of sevoflurane on viability of T98G and LN229 cells was determined by CCK-8 assay. **C,** and **D,** Impact of sevoflurane on colony formation ability of T98G and LN229 cells was evaluated *via* colony formation assay. **E,** and **F,** Influence of sevoflurane on apoptosis of T98G and LN229 cells was detected with flow cytometry assay. **G,** and **H,** PI single staining assay was performed to assess the effect of sevoflurane on cell cycle in T98G and LN229 cells. **I,** and **J,** Western blot analysis was executed to examine the protein levels of CDK2 and CDK4 in sevoflurane treated T98G and LN229 cells. **K-N,** The effects of sevoflurane on cell migration and invasion of T98G and LN229 cells were detected by transwell assay (100×). **O,** and **P,** The protein levels of E-cadherin, N-cadherin, and Vimentin in sevoflurane-treated T98G and LN229 cells were examined *via* Western blot analysis. **Q,** and **R,** The levels of glucose consumption and lactate production of sevoflurane treated T98G and LN229 cells were detected by special commercial kits. $*p < 0.05$.

apoptosis and cell cycle of glioma cells. The results of flow cytometry assay exhibited that the apoptosis rate of T98G and LN229 cells in 1.7% sevoflurane, 3.4% sevoflurane, and 5.1% sevoflurane were strikingly increased (Figure 1E and 1F). PI single staining assay presented an apparent cell cycle arrest at the G0/G1 phase in T98G and LN229 cells with increasing of sevoflurane (Figure 1G and 1H). Western blot analysis exhibited that the protein levels of CDK2 and CDK4 in sevoflurane-treated T98G and LN229 cells were remarkably decreased in a concentration-dependent manner (Figure 1I and 1J). Furthermore, the effects of different concentrations of sevoflurane on cell migration and invasion in T98G and LN229 cells were assessed through transwell assay. We discovered that the migration and invasion abilities of sevoflurane-treated T98G and LN229 cells were dramatically curbed in a concentration-dependent manner (Figure 1K-1N). We also detected the expression of epithelial-mesenchymal transition marker proteins E-cadherin, N-cadherin, and Vimentin in sevoflurane-treated T98G and LN229 cells. The results demonstrated that as concentration of sevoflurane increasing, the protein levels of N-cadherin and Vimentin were gradually declined, while E-cadherin protein level was progressively enhanced in T98G and LN229 cells (Figure 1O and 1P). Additionally, the glucose consumption and lactate production of T98G and LN229 cells were lower with the increase of sevoflurane concentration (Figure 1Q and 1R). These data revealed that sevoflurane curbed viability, colony formation, cell cycle, migration, and invasion and accelerated apoptosis of glioma cells.

Sevoflurane Downregulated the Expression of Circ_0012129 in Glioma Cells

To probe into the possible role of circ_0012129 in glioma, the expression of circ_0012129 in 30 glioma tissues and 30 normal brain tissues was examined through qRT-PCR. We observed that circ_0012129 was robustly upregulated in glioma tissues compared to the normal brain tissues (Figure 2A). Moreover, the expression of circ_0012129 in T98G and LN229 cells was aberrantly enhanced compared to the NHA cells and the trend was consistent with that in glioma tissues (Figure 2B). Subsequently, the impact of different concentrations of sevoflurane on the expression of circ_0012129 of glioma cells was analyzed *via* qRT-PCR. In comparison to the control group, the expression of circ_0012129 was conspicuously downregulated in sevoflurane-treated T98G and LN229 cells in a concentration-dependent manner (Figure 2C and D). These results indicated that the inhibition of the biological characteristics of glioma cells by sevoflurane might be related to the reduction of circ_0012129 caused by it.

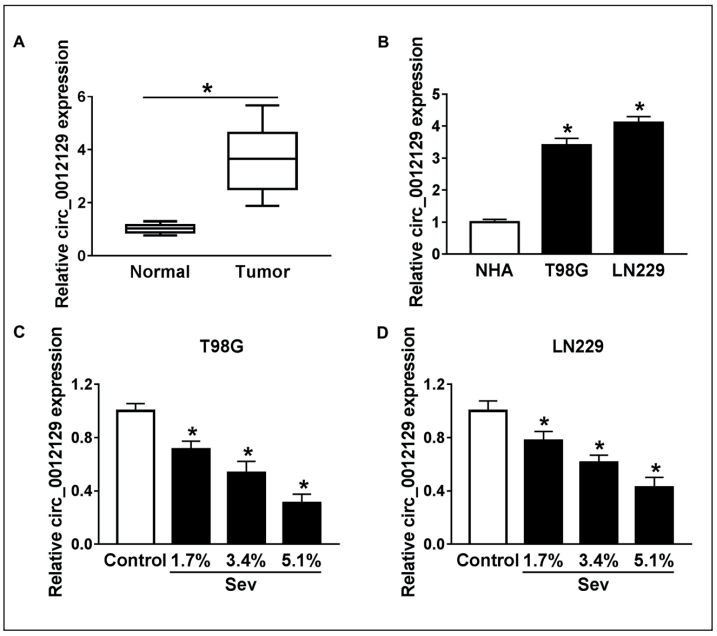

Figure 2. Effect of sevoflurane on circ_0012129 expression of glioma cells. **A,** and **B,** QRT-PCR was executed to assess the expression of circ_0012129 in 30 glioma tissues, 30 normal brain tissues, glioma cells (T98G and LN229 cells), and NHA cells. **C,** and **D,** The effect of different concentrations of sevoflurane on the expression of circ_0012129 of glioma cells was determined by qRT-PCR. *$p < 0.05$.

Circ_0012129 Elevation Recovered Sevoflurane-Mediated the Biological Characteristics of Glioma Cells

Subsequently, we selected glioma cells treated with sevoflurane (5.1%) for 6 h for further studies according to the above results. In the beginning, T98G and LN229 cells were transfected with circ_0012129 or vector and were treated with sevoflurane. The results of qRT-PCR displayed that the reduction of circ_0012129 in T98G and LN229 cells caused by sevoflurane was restored by the introduction of circ_0012129 compared to the vector group (Figure 3A and 3B). After that, the impact of circ_0012129 overexpression on sevoflurane-mediated viability, colony formation, apoptosis, cell cycle, migration, and invasion of glioma cells was further assessed. CCK-8 and colony formation assays disclosed that circ_0012129 upregulation abolished the repression of viability and colony formation ability

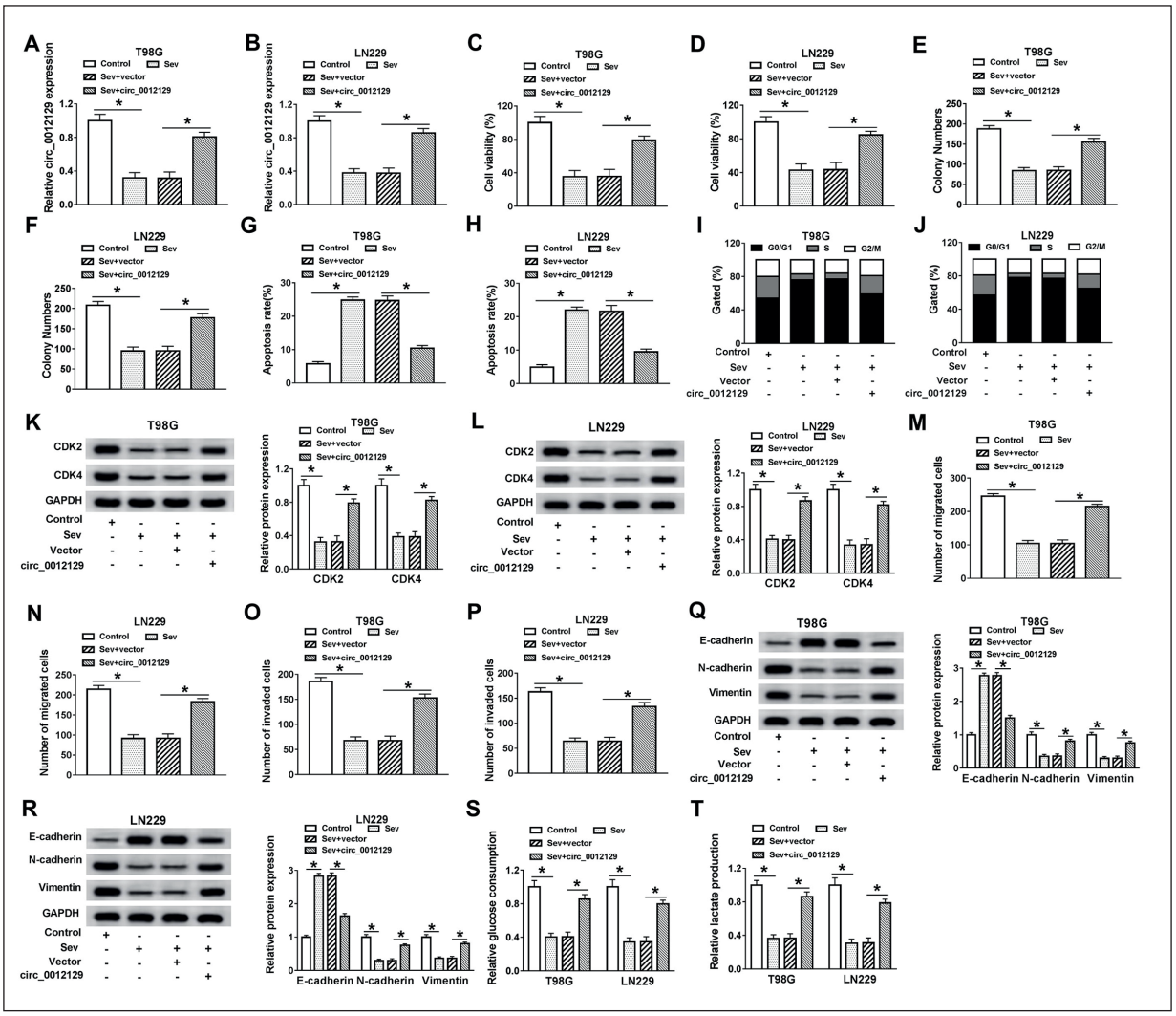

Figure 3. Effects of circ_0012129 enhancement on sevoflurane-mediated the biological characteristics of glioma cells. **A-T,** T98G and LN229 cells were transfected with circ_0012129 or vector and induced with sevoflurane. **A,** and **B,** QRT-PCR was executed to measure the expression of circ_0012129 in T98G and LN229 cells. **C-F,** Effects of circ_0012129 enhancement on sevoflurane-mediated cell viability and colony formation ability of T98G and LN229 cells were determined by CCK-8 or colony formation assays. **G,** and **H,** Flow cytometry assay was performed to assess the influence of circ_0012129 upregulation on sevoflurane-mediated apoptosis of T98G and LN229 cells. **I,** and **J,** Impact of circ_0012129 elevation on sevoflurane-mediated cell cycle was evaluated through PI single staining assay. **K,** and **L,** Western blot analysis was utilized to detect the protein levels of CDK2 and CDK4 in T98G and LN229 cells. **M-P,** Influence of circ_0012129 augmentation on sevoflurane-mediated migration and invasion was analyzed through transwell assay. **Q,** and **R,** Western blot analysis was carried out for the assessment of the expression of E-cadherin, N-cadherin, and Vimentin protein levels in T98G and LN229 cells. **S,** and **T,** Glucose consumption and lactate production of T98G and LN229 cells were measured using special commercial kits. *$p < 0.05$.

of T98G and LN229 cells caused by sevoflurane (Figure 3C-3F). Flow cytometry assay showed that the facilitation of apoptosis of T98G and LN229 cells induced by sevoflurane was abrogated by circ_0012129 elevation (Figure 3G and 3H). The arrest of cell cycle at the G0/G1 phase in T98G and LN229 cells caused by sevoflurane was recovered by the introduction of circ_0012129 (Figure 3I and 3J). Also, elevated circ_0012129 expression overturned the repressive effect of sevoflurane on the protein levels of CDK2 and CDK4 (Figure 3K and 3L). Besides, the inhibitory effect of sevoflurane on migration and invasion of T98G and LN229 cells was reversed by the enhancement of circ_0012129 (Figure 3M-3P). Moreover, both the upregulation of E-cadherin protein and the downregulation of N-cadherin and Vimentin proteins in sevoflurane-treated T98G and LN229 cells were recuperated by the upregulation of circ_0012129 (Figure 3Q and 3R). Furthermore, enhanced circ_0012129 expression recovered the inhibition of the glucose consumption and lactate production of T98G and LN229 cells caused by sevoflurane (Figure 3S and 3T). In sum, these data indicated that the overexpression of circ_0012129 could restore the repressive effect of sevoflurane on the biological characteristics of glioma cells.

MiR-761 Acted as a Target for Circ_0012129 in Glioma Cells

To further clarify how sevoflurane played its function *via* circ_0012129 in glioma cells, we applied the starBase v2.0 to predict the possible targets for circ_0012129. We uncovered that miR-761 possessed the possible binding sites for circ_0012129, as exhibited in Figure 4A. To verify the underlying binding sites between miR-761 and circ_0012129, we employed the pGL3-control vector to construct the Luciferase reporter vectors circ_0012129 WT and circ_0012129 MUT. The results of the Dual-Luciferase reporter assay manifested that miR-761 elevation effectively reduced the Luciferase intensity controlled by circ_0012129 WT in comparison to the miR-NC group in T98G and LN229 cells, while miR-761 upregulation did not affect the Luciferase intensity of the reporter vector circ_0012129 MUT in T98G and LN229 cells (Figure 4B and 4C). Furthermore, reduced circ_0012129 expression augmented miR-761 expression level in T98G and LN229 cells than that in the si-NC group (Figure 4D). Besides, qRT-PCR revealed that a distinct downregulation of miR-761 was observed in glioma tissues and cells (T98G and LN229) in contrast to the normal brain tissues or NHA cells (Figure 4E and 4F). In addition, we observed an evident acceleration of miR-761 in sevoflurane-treated T98G and LN229 cells in a concentration-dependent manner (Figure 4G and 4H). Taken together, these findings demonstrated that miR-761 was negatively regulated by circ_0012129 in glioma cells.

MiR-761 Overexpression Reversed Circ_0012129 Enhancement-Mediated Biological Characteristics in Sevoflurane-Treated Glioma Cells

In view of the above findings, we further explored whether circ_0012129 affected sevo-

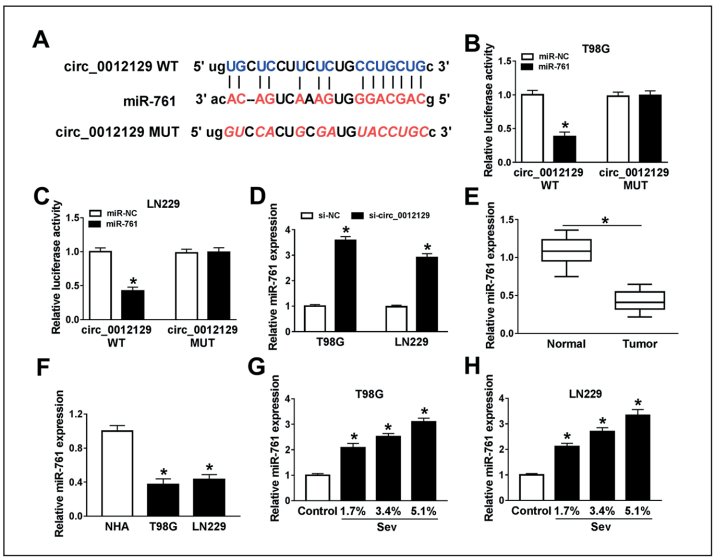

Figure 4. MiR-761 was a target of circ_0012129 in glioma cells. **A,** The binging sites of circ_0012129 in miR-761 were predicted via starBase v2.0 **B,** and **C,** Effect of miR-761 or miR-NC on the Luciferase intensity of the Luciferase reporter vectors circ_0012129 WT and circ_0012129 MUT in T98G and LN229 cells was assessed through Dual-Luciferase reporter assay. **D,** Effect of circ_0012129 inhibition on miR-761 expression level was analyzed by qRT-PCR. **E,** and **F,** The expression of miR-761 in glioma tissues and normal brain tissues as well as glioma cells (T98G and LN229) and NHA cells was examined by qRT-PCR. **G,** and **H,** Impact of different concentrations of sevoflurane on miR-761 expression level of glioma cells was evaluated with qRT-PCR. *$p < 0.05$.

flurane-mediated the biological characteristics of glioma cells *via* miR-761. We found that circ_0012129 augmentation restored the upregulation of miR-761 of T98G and LN229 cells induced by sevoflurane, while this effect was partly abolished by the introduction of miR-761 (Figure 5A and 5B). Afterward, CCK-8 and colony formation assays exhibited that the elevation of viability and colony formation capacity of sevoflurane-treated T98G and LN229 cells induced by circ_0012129 elevation were overturned by miR-761 upregulation (Figure 5C-5F). The sup-

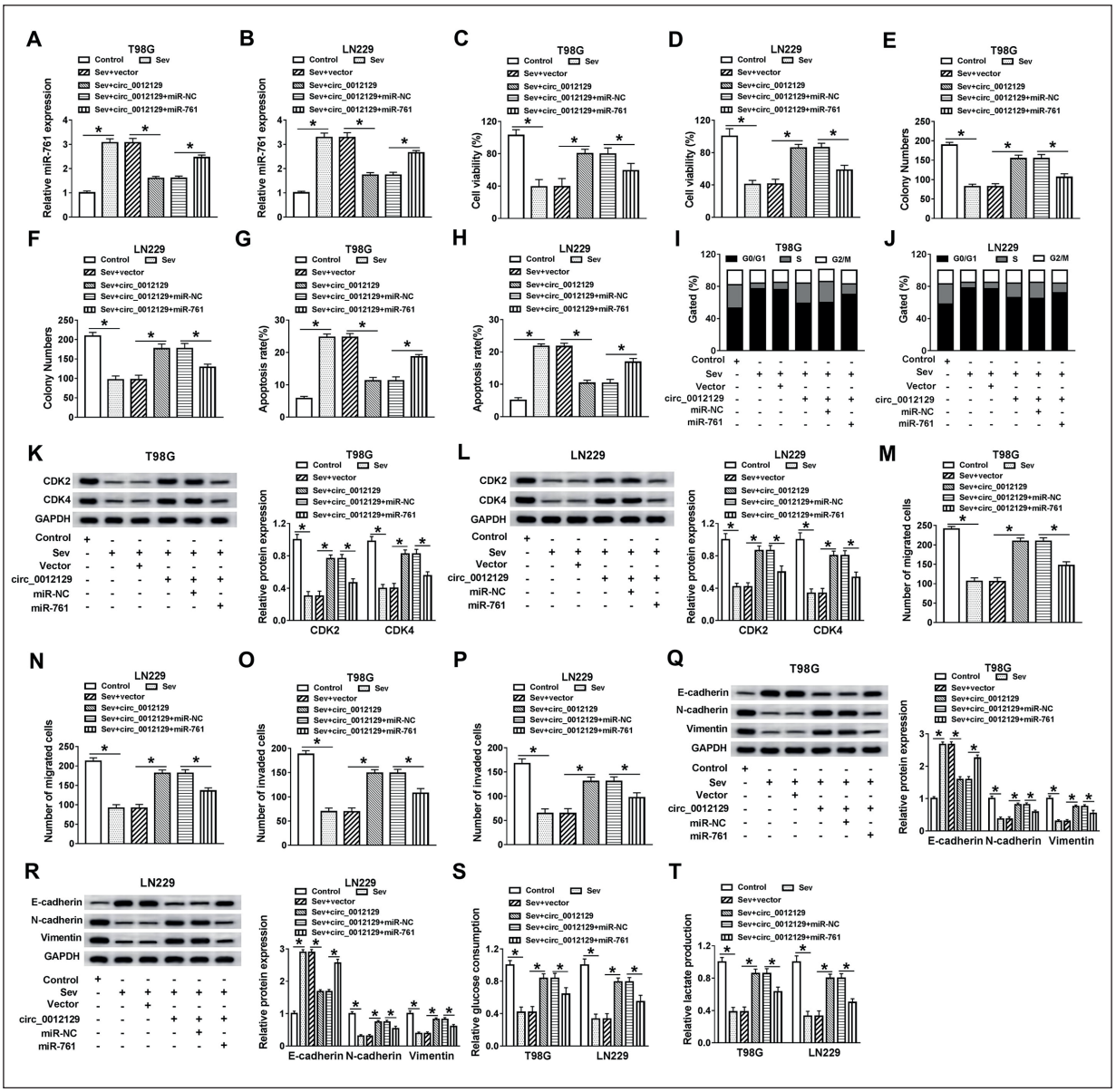

Figure 5. Effects of miR-761 upregulation on circ_0012129 enhancement-mediated biological characteristics of sevoflurane-treated glioma cells. **A-T,** T98G and LN229 cells were transfected with circ_0012129, vector, circ_0012129+miR-761, or circ_0012129+miR-NC and induced with sevoflurane. **A,** and **B,** The expression of miR-761 in T98G and LN229 cells was detected through qRT-PCR. **C-F,** CCK-8 and colony formation assays were carried out to assess the viability and colony formation ability of T98G and LN229 cells. **G,** and **H,** Flow cytometry assay was executed for analysis of the apoptosis rate of T98G and LN229 cells. **I,** and **J,** PI single staining assay was used to analyze the cell cycle of T98G and LN229 cells. **K,** and **L,** Protein levels of CDK2 and CDK4 in T98G and LN229 cells were evaluated *via* Western blot analysis. **M-P,** The migration and invasion capacities of T98G and LN229 cells were assessed through transwell assay. **Q,** and **R,** Protein levels of E-cadherin, N-cadherin, and Vimentin in T98G and LN229 cells were detected by Western blot analysis. **S,** and **T,** The levels of glucose consumption and lactate production of T98G and LN229 cells were examined with special commercial kits. *$p < 0.05$.

pressive effect of circ_0012129 introduction on apoptosis of sevoflurane-treated T98G and LN229 cells was abolished by the elevation of miR-761 (Figure 5G and 5H). Besides, the restoration of cell cycle arrest of sevoflurane-treated T98G and LN229 cells caused by circ_0012129 overexpression was overturned by miR-761 augmentation (Figure 5I and 5J). The elevation of CDK2 and CDK4 protein levels of sevoflurane-treated T98G and LN229 cells induced by elevating of circ_0012129 were recuperated by miR-761 upregulation (Figure 5K and 5L). Additionally, upregulated miR-761 expression reversed the promotive effect of circ_0012129 enhancement on migration and invasion of sevoflurane-treated T98G and LN229 cells (Figure 5M-5P). Also, augmented miR-761 expression restored both the downregulation of E-cadherin protein and the upregulation of N-cadherin and Vimentin of sevoflurane-treated T98G and LN229 cells caused by enhancing circ_0012129 (Figure 5Q and 5R). Moreover, the accelerative effects of circ_0012129 overexpression on glucose consumption and lactate production of sevoflurane-treated T98G and LN229 cells were abolished by upregulating miR-761 (Figure 5S and 5T). Therefore, these findings demonstrated that circ_0012129 affected sevoflurane-mediated the biological characteristics of glioma cells through miR-761.

TGIF2 Was a Target of MiR-761 in Glioma Cells

To gain insight into the molecular mechanisms by which circ_0012129 affected the sevoflurane-mediated biological characteristics of glioma cells, we further explored the possible targets of miR-761. We found that TGIF2 might be a target of miR-761, as exhibited in Figure 6A. Also, Dual-Luciferase reporter assay indicated that the Luciferase activity of TGIF2 3'UTR WT was attenuated in T98G and LN229 cells transfected with miR-761 in contrast to the miR-NC group, while there was no striking difference in the Luciferase activity of TGIF2 3'UTR MUT (Figure 6B and 6C). The protein level of TGIF2 in T98G and LN229 cells transfected with anti-miR-761 was conspicuously enhanced compared to the anti-miR-NC group (Figure 6D). Furthermore, TGIF2 was upregulated in glioma tissues and cells compared to the control group (Figure 6E and 6F). Moreover, the protein level of TGIF2 in sevoflurane-treated T98G and LN229 cells was markedly declined in a concentration-dependent manner (Figure 6G and 6H). In addition, inhibition of circ_0012129 reduced the expression of TGIF2 protein, while this reduction was reversed by miR-761 silencing (Figure 6I and 6J). Together, these data disclosed that circ_0012129 regulated the expression of TGIF2 *via* miR-761 in glioma cells.

TGIF2 Silencing Recuperated the Promotive effect of Circ_0012129 Upregulation on Biological Characteristics of Sevoflurane-Treated Glioma Cells

Since TGIF2 was a target of miR-761 in glioma cells, we further concluded that circ_0012129 affected the sevoflurane-mediated biological characteristics of glioma cells *via* TGIF2. We observed that the upregulation of TGIF2 protein in sevoflurane-treated T98G and LN229 cells induced by circ_0012129 overexpression was reversed by the introduction of si-TGIF2 (Figure 7A and 7B). Moreover, inhibition of TGIF2 abolished the promotive effect of circ_0012129 elevation on viability and colony formation ability of sevoflurane-treated T98G and LN229 cells (Figure 7C-7F). Also, the suppressive effect of circ_0012129 upregulation on apoptosis of sevoflurane treated T98G and LN229 cells was restored by the knockdown of TGIF2 (Figure 7G and 7H). Besides, TGIF2 suppression restored the facilitation of cell cycle of sevoflurane treated T98G and LN229 cells induced by circ_0012129 upregulation (Figure 7I and 7J). Moreover, the inhibited TGIF2 expression abrogated the augmentation of CDK2 and CDK4 of sevoflurane treated T98G and LN229 cells induced by circ_0012129 introduction (Figure 7K and 7L). Besides, TGIF2 repression overturned circ_0012129 enhancement-mediated migration and invasion of sevoflurane-treated T98G and LN229 cells (Figure 7M-7P). The protein levels of E-cadherin, N-cadherin, and Vimentin of sevoflurane-treated T98G and LN229 cells mediated by circ_0012129 introduction were recovered by TGIF2 depletion (Figure 7Q and 7R). Additionally, TGIF2 silencing recuperated the elevation of glucose consumption and lactate production of sevoflurane treated T98G and LN229 cells mediated by circ_0012129 overexpression (Figure 7S and 7T). Therefore, these findings revealed that circ_0012129 modulated the biological characteristics of glioma cells mediated by sevoflurane through TGIF2.

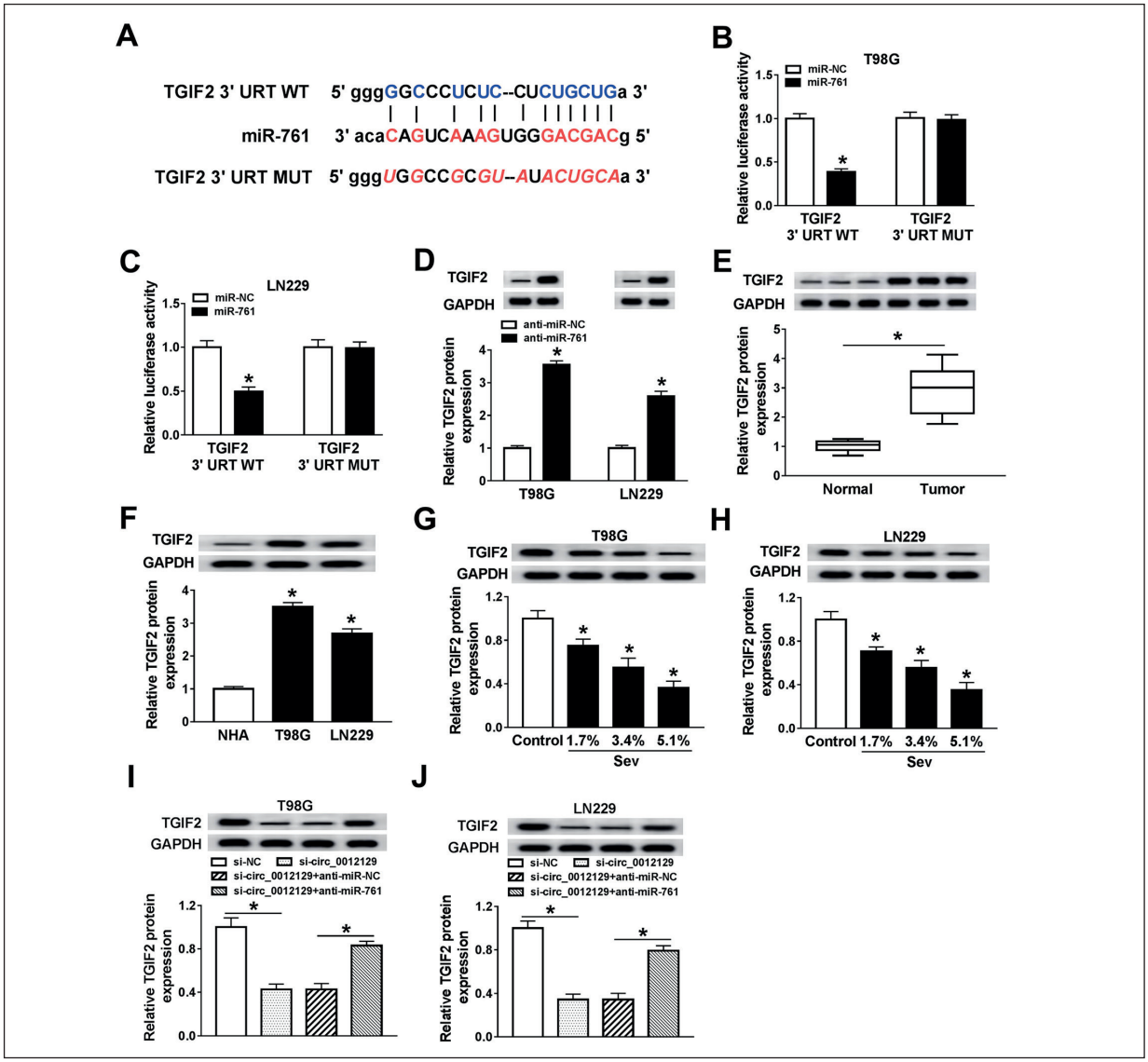

Figure 6. TGIF2 served as a target for miR-761 in glioma cells. **A,** The binding sites between TGIF2 and miR-761 were predicted through starBase v2.0. **B,** and **C,** Dual-Luciferase reporter assay was conducted to assess the impact of miR-761 on Luciferase intensity of the Luciferase reporter vector TGIF2 3'UTR WT and TGIF2 3'UTR MUT in T98G and LN229 cells. **D,** Protein level of TGIF2 in T98G and LN229 cells transfected with anti-miR-761 or anti-miR-NC was measured using Western blot analysis. **E,** and **F,** Expression level of TGIF2 protein in glioma tissues and normal brain tissues as well as glioma cells (T98G and LN229) and NHA cells was examined through Western blot analysis. **G,** and **H,** Effect of different concentrations of sevoflurane (1.7%, 3.4%, and 5.1%) on the expression of TGIF2 protein was determined *via* Western blot analysis. **I,** and **J,** The expression of TGIF2 protein in T98G and LN229 cells transfected with si-NC, si-circ_0012129, si-circ_0012129+anti-miR-NC, or si-circ_0012129+anti-miR-761 was measured with Western blot analysis. *$p < 0.05$.

Circ_0012129 Upregulation Overturned the Repressive Effect of Sevoflurane on Tumor Growth In Vivo

Taking into consideration that sevoflurane affected the biological characteristics of glioma cells through the circ_0012129/miR-761/TGIF2 axis *in vitro*, we further verified this result *in vivo via* xenograft model. T98G cells stably transfected with circ_0012129 or vector and stimulated with sevoflurane. 8 nude mice were subcutaneously inoculated with T98G cells. The results exhibited that sevoflurane impeded tumor volume and weight, while this repression was restored by the introduction of circ_0012129 (Figure 8A and 8B). Circ_0012129 was upregulated and miR-761 was downregu-

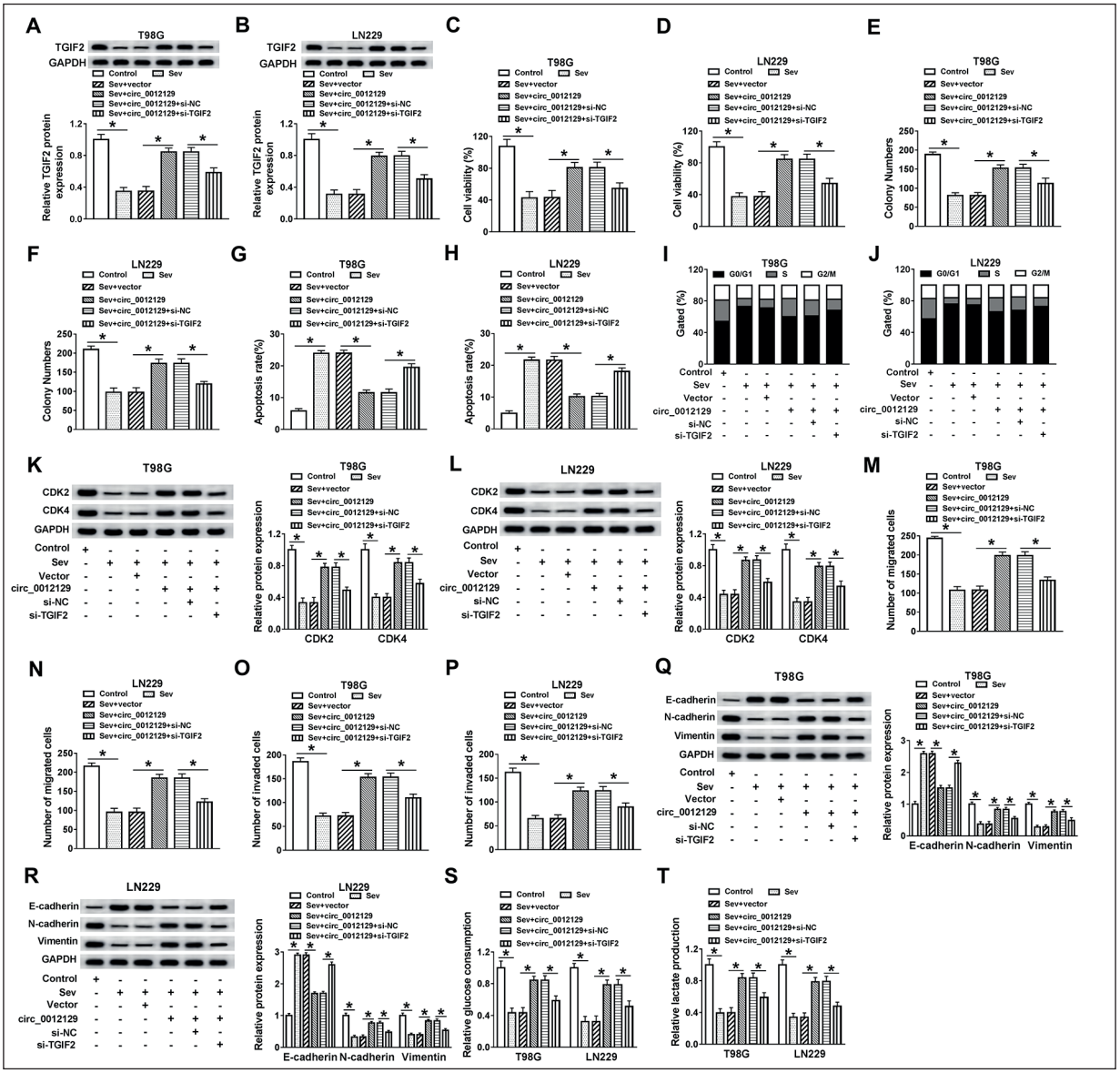

Figure 7. Effects of TGIF2 depletion on circ_0012129 overexpression-mediated biological characteristics of sevoflurane-treated glioma cells. **A-T,** T98G and LN229 cells were transfected with circ_0012129, vector, circ_0012129+si-TGIF2, or circ_0012129+si-NC, and then, stimulated with sevoflurane. **A,** and **B,** Western blot analysis was employed to assess the expression of TGIF2 protein in T98G and LN229 cells. **C-F,** The viability and colony formation ability of T98G and LN229 cells were determined *via* CCK-8 assay or colony formation assay. **G,** and **H,** The apoptosis rate of T98G and LN229 cells was examined with flow cytometry assay. **I,** and **J,** Cell cycle of T98G and LN229 cells was analyzed through PI single staining assay. **K,** and **L,** Western blot analysis was applied to assess the expression of CDK2 and CDK4 proteins in T98G and LN229 cells. **M-P,** The migration and invasion abilities of T98G and LN229 cells were determined with transwell assay. **Q,** and **R,** Western blot analysis was used to detect the protein levels of E-cadherin, N-cadherin, and Vimentin in T98G and LN229 cells. **S,** and **T,** Glucose consumption and lactate production were measured with special commercial kits in T98G and LN229 cells. *$p < 0.05$.

lated in the sevoflurane+circ_0012129 group than that in the sevoflurane+vector group (Figure 8C and 8D). Additionally, TGIF2 mRNA and protein levels were augmented in the sevoflurane+circ_0012129 group in contrast to the sevoflurane+vector group (Figure 8E and 8F). Collectively, these results suggested that circ_0012129 affected the effect of sevoflurane on tumor growth *in vivo via* the miR-761/TGIF2 axis.

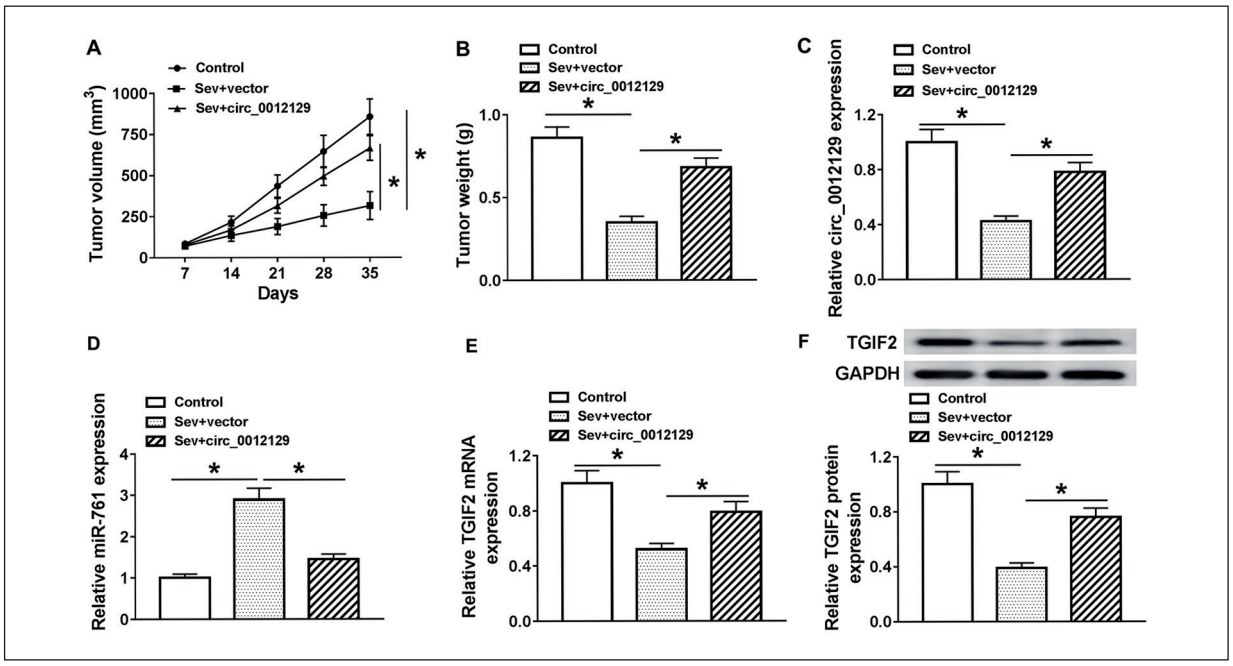

Figure 8. Effects of circ_0012129 upregulation on sevoflurane-mediated the growth of on tumor *in vivo*. A, Tumor volume was measured with a digital caliper every 7 days. **B,** On day 35, xenograft mice were dissected, and their tumors were weighted. **C,** and **D,** The expression levels of circ_0012129 and miR-761 in tumors of mice with sevoflurane+circ_0012129 or sevoflurane+vector were evaluated by qRT-PCR. **E,** and **F,** The mRNA and protein levels of TGIF2 in tumors of mice with sevoflurane+circ_0012129 or sevoflurane+vector were assessed *via* qRT-PCR or Western blot analysis. *$p < 0.05$.

Discussion

Perioperative periods might exert a crucial role in postoperative cancer recurrence, including anesthesia used, anesthesia techniques, and surgical stress[23,24]. A some studies[5,6,25,26] have shown that sevoflurane could trigger cell apoptosis and curb cell invasion, proliferation, and migration in glioma cells. Therefore, exploring the molecular mechanism of the effects of sevoflurane on glioma cells will enhance our understanding of postoperative tumor recurrence.

The dysregulation of circRNA was connected with the pathogenesis of glioma[11,12]. Meng et al[27] revealed that circRNA SCAF11 accelerated invasion, proliferation, and cell cycle in glioma cells via the miR-421/VEGFA axis. Moreover, circRNA SHKBP1 facilitated cell migration, viability, and tube formation in U87 glioma-exposed endothelial cells through miR-379/FOXP2 and miR-544a/FOXP1 pathways[28]. In our study, circ_0012129 was upregulated in glioma tissues and cells. Furthermore, sevoflurane repressed the expression of circ_0012129 in glioma cells in a concentration-dependent manner. Also, sevoflurane promoted apoptosis and repressed viability, colony formation, cell cycle, migration, and invasion of glioma cells in vitro and suppressed tumor growth in vivo, while these effects were abolished by circ_0012129 elevation. Xie et al[13] claimed that circ_0012129 expedited migration, proliferation, and invasion of glioma cells through sponging miR-661. These data indicated that sevoflurane repressed the development of glioma by downregulating circ_0012129.

To further elucidate the molecular mechanism by which sevoflurane repressed the development of glioma via circ_0012129, we predicted the target of circ_0012129 through the starBase v2.0. We uncovered and confirmed that miR-761 was a target of circ_0012129 in glioma cells. MiR-761 could induce cell apoptosis and suppress cell invasion, proliferation, and migration in osteosarcoma cells by deactivating FGFR1/PI3K/AKT pathway[29]. Xiong et al[17] suggested that miR-761 targeted HDAC1 to suppress the invasion and proliferation of colorectal cancer cells. MiR-761 could inhibit cell invasion and proliferation in glioma cells by repressing circ_0007534[18]. Herein, miR-761 was downregulated in glioma tissues and cells. Sevoflurane boosted the expression of miR-761 in glioma

cells. Both the inhibition of apoptosis and the facilitation of viability, colony formation, cell cycle, migration, and invasion of sevoflurane-treated glioma cells caused by circ_0012129 elevation were reversed by the overexpression of miR-761. These results indicated that miR-761 played an anti-cancer role in glioma. Additionally, miR-761 was pointed out to expedite the development of hepatocellular cancer, breast cancer, and non-small cell lung cancer, which might be related to tissue specificity and microenvironment differences[16,30,31]. Hence, we inferred that sevoflurane mediated the progression of glioma via circ_0012129/miR-761.

Subsequently, TGIF2 was verified to be a target for miR-761 *via* starBase v2.0 and Dual-Luciferase reporter assay. Diao et al[21] revealed that TGIF2 inhibition curbed invasion, proliferation, migration, and cell cycle and induced apoptosis of glioma cells. Besides, TGIF2 could contribute to migration and EMT in glioblastoma cells[32]. In the current study, the TGIF2 expression level was augmented in glioma tissues and cells and was reduced in sevoflurane-treated glioma cells. Also, TGIF2 reduction reversed circ_0012129 upregulation-mediated viability, colony formation, cell cycle, apoptosis, migration, and invasion of sevoflurane-treated glioma cells. Circ_0012129 modulated the expression level of TGIF2 in glioma cells through miR-761. From all the data above, sevoflurane inhibited the development of glioma *via* modulating the circ_0012129/miR-761/TGIF2 axis.

Conclusions

In sum, sevoflurane promoted apoptosis and curbed viability, colony formation, cell cycle, migration, and invasion of glioma cells in vitro, and repressed tumor growth in vivo, and these impacts were mediated by the circ_0012129/miR-761/TGIF2 axis. This study provided a novel theoretical basis for the application of sevoflurane in glioma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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