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circRNA-SMO upregulates CEP85 to promote proliferation and migration of glioblastoma via sponging miR-326

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Summary. Circular RNAs (circRNAs) play an important role in cancer development by sponging microRNAs (miRNAs) to regulate the signaling axis. However, more comprehensive mechanisms of circRNAs in glioblastoma need to be elucidated.

RT-qPCR was used to detect the expression levels of circRNA-SMO and miR-326. Dual-luciferase reporter assays were conducted to verify the interaction among circRNA-SMO, miR-326, and CEP85. Flow cytometric analysis was performed to detect apoptosis. Western blotting was used to determine the protein levels of the different molecules. Animal xenograft experiments were performed to evaluate the role of circRNA-SMO *in vivo*.

CircRNA-SMO was upregulated in glioblastoma tissues and glioblastoma cells. CircRNA-SMO downregulation inhibited the viability and colony-forming ability of the glioblastoma cells. In addition, miR-326 was downregulated in glioblastoma cells, which was verified to sponge circRNA-SMO and interact with CEP85. Moreover, circRNA-SMO inhibition induced the elevation of miR-326 and apoptosis, accompanied by a decrease in CEP85. CircRNA-SMO knockdown-mediated tumor inhibition was prevented by an miR-326 inhibitor. Furthermore, circRNA-SMO inhibition inhibited tumor growth *in vivo*, accompanied by an increase in miR-326 and a decline in CEP85 in tumor tissues.

Conclusions. CircRNA-SMO sponges miR-326 to promote glioblastoma proliferation and migration by upregulating CEP85 expression. This study clarified the role of circRNA-SMO in the development of glioblastoma, providing novel insights for its treatment.

Key words: Glioblastoma, CircRNA-SMO, MiR-326, CEP85

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Introduction

Malignant glioblastoma is a malignant neuroepithelial tumor, and its incidence ranks first among all primary types of intracranial malignant tumors, accounting for approximately 40-50% of all intracranial tumors, with a high degree of infiltration into the peripheral brain tissue and poor prognosis (Omuro and DeAngelis, 2013; Weller et al., 2015; Bach et al., 2018). Regular methods like imaging, surgery, and radiotherapy have been applied to treat glioblastoma (Li et al., 2013; Mirimanoff, 2014). However, there is currently no specific therapy for glioblastoma. Therefore, exploring the deep-seated mechanisms of glioblastoma could guide its prevention, diagnosis, and treatment.

Circular RNAs (circRNAs) are a kind of special non-coding RNA molecules (Yao et al., 2017). CircRNAs have a closed loop structure which cannot be affected by RNA exonucleases, leading to a more stable expression (Pamudurti et al., 2017). Of note, circRNA plays an important regulatory role in glioblastoma (Liu

Abbreviations. NOB1, Human Nin one binding protein; CCDC21, Coiled-coil domain-containing protein 21; STIL, SCL-interrupting locus protein; PLK4, Polo Like Kinase 4; UTR, untranslated regions.

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et al., 2019). circHIPK3 serves as a prognostic marker to promote glioblastoma progression by regulating miR-654/IGF2BP3 signaling (Jin et al., 2018). In addition, many studies have reported that circular RNA inhibition can restrain the progression, migration, and invasion of glioblastoma. For instance, circITGA7 has been shown to accelerate glioblastoma progression via the miR-34a-5p/VEGFA axis and Circ0001367 sponges miR-431, thus perturbing NRXN3 to suppress glioblastoma proliferation, migration, and invasion (Liu et al., 2021; Qi et al., 2021). Moreover, circRNA-SMO sponges miR-338-3p to promote glioblastoma growth by enhancing SMO expression (Xiong et al., 2019). In addition, recent research has revealed that circular RNA SMO can encode a novel protein, SMO-193a.a., which is essential for Hedgehog signaling activation and glioblastoma tumorigenicity (Wu et al., 2021). Although circRNA-SMO is linked to glioblastoma progression, the detailed regulatory mechanism remains largely unknown.

CircRNAs are believed to be rich in microRNA (miRNA) binding sites, acting as miRNA sponges in cells, thereby releasing the inhibitory effect of miRNA and increasing the expression level of target genes. This is called the competitive endogenous RNA (ceRNA) mechanism (Bolha et al., 2017; Han et al., 2017; Barbagallo et al., 2022). miR-326 acts as a tumor suppressor in glioblastoma and can regulate the metabolic activity of glioblastoma stem cells (Kefas et al., 2010; Nawaz et al., 2016b; Wang et al., 2013). For example, miR-326 has been reported to directly target Human Nin one binding protein (NOB1), a potential oncogene in human glioblastoma, thus serving as a tumor suppressor (Zhou et al., 2013). Moreover, miR-326 knockdown can reverse the suppression of glioblastoma progression caused by circ0082374 silencing (Wang et al., 2020). Additionally, miR-326 has been demonstrated to play a role in critical signaling axes in glioblastoma, such as the PI3 kinase pathway (Nawaz et al., 2016a). The interaction between circ_0001730 and miR326 promotes glioblastoma cell proliferation by influencing the Wnt axis (Lu et al., 2019). Therefore, the ceRNA mechanism involved in miR-326 expression plays a critical role in glioblastoma progression.

Centrosomal protein 85 (CEP85) encodes a protein that belongs to the centrosome-associated family of proteins, also called coiled-coil domain-containing protein 21 (CCDC21) (Chen et al., 2015). Although the role of CEP85 in cancer has not been clearly clarified, it has been demonstrated that CEP85 can cooperate with the SCL-interrupting locus protein (STIL) to mediate polo-like kinase 4 (PLK4)-driven directed cancer cell migration (Liu et al., 2020). Thus, CEP85 might be a key regulator of cell growth. However, whether CEP85 participates in the proliferation and growth of glioblastoma cells remains unclear.

In this study, circRNA-SMO expression in glioblastoma tissues and cells was investigated. The ceRNA mechanism involving miR-326 and circRNA-SMO has been demonstrated in glioblastoma cells. Furthermore, the downstream target gene of circRNA-SMO/miR-326 in glioblastoma was identified. It is possible that circRNA-SMO regulates glioblastoma progression via the ceRNA mechanism. This research helped further understand the role of circRNA-SMO in the development of glioblastoma and provides evidence for the treatment prospects of circRNA-SMO/miR326/CEP85 in glioblastoma.

Materials and methods

Subjects

Twenty patients with glioblastoma and paired para-carcinoma tissues were enrolled from the Biobank of Zhejiang Cancer Hospital. Glioblastoma tissues were subjected to quantitative real-time PCR (qRT-PCR) to detect circRNA_SMO expression. Informed consent was obtained from all patients, and the study was approved by the local ethics committee of The Cancer Hospital of the University of Chinese Academy of Sciences, Zhejiang Cancer Hospital (Approval number: IRB-2021-241). Basic clinical information of the patients is presented in Table 1.

RNA extraction and RT-PCR

Total RNA was extracted from glioblastoma tissues using TRIzol reagent (15596-026, Ambion) and the miRNeasy Mini Kit (Qiagen, Germany). For cDNA synthesis, 1,000 ng of total RNA was used for reverse transcription in a 20 µl reaction volume using the

Table 1. Clinical information of glioblastoma used in this study.

Clinicopathological Features	No. of patients	circ_SMO expression high	circ_SMO expression low
Sex			
Male	9	4	5
female	11	6	5
Age			
<45	5	2	3
≥45	15	9	6
Tumor location			
frontal lobe	9	4	5
Temporal lobe	7	2	5
Occipital lobe	4	1	3
Tumor size			
<3 cm	7	4	3
≥3 cm	13	9	4
WHO grade			
III	2	1	1
IV	18	9	9
Histology			
astrocytoma	2	1	1
glioblastoma	18	9	9

HiScript Reverse Transcriptase kit (R101-01/02, VAZYME). qRT-PCR was performed using SYBR Green Master Mix (Q111-02, VAZYME) and a Real-Time PCR Detection System (ABI, Quant Studio 6). Primer sequences were as follows: GAPDH-Forward-5‘-TCAAGAAGGTGGTGAAGCAGG-3’, Reverse-5‘-TCAAAGGTGGAGGAGTGGGT-3’; circRNA-SMO-Forward-5‘-CCCTTGGTTCGGACAGACA-3’, Reverse-5‘-CAAAGAAGCACGCATTGACG-3’; stem-loop-specific primer method was applied to measure the expression levels of miR326, and U6 was used as a control. The primers used were as follows. MiR-326-Loop primer-5‘-GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGAC CTGGAGGA-3’, Forward-5‘-TGCGCCCTCTGGGCCCTTC-3’, Reverse-5‘-CCAGTGCAGGGTCCGAGGTATT-3; U6-Forward-5‘-CGCTTCGGCAGCACATATAC-3’, Reverse-5‘-AAATATGGAACGCTTCACGA-3’. GAPDH and U6 expression levels were used as internal references. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total protein from cells and glioblastoma tissues was extracted using RIPA lysis buffer. Protein concentrations were determined using a Bicinchoninic Acid Assay Kit (P0010; Beyotime Biotechnology). The supernatants were collected and loaded into SDS-PAGE following by a transmembrane PVDF (Millipore, IPVH00010). The membranes were blocked in Tris-buffered saline and Tween 20 (TBST, pH 7.6) containing 5% non-fat milk powder at room temperature for 1.5 h, and subsequently incubated with primary antibodies against CEP85 (1:1,000, Bioss, Bs-7988r), PLAM (1:1,000, Santa, Sc-365869), ZNF322 (1:1,000, Novus, Nbp2-33384) at 4°C overnight. The membranes were washed thrice with TBST and incubated with secondary goat anti-mouse (1:5,000, BOSTER Biological Technology, BA1051) or goat anti-rabbit (1:5,000, BOSTER Biological Technology, BA1054) conjugated to HRP for 1 h at room temperature. The bands were then covered with an ECL detection kit (Applygen, Beijing, P1050) and visualized using a ChemiDoc XRS+S system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using the ImageJ software (NIH, USA).

Cell culture

The normal human astrocyte cell line NHA was purchased from ATCC, and the glioblastoma cell lines (U118, U251, U87MG, and LN229) were purchased from Tongpai (Shanghai) Biotechnology Co., Ltd. Cells were routinely cultured in high-glucose Dulbecco’s Modified Eagle Medium (Gibco,11965-092) supplemented with 10% Fetal Bovine Serum (Gibco,10099-141) and 1% penicillin-streptomycin (Gibco,15070-063) at 37°C in a 5% CO_2-humidified air atmosphere incubator (SANYO, MCO-15AC).

Plasmid construction and transfection

CircRNA-SMO small interfering RNAs (siRNAs) were constructed using the following primers: siRNA1 GCAGAACATCAAGTTCAACAGTTCA, siRNA2 GAACTCGAATCGCTACCCTGCTGTT, siRNA3 ACTCGAATCGCTACCCTGCTGTTAT, and siRNA-NC UUCUCCGAACGUGUCACGUTT). SiRNAs were synthesized by the Tsingke Company (Beijing). siRNA (20 mmol/L) was transfected into LN229 or U251 cells for 48h in a 6-well plate using Lipofectamine^{TM} 2000 (Invitrogen,11668-019).

CCK8 assay

The Cell Counting Kit-8 reagent (MCE, HY-K0301) was used to monitor cell viability. Transfected cells were inoculated at 0, 24, 48, and 72h (1×10^3 cells per well). At the time point set, 10 μL CCK-8 solution was added to each well and then incubated for 4h at 37°C. The absorbance of each sample at 450 nm was measured using a microplate reader (Thermo, MULTISKAN MK3). The cell viability was calculated using the following formula: experimental group/control group ×100%.

Cloning formation assay

LN229 and U251 cells were inoculated in 6-well plates (300 cells/well). After incubation in an incubator for two weeks, the cells were washed twice with PBS and subsequently fixed with 70% ethanol for 30 min. The cells were then dyed with 0.3% purple crystals for 30 min. Finally, the plates were washed with PBS and processed using the scanner.

Dual luciferase reporter assays

CircRNA-SMO and CEP85, which contain the predicted miR-326 binding sites, were amplified. PCR was used to construct wild-type (WT) plasmids, while site-directed mutagenesis was used to generate mutant type (MUT) plasmids, replacing the first six ribonucleotides of the miR-326 complementary sequence. Co-transfection of LN229 cells with plasmids was performed using the Lipofectamine 2000 transfection system (Invitrogen,11668-019) following the manufacturer’s instructions. After treatment with the indicated reagents, cells were lysed in reporter lysis buffer (Promega, E1910). Firefly luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) on a single-channel luminometer. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Apoptosis assay

LN229 and U251 cells transfected for 48h were inoculated into a 6-well plate at approximately 1×10^5

cells/well. The cells were collected for apoptosis assay. Apoptosis analysis was conducted according to the manufacturer's protocols (BD Bioscience). In summary, the cells were fixed with binding buffer and stained with FITC Annexin V/PI. Finally, apoptosis status was monitored using a Beckman Epics Altra Culter. The proportion of early apoptosis (annexin V-positive and PI-negative) and late apoptosis (annexin V-positive and PI-positive) were determined using the EXP032 software.

Cell migration and invasion

The migration and invasion of glioblastoma cells were determined using transwell chamber inserts (BD Biosciences, 353097) with or without Matrigel (for invasion assays, Corning, 356234). For the migration assay, 5×10^3 transfected cells were inoculated in the upper chamber without serum. Medium mixed with 10% FBS was added to the lower chamber of the inserts and incubated at 37°C for 36h. The chambers were washed with PBS and stained with 0.5% crystal violet for 20 min at room temperature. For invasion assay, before the cells were inoculated into the chambers, 1 mg/mL Matrigel was added to the upper chamber and then solidified into gelatinous. After cells were cultured for 48h, the chamber inserts were removed, washed with PBS, fixed with 70% ethanol, and finally stained with 0.5% crystal violet for 20 min at room temperature. Images were captured using a microscope (Leica DMIRB Inverted Fluorescence Microscope, Leica Microsystems GmbH, Wetzlar, Germany).

Tumor xenograft

BALB/c female nude mice aged four weeks were used for the experimental procedures. Normal cells, negative control siRNA-transfected cells, and circRNA-SMO siRNA-transfected LN229 cells were sub-cutaneously and intracranially implanted into nude mice. Mice were randomly and equally divided into three groups: Negative Control, LN229 cells transfected with circRNA_SMO siRNA NC, and LN229 cells transfected with circRNA_SMO siRNA (n=6/each group). Cell suspensions (2×10^6) were subcutaneously inoculated into the flank of nude mice, and si-circRNA-SMO was implanted into the mice every four days. Tumor size was measured every three days. Tumor size and weight were separately calculated 28 days after the initial injection, and tumor weight was measured. This study was approved by the Animal Ethics Committee of The Second Affiliated Hospital of the Medical College of Zhejiang University.

Histochemistry

Tumor tissue samples were dehydrated and

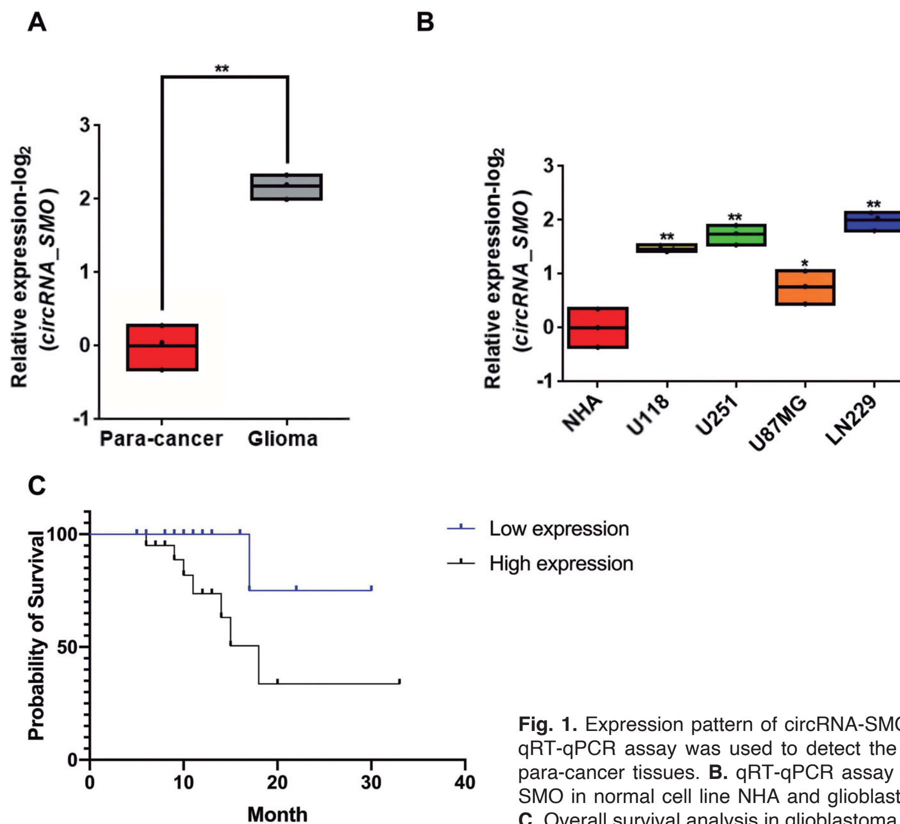

Fig. 1. Expression pattern of circRNA-SMO in glioblastoma tissues and cancer cell lines. **A.** qRT-qPCR assay was used to detect the expression of circRNA-SMO in glioblastoma and para-cancer tissues. **B.** qRT-qPCR assay was applied to detect the expression of circRNA-SMO in normal cell line NHA and glioblastoma cell lines (U118, U251, U87MG and LN229). **C.** Overall survival analysis in glioblastoma patients. *P<0.05 and **P<0.01.

paraffinized following routine procedures. The paraffin sections were rinsed in ddH_2O for 3-5 min and blocked with 3% peroxide to ablate endogenous peroxidase. Slices were incubated with anti-Ki67 antibody (1:100; Abcam, Ab16667) overnight at 4°C. Subsequently, the slices were washed four times with PBS and incubated with the secondary antibody for 20 min at room temperature in the dark. Finally, after the slices were dried, images were screened with a microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0, and the results are shown as the mean ± SEM. The Student's two-tailed unpaired t-test for two groups was used to determine significance. Multiple group analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Statistical significance was set at P<0.05.

Results

CircRNA-SMO is aberrantly upregulated in glioblastoma tissues and glioblastoma cells

In clinical glioblastoma tumor samples, circRNA-SMO displayed a significant elevation in tumor sample as compared to paired para-cancer tissues (P<0.01; Fig. 1A). The human brain astrocyte cell line NHA and four glioblastoma cancer cell lines (U118, U251, U87MG, and LN229) were selected for the detection of circRNA-SMO expression. The results showed that circRNA-SMO was highly expressed in glioblastoma cancer cell lines compared with that in NHA cell lines, among which U251 and LN229 showed the most prominent overexpression (Fig. 1B). Importantly, patients with high

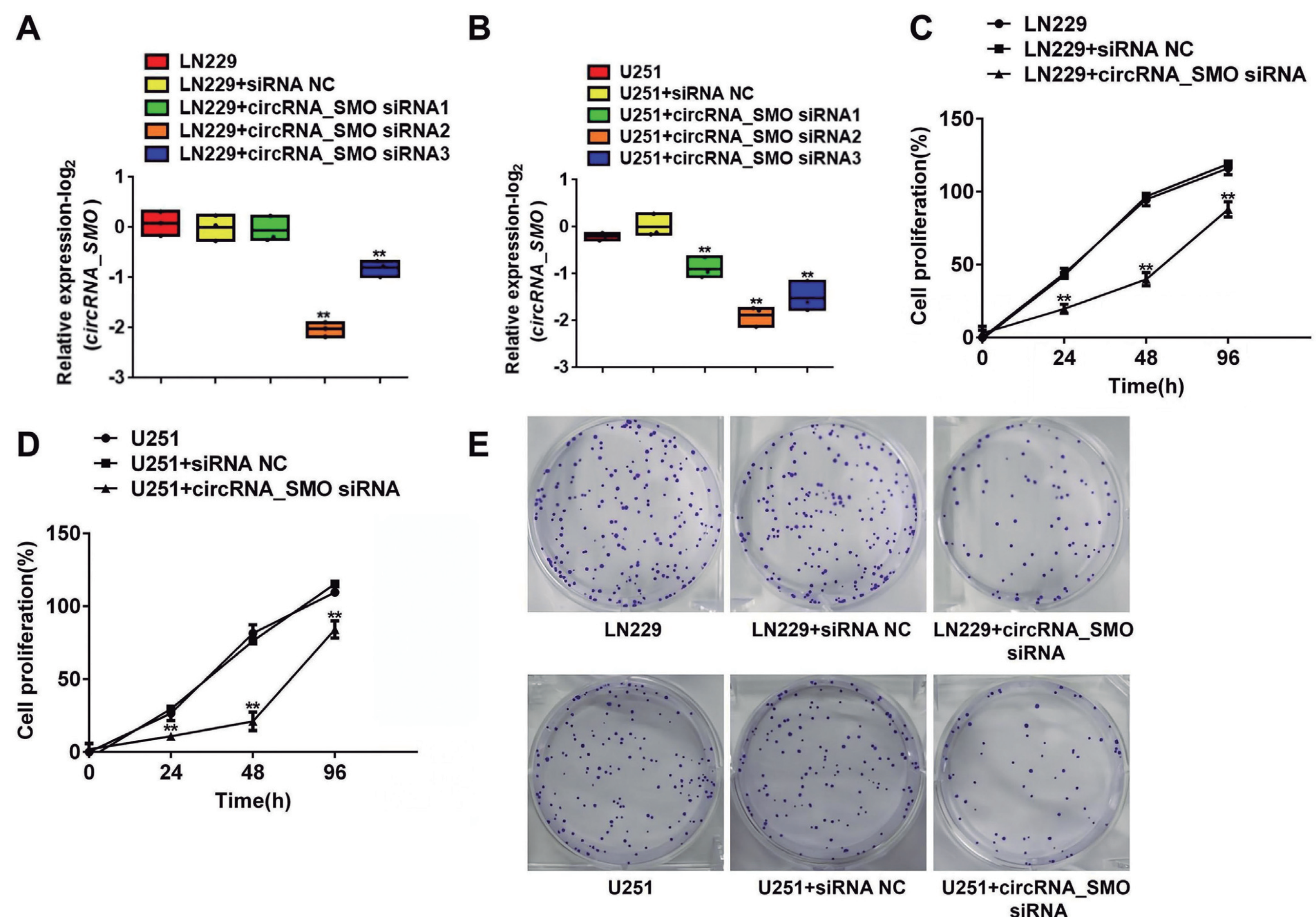

Fig. 2. Effects of circRNA-SMO on proliferation and growth of glioblastoma cells. **A, B.** RT-qPCR assay was conducted to screen the efficiency of circRNA-SMO siRNAs in LN229 cell lines **(A)** and U251 cell lines **(B)**. **C, D.** CircRNA SMO siRNA or control siRNA were transfected into LN229 and U251 cell lines. Subsequently, cell viability in LN229 cell line **(C)** and U251 cell line **(D)** as monitored by CCK8 assay. LN229: LN229 control group, LN229+siRNA NC: negative siRNA-transfected LN229 cells; LN229+circRNA-SMO siRNA: circRNA-SMO siRNA-transfected LN229 cells. **E.** CircRNA SMO siRNA or control siRNA were transfected into LN229 and U251 cell lines. Then, colony formation assays were conducted to detect the clonality of LN229 and U251 cells. Data analysis used One-Way ANOVA. Data are shown as the mean ± SD, n=3. *P<0.05 and **P<0.01 compared with the corresponding.

circRNA-SMO presented a poor prognosis compared to patients with low circRNA-SMO (Fig. 1C). These data further prove that circRNA-SMO is present at a high level in glioblastoma.

CircRNA-SMO promotes the proliferation of glioblastoma cells

CircRNAs are known to play a critical role in the regulation of normal physical activity. To verify whether circRNA-SMO participated in the cellular process of glioblastoma cells, we constructed three siRNAs to achieve circRNA-SMO-knockdown in LN229 and U251 cells. Combined with the qRT-PCR results, siRNA2 targeting circRNA-SMO showed the best knockdown efficiency and was chosen for subsequent experiments (Fig. 2A,B). As shown in Fig. 2C,D, circRNA-SMO inhibition led to a slower proliferation rate than that of the control group in LN229 and U251 cells, implying that circRNA-SMO was essential for the normal proliferation of glioblastoma cancer cells (Fig. 2C,D). Additionally, a clone formation assay was conducted to evaluate the role of circRNA-SMO in glioblastoma cell growth. As expected, the number of clones was significantly reduced in circRNA-SMO-knockdown LN229 and U251 cells, further confirming that circRNA-SMO serves as a pathogenic factor that promotes the proliferation and growth of glioblastoma cancer cells (Fig. 2E).

CircRNA-SMO sponges miR-326

CircRNAs typically act as sponges for miRNAs. However, the miRNAs that interact with circRNA-SMOs remain unclear. Previous studies have demonstrated that miR-326 plays a key role in glioblastoma, and its downregulation may accelerate glioblastoma progression (Wang et al., 2013). As previously reported, miR-326 was drastically decreased in glioblastoma samples compared to para-cancer samples (Fig. 3A). The dual-luciferase reporter gene assay indicated that miR-326 mimics significantly reduced the activation of circRNA_SMO, which had no effect on cells transfected with the mutated circRNA_SMO reporter plasmid (Fig. 3B). Interestingly, circRNA-SMO downregulation was not relieved by

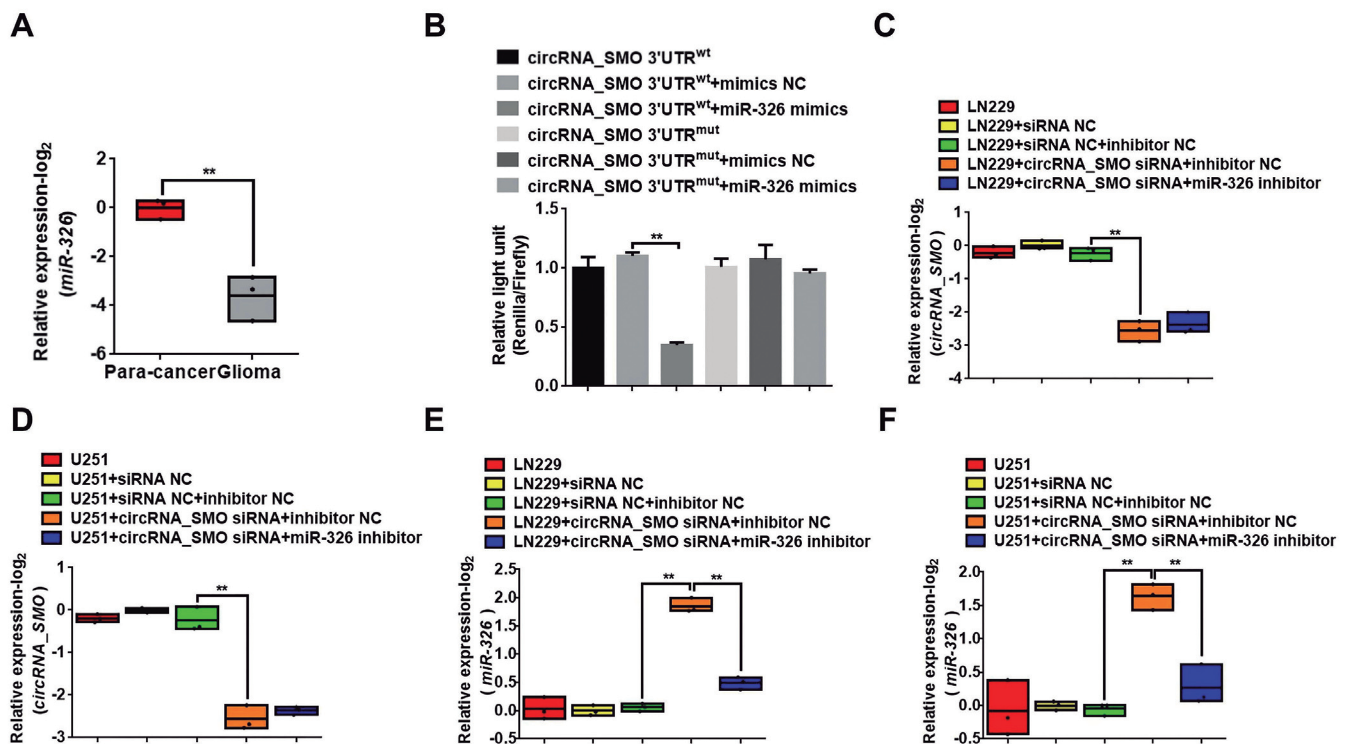

Fig. 3. Association between circRNA-SMO and miR-326. **A.** RT-qPCR assay was carried out to detect the relative expression of miR-326 in glioblastoma and para-cancer samples. **B.** Cell was co-transfected with circRNA SMO 3'-UTRwt or circRNA SMO 3'-UTRmut and miR-326 mimics or mimics control. Dual luciferase reporter gene assay was conducted to evaluate the binding of circRNA SMO with miR-326. **C.** LN229 and U215 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Then, relative expression of circRNA SMO was detected with RT-qPCR. **D.** Relative expression of miR-326 was detected with RT-qPCR in LN229 and U215 cells co-transfected with circRNA SMO siRNA or siRNA control. Data were shown as the mean ± SD, n=3. Values were significantly different compared with the corresponding control value at *P<0.05 and **P<0.01.

miR-326 inhibition in U251 and LN229 cells, and miR-326 mimics did not affect the expression of circRNA SMO (Fig. 3C). However, miR-326 was significantly upregulated in the presence of circRNA-SMO siRNA and decreased to normal levels upon miR-326 inhibition (Fig. 3D). These data indicate that circRNA-SMO may be a sponge for miRNA-326.

Regulation between circRNA-SMO, miR326 and CEP85

Interestingly, increased expression of CEP85 and PALM proteins was detected in glioblastoma compared to para-cancer samples, and ZNF322 showed no changes in the two tissue types (Fig. 4A). The data showed that CEP85 was prominently elevated in glioblastoma samples, whereas PALM was only slightly increased (Fig. 4A). Furthermore, significant inhibition of luciferase activity by CEP85 was observed in miR-326 mimic-challenged LN229 cells, indicating that miR-326 might bind to CEP85 in glioblastoma cells (Fig. 4B). Subsequently, the content of CEP85 was reduced after circRNA-SMO knockdown, which was abolished by miR-326 inhibition in circRNA-SMO-knockdown cells (Fig. 4C,D). The data demonstrated that circRNA-SMO/miR-326/CEP85 exists as a signaling cascade in glioblastoma cells.

CircRNA-SMO/miR-326 signaling axis regulates glioblastoma tumor behaviors

MiR-326 has been reported to function as a tumor suppressor in cancer (Kefas et al., 2009; Li et al., 2015). To illustrate its function in glioblastoma, miR-326 mimics and inhibitors, which were proven to have high efficiency in LN229 and U251 cancer cell lines, were constructed (Fig. 5A,B). In our data, miR-326 inhibition accelerated the proliferation rate of glioblastoma cells, which was decreased by circRNA-SMO siRNA transfection (Fig. 5C,D). In addition, the suppression of miR-326 abrogated the circRNA-SMO siRNA-mediated decline in clone number (Fig. 5E,F). These results confirmed that miR-326 inhibition could restore the circRNA-SMO inhibition-mediated decrease in glioblastoma cell viability, accelerating the proliferation and growth of glioblastoma cells. Additionally, cell apoptosis was assessed in the presence of miR-326 inhibitor and circRNA-SMO siRNA. CircRNA-SMO knockdown induced glioblastoma cell apoptosis from 8.04% to 28.85%, whereas miR-326 suppression decreased the ratio to 13.52% in the LN229 cell line (Fig. 6A,B), which was also observed in the U251 cell line (Fig. 6C,D). Subsequently, the roles of circRNA-SMO and miR-326 in glioblastoma cell migration and

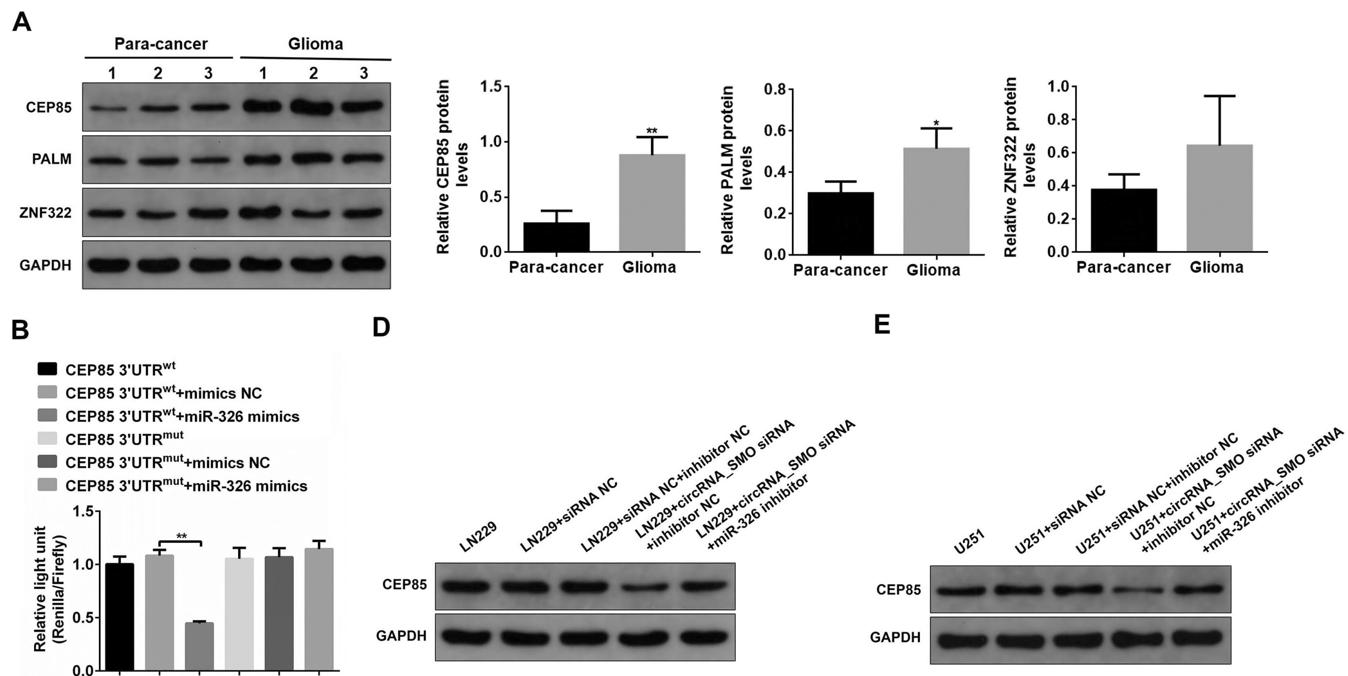

Fig. 4. Regulation among circRNA-SMO, miR326 and CEP85. **A.** Immunoblot assay was carried out using antibodies for CEP85, PALM and ZNF322 in glioblastoma and para-cancer samples. Grayscale analysis of protein levels in panel A was conducted. **B.** LN229 cells were co-transfected with CEP85 3'-UTRwt or CEP85 3'-UTRmut and miR-326 mimics or mimics control. Dual luciferase reporter gene assay was conducted to evaluate the binding of CEP85 with miR-326. **C, D.** LN229 and U215 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Protein levels of CEP85 was detected using Western Blot with different treatment in LN229 **(C)** and U251 **(D)** cells. All data analysis above used One-Way ANOVA. Data are shown as the mean ± SD, n=3. *P<0.05 and **P<0.01.

invasion were investigated. The high migration and aggressiveness of glioblastoma cells could be relieved by circRNA-SMO knockdown, and miR-326 inhibition significantly abolished the role of circRNA-SMO removal (Fig. 7A-H). Therefore, miR326 is a downstream sponge of circRNA-SMO, and its inhibition could prevent the improvement of circRNA-SMO siRNA on glioblastoma tumor behavior.

CircRNA_SMO interfere inhibits tumor growth

As shown in Figure 8A, xenograft tumor size was notably reduced in circRNA-SMO siRNA-challenged tumors (Fig. 8A). Tumor weight and volume were also decreased in the circRNA-SMO knockdown mice in comparison with the controls, thus confirming that circRNA-SMO knockdown suppressed tumor growth (Fig. 8B,C). In tumor tissues, the level of Ki-67, a marker of cell proliferation, was downregulated in the circRNA-SMO siRNA-exposed tumors compared to that in the control group (Fig. 8D). Combined with the qRT-PCR results, circRNA-SMO expression was significantly downregulated and miR-326 was notably upregulated in the circRNA-SMO-knockdown group compared to those in the control group (Fig. 8E,F). Notably, CEP85 was also inhibited along with the elevation of miR-326 and a decrease in circRNA-SMO *in vivo* (Fig. 8G,H). These observations confirmed that circRNA-SMO inhibition promoted miR-326-CEP85 signal transduction, exerting an inhibitory effect on tumor growth.

Discussion

Glioblastoma is the most common primary tumor of the central nervous system and has a high postoperative recurrence rate and resistance to chemotherapy (Geng et al., 2020). The association of circRNAs and miRNAs

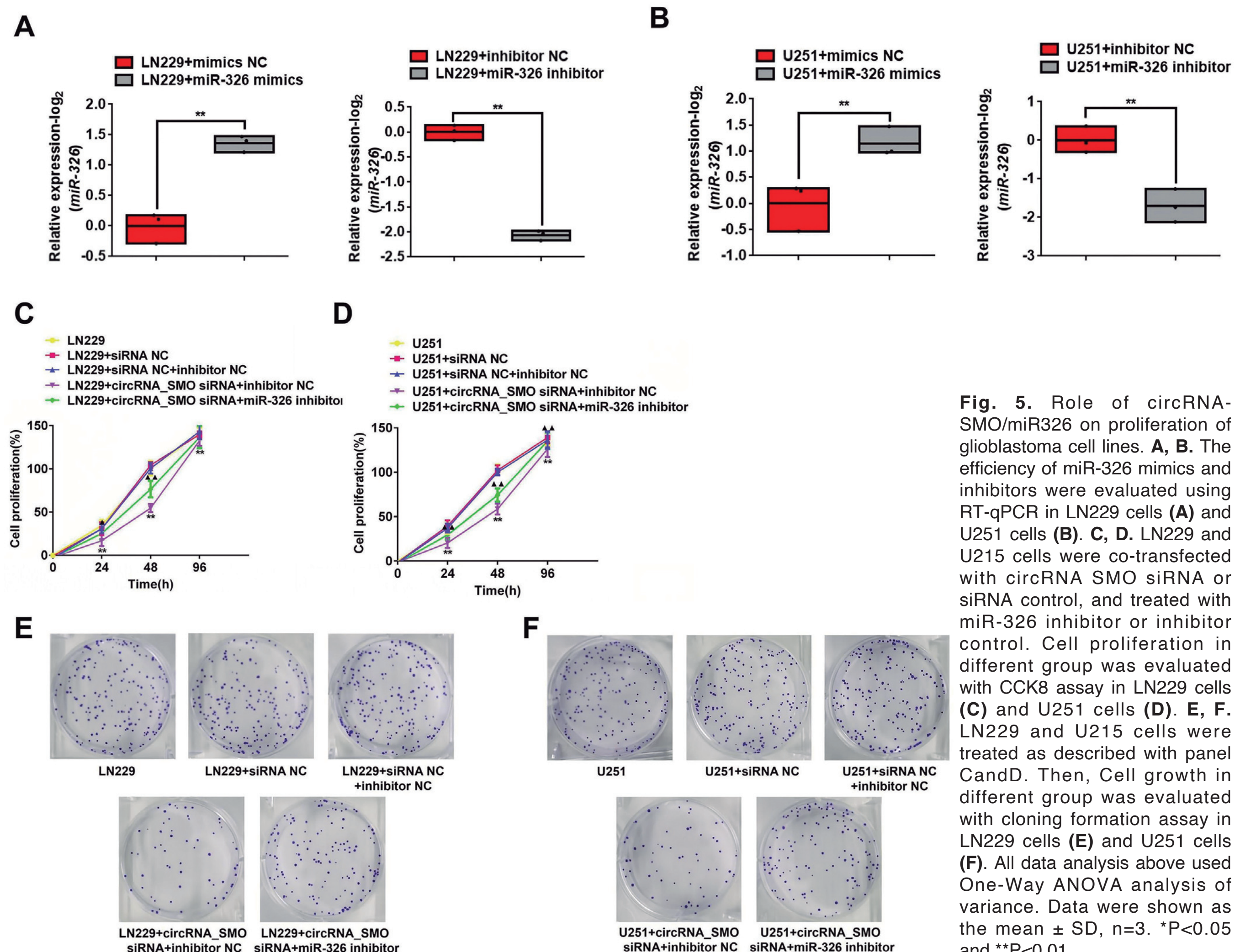

Fig. 5. Role of circRNA-SMO/miR326 on proliferation of glioblastoma cell lines. **A, B.** The efficiency of miR-326 mimics and inhibitors were evaluated using RT-qPCR in LN229 cells **(A)** and U251 cells **(B)**. **C, D.** LN229 and U215 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Cell proliferation in different group was evaluated with CCK8 assay in LN229 cells **(C)** and U251 cells **(D)**. **E, F.** LN229 and U215 cells were treated as described with panel CandD. Then, Cell growth in different group was evaluated with cloning formation assay in LN229 cells **(E)** and U251 cells **(F)**. All data analysis above used One-Way ANOVA analysis of variance. Data were shown as the mean ± SD, n=3. *P<0.05 and **P<0.01.

with glioblastoma development has recently gained attention (Banelli et al., 2017; Liu et al., 2019; Sun et al., 2020). In this study, we demonstrated that circRNA-SMO acts as a pathological factor in glioblastoma, which promotes the proliferation of glioblastoma cells. In addition, circRNA-SMO/miR-326/CEP85 signaling cascades were verified to mediate the progression, growth, and invasion of glioblastoma. CircRNA inhibition diminished the proliferation, growth, migration, and invasion of glioblastoma *in vitro*. Simultaneously, *in vivo* animal experiments supported our conclusion that circRNA-SMO knockdown could inhibit tumor growth by interacting with the miR-326-CEP85 signal axis. These results deepen our understanding of the mechanisms of glioblastoma development and offer suggestions for clinical therapy and new drug development.

CircRNAs have been found to play an important role in glioblastoma progression. For instance, circular RNA PRKCI has been reported to promote glioblastoma cell progression by inhibiting miRNA-545, and targeting the circPRKCI-miR-545 cascade might be a promising approach to efficiently inhibit human glioblastoma cells (Zhang et al., 2019). In addition, high Circ-TTBK2

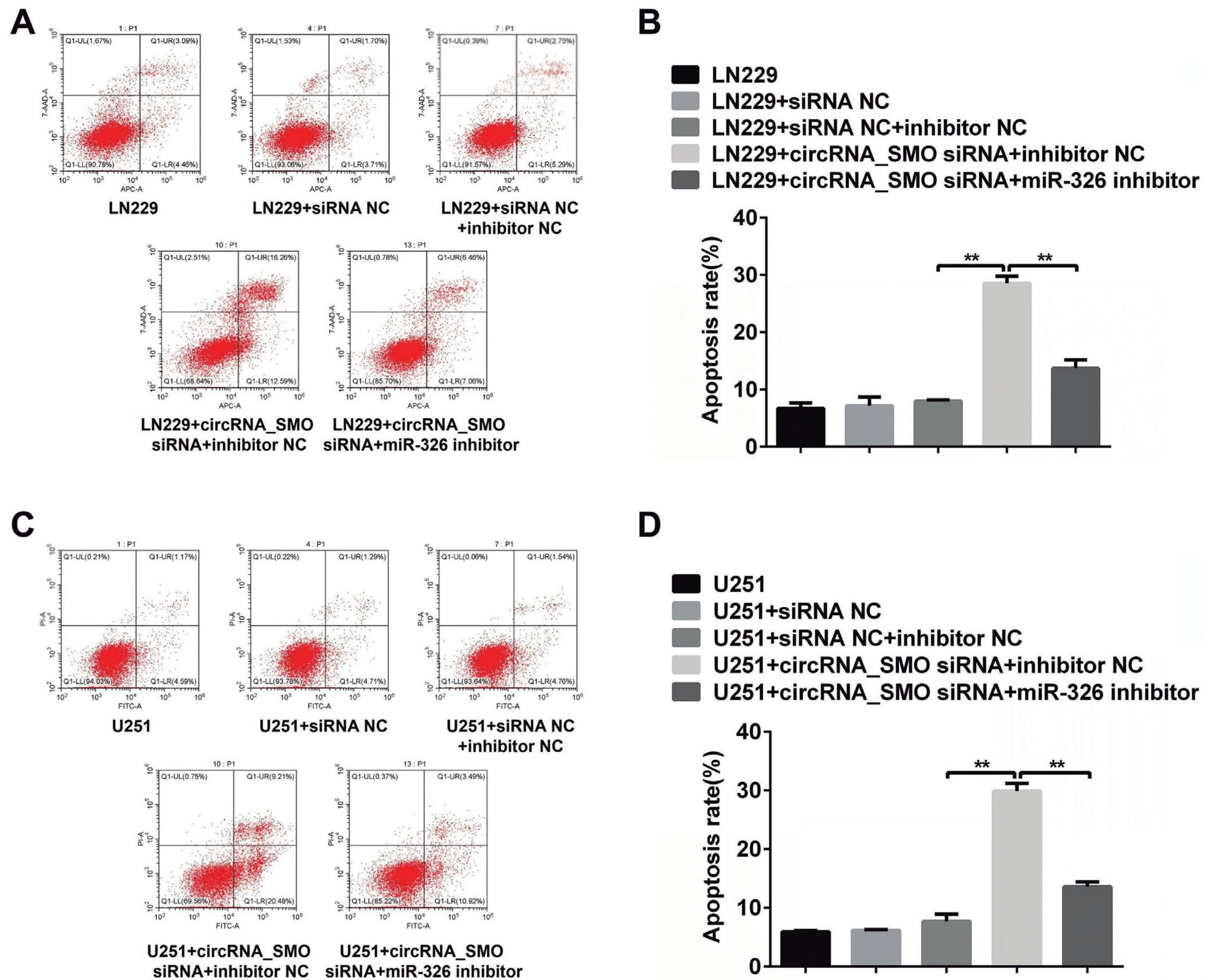

Fig. 6. Cell apoptosis detection in circRNA-SMO and/or miR326-transfected glioblastoma cells. **A.** LN229 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Then, flow cytometry assay was used to calculate the apoptotic cells in LN229. **B.** Quantification of apoptotic cells in panel A. **C.** U251 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Flow cytometry assay was used to calculate the apoptotic cells in U251. **D.** Quantification of apoptotic cells in panel C. All data analysis above used One-Way ANOVA. Data are shown as the mean ± SD, n=3. *P<0.05 and **P<0.01.

expression in glioblastoma promotes tumor growth by sponging miR-217 (Zheng et al., 2017). It has been reported that circRNA-SMO is upregulated to promote glioblastoma growth by sponging miR-338-3p in glioblastoma cells (Xiong et al., 2019). In our study, circRNA-SMO was found to have higher expression levels in glioblastoma tissues and cell lines. Our data are consistent with the previous results. Furthermore, inhibition of circRNA-SMO effectively restrained the proliferation and growth of glioblastoma cells *in vitro* and suppressed tumor growth *in vivo*. These findings further prove the potential therapeutic action of circRNA-SMO inhibition in glioblastoma. However, the clinical value of circRNA-SMO should be explored in a large-scale glioblastoma cohort. Whether circRNA-SMO is correlated with the clinical characteristics of glioblastoma, such as pathological grade and overall survival rate, needs to be explored using large samples and online databases.

MiR-326 associated signal cascades play a vital role in glioblastoma. miR-326 is downregulated in glioblastoma and mediates several signaling cascades (Lu et al., 2019). MiR-326 can target the SMO oncogene, thus inhibiting glioblastoma biological behavior and stemness (Du et al., 2015). In our data, miR-326 was also shown to be at a low level in glioblastoma tissues compared to normal tissues. Various miRNAs that sponge miR-326 participate in cancer progression, such as circ_POLA2 in cervical squamous cell carcinoma and circPUM1 in lung adenocarcinoma (Chen et al., 2019; Cao et al., 2020). Another study showed that circ0082374 can bind to miR-326, exerting an inhibitory role in glioblastoma progression (Wang et al., 2020). Here, circRNA-SMO was shown to interact with the 3'-untranslated region (UTR) of miR-326 and subsequently inhibit its expression. Notably, miR-326

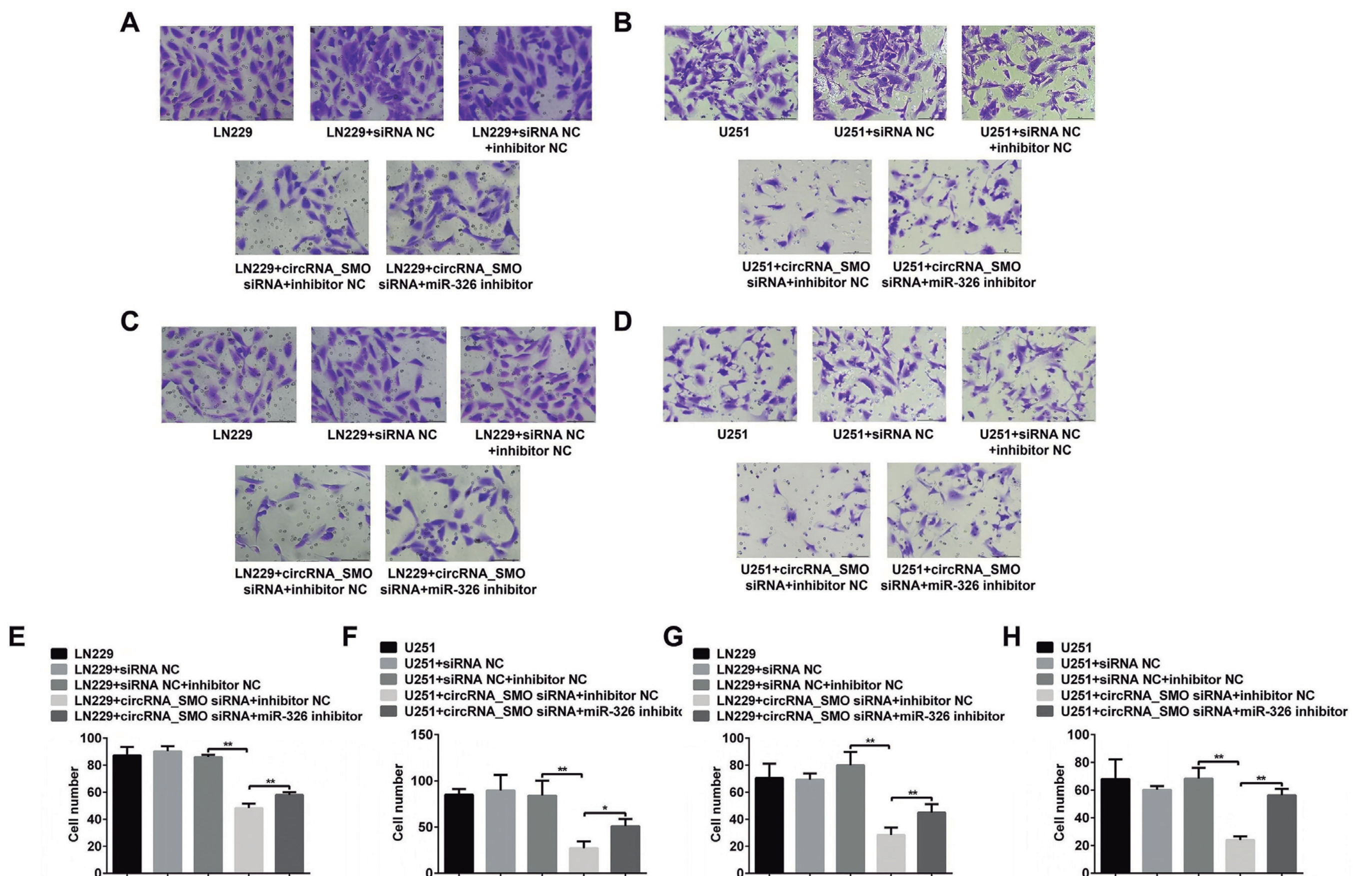

Fig. 7. Cell migration measurement in circRNA-SMO and/or miR326-transfected glioblastoma cells. **A.** LN229 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Then, transwell migration assay was used to measure migration ability of LN229 cells with different treatment. **B.** Quantification of migrating cells in panel A. **C.** U251 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Transwell migration assay was used to measure migration ability of U251 cells with different treatment. **D.** Quantification of migrating cells in panel C. **E.** LN229 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Transwell invasion assay was applied to evaluate the invasive ability of MDA-MB-231 cells. **F.** Quantification of migrating cells in panel E. **G.** U251 cells were co-transfected with circRNA SMO siRNA or siRNA control, and treated with miR-326 inhibitor or inhibitor control. Matrigel was applied to measure invasion ability of U251 cells with different treatment. **H.** Quantification of migrating cells in panel G. All data analysis above used One-Way ANOVA. Data are shown as the mean ± SD, n=3. *P<0.05 and **P<0.01.

knockdown attenuated the effect of circRNA-SMO inhibition in glioblastoma. Additionally, circRNA-SMO removal significantly promoted the expression of miR-326, while miR-326 inhibition did not affect circRNA-SMO expression. Collectively, these results suggest that circRNA-SMO sponges miR-326 to exert biological functions in glioblastoma development. This might be a promising strategy to target circRNA-SMO and miR326 to improve glioblastoma.

CEP85 has been reported to serve as PLK4-associated centriole duplication factors and directly regulates cell migration (Liu et al., 2020). In this study, higher levels of CEP85 were observed in glioblastoma tissues than in para-cancer tissues. However, miRNAs that interact with CEP85 have not yet been profiled. As speculated, we verified that miR-326 interacts with the 3'-UTR of CEP85 and negatively regulates its expression. Several circRNAs can act as competing molecules with miRNA response elements and affect the efficiency of miRNAs, thus regulating downstream mRNA expression (Bartel, 2009; Hansen et al., 2013; Liang et al., 2020). Here, circRNA-SMO inhibition induced miR-326 upregulation and subsequently inhibited CEP85 expression. These results also confirmed *in vivo* that circRNA-SMO knockdown negatively modulated the miR-326/CEP85 signaling axis, thereby suppressing the tumor growth of glioblastoma. The circRNA-miRNA axis plays a critical role in the development of several cancers, and some circRNAs have been used as prognostic or diagnostic biomarkers and may be potential molecular targets for glioblastoma treatment (Hao et al., 2019; Stella et al., 2021). Taken together, this study further explored the mechanism of circRNA-miRNA-mediated glioblastoma progression and elucidated the role of circRNA-SMO in glioblastoma, thereby offering helpful ideas for the screening and treatment of glioblastoma by considering circRNA-SMO, miR326, and CEP85 as gene signatures.

In conclusion, the results confirmed that circRNA-SMO is aberrantly upregulated in glioblastoma tissues

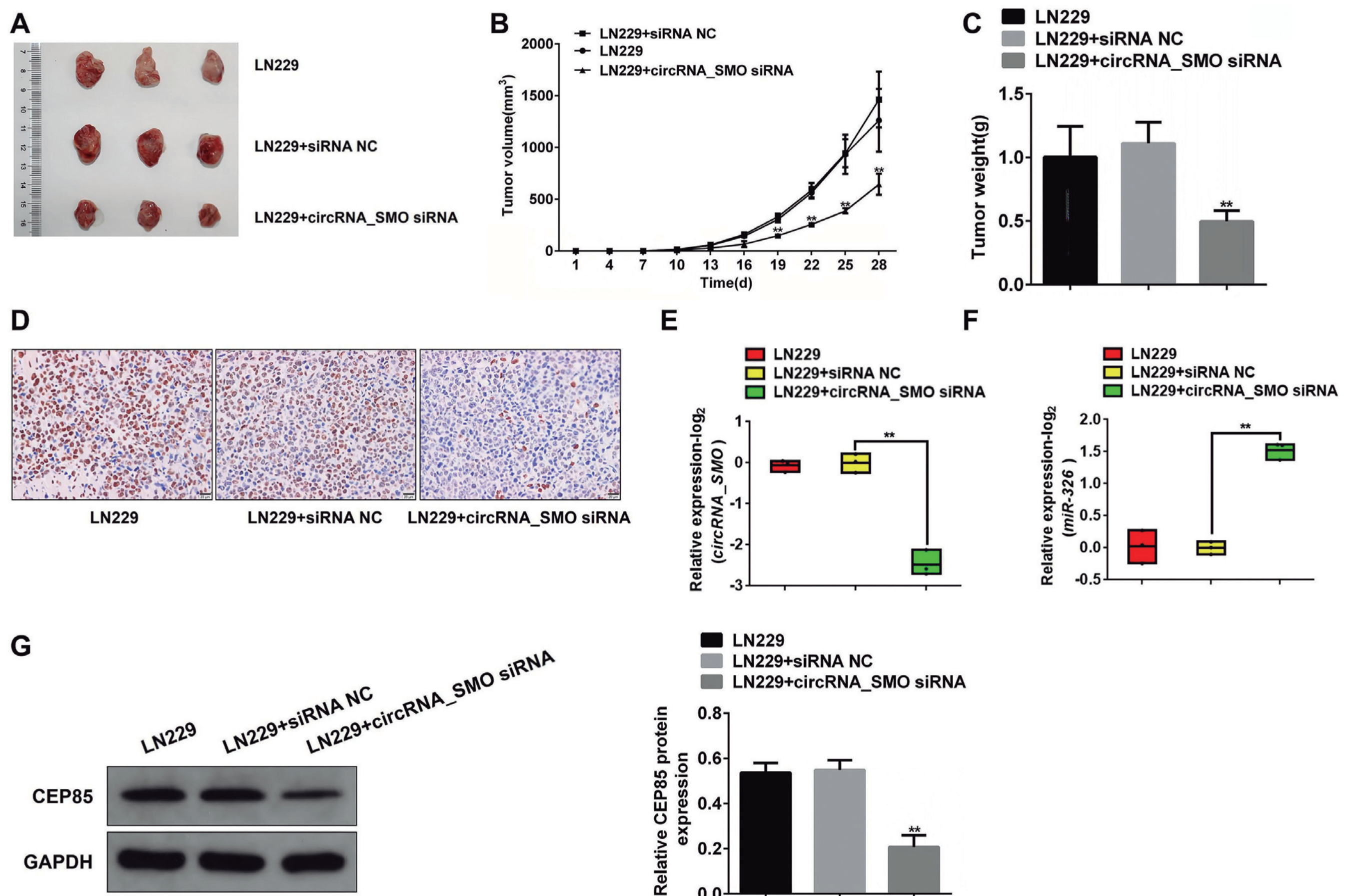

Fig. 8. Effects of circRNA-SMO inhibition on tumor growth. **A.** Mice were challenged with circRNA SMO siRNA or siRNA control. Tumor volume in each group were shown. **B, C.** The change in tumor volume **(B)** and weight **(C)** was determined in each group for a 28-day observation. **D.** Ki67 staining was used to detect the proliferation ability of tumors described in panel A. **E, F.** Expressions of circRNA-SMO **(E)** and miR-326 **(F)** of tumor tissue in each group were detected with RT-qPCR. **G.** Protein levels of CEP85 in tumor tissues was monitored by WB assay. And quantification of protein level was shown in right panel. All data analysis above used One-Way ANOVA. Data are shown as the mean ± SD, n=3. *P<0.05 and **P<0.01.

and cancer cell lines. CircRNA-SMO downregulation inhibits proliferation and tumor growth of glioblastoma cells. Mechanistically, circRNA-SMO sponges miR326, which subsequently suppresses the expression of CEP85 by directly binding to its 3'UTR, resulting in the development of glioblastoma. In summary, this study clarifies the role of circRNA-SMO and miR236 in glioblastoma and provides a meaningful strategy for glioblastoma treatment and drug development.

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Ethics approval and consent to participate. All animal studies were approved according to institutional guidelines for laboratory animals. This study was approved by the Animal Ethics Committee of the second Affiliated Hospital of Medical College of Zhejiang University.

Conflicts of interest. The authors declare that they have no competing interests.

Data Availability. The data used to support the findings of this study are included within the article.

Consent for publication. Not applicable.

References

Bach D.H., Long N.P., Luu T.T., Anh N.H., Kwon S.W. and Lee S.K. (2018). The dominant role of forkhead box proteins in cancer. Int. J. Mol. Sci. 19, 3279.

Banelli B., Forlani A., Allemanni G., Morabito A., Pistillo M.P. and Romani M. (2017). MicroRNA in glioblastoma: An overview. Int. J. Genomics. 2017, 7639084.

Barbagallo D., Palermo C.I., Barbagallo C., Battaglia R., Caponnetto A., Spina V., Ragusa M., Di Pietro C., Scalia G. and Purrello M. (2022). Competing endogenous RNA network mediated by circ_3205 in SARS-CoV-2 infected cells. Cell Mol. Life Sci. 79, 75.

Bartel D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell 136, 215-233.

Bolha L., Ravnik-Glavac M. and Glavac D. (2017). Circular RNAs: Biogenesis, function, and a role as possible cancer biomarkers. Int. J. Genomics 2017, 6218353.

Cao Y., Li J., Jia Y., Zhang R. and Shi H. (2020). CircRNA circ_POLA2 promotes cervical squamous cell carcinoma progression via regulating miR-326/ GNB1. Front. Oncol. 10, 959.

Chen C., Tian F., Lu L., Wang Y., Xiao Z., Yu C. and Yu X. (2015). Characterization of Cep85 - a new antagonist of Nek2A that is involved in the regulation of centrosome disjunction. J. Cell Sci. 128, 3290-3303.

Chen J., Xu S., Chen S., Zong Z., Han X., Zhao Y. and Shang H. (2019). CircPUM1 promotes the malignant behavior of lung adenocarcinoma by regulating miR-326. Biochem. Biophys. Res. Commun. 508, 844-849.

Du W., Liu X., Chen L., Dou Z., Lei X., Chang L., Cai J., Cui Y., Yang D., Sun Y., Li Y. and Jiang C. (2015). Targeting the SMO oncogene by miR-326 inhibits glioma biological behaviors and stemness. Neuro Oncol. 17, 243-253.

Geng X., Zhang Y., Li Q., Xi W., Yu W., Shi L., Lin X., Sun S. and Wang H. (2020). Screening and functional prediction of differentially expressed circular RNAs in human glioma of different grades. Aging (Albany NY) 13, 1989-2014.

Han C., Seebacher N.A., Hornicek F.J., Kan Q. and Duan Z. (2017). Regulation of microRNAs function by circular RNAs in human cancer. Oncotarget 8, 64622-64637.

Hansen T.B., Jensen T.I., Clausen B.H., Bramsen J.B., Finsen B., Damgaard C.K. and Kjems J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature 495, 384-388.

Hao Z., Hu S., Liu Z., Song W., Zhao Y. and Li M. (2019). Circular RNAs: Functions and prospects in glioma. J. Mol. Neurosci. 67, 72-81.

Jin P.C., Huang Y.N., Zhu P.L., Zou Y., Shao T.T. and Wang O. (2018). CircRNA circHIPK3 serves as a prognostic marker to promote glioma progression by regulating miR-654/IGF2BP3 signaling. Biochem. Biophys. Res. Commun. 503, 1570-1574.

Kefas B., Comeau L., Floyd D.H., Seleverstov O., Godlewski J., Schmittgen T., Jiang J., diPierro C.G., Li Y., Chiocca E.A., Lee J., Fine H., Abounader R., Lawler S. and Purow B. (2009). The neuronal microRNA miR-326 acts in a feedback loop with notch and has therapeutic potential against brain tumors. J. Neurosci. 29, 15161-15168.

Kefas B., Comeau L., Erdle N., Montgomery E., Amos S. and Purow B. (2010). Pyruvate kinase M2 is a target of the tumor-suppressive microRNA-326 and regulates the survival of glioma cells. Neuro Oncol. 12, 1102-1112.

Li W., Holsinger R.M., Kruse C.A., Flugel A. and Graeber M.B. (2013). The potential for genetically altered microglia to influence glioma treatment. CNS Neurol. Disord. Drug Targets 12, 750-762.

Li Y., Gao Y., Xu Y., Ma H. and Yang M. (2015). Down-regulation of miR-326 is associated with poor prognosis and promotes growth and metastasis by targeting FSCN1 in gastric cancer. Growth Factors 33, 267-274.

Liang Z.Z., Guo C., Zou M.M., Meng P. and Zhang T.T. (2020). circRNA-miRNA-mRNA regulatory network in human lung cancer: an update. Cancer Cell Int. 20, 173.

Liu J., Zhao K., Huang N. and Zhang N. (2019). Circular RNAs and human glioma. Cancer Biol. Med. 16, 11-23.

Liu Y., Kim J., Philipt R., Sridhar V., Chandrashekhar M., Moffat J., van Breugel M. and Pelletier L. (2020). Direct interaction between CEP85 and STIL mediates PLK4-driven directed cell migration. J. Cell Sci. 133, jcs238352.

Liu L., Zhang P., Dong X., Li H., Li S., Cheng S., Yuan J., Yang X., Qian Z. and Dong J. (2021). Circ_0001367 inhibits glioma proliferation, migration and invasion by sponging miR-431 and thus regulating NRXN3. Cell Death Dis. 12, 536.

Lu Y., Deng X., Xiao G., Zheng X., Ma L. and Huang W. (2019). circ_0001730 promotes proliferation and invasion via the miR-326/Wnt7B axis in glioma cells. Epigenomics 11, 1335-1352.

Mirimanoff R.O. (2014). High-grade gliomas: reality and hopes. Chin. J. Cancer 33, 1-3.

Nawaz Z., Patil V., Paul Y., Hegde A.S., Arivazhagan A., Santosh V. and Somasundaram K. (2016a). PI3 kinase pathway regulated

miRNome in glioblastoma: identification of miR-326 as a tumour suppressor miRNA. Mol. Cancer 15, 74.

Nawaz Z., Patil V., Paul Y., Hegde A.S., Arivazhagan A., Santosh V. and Somasundaram K. (2016b). PI3 kinase pathway regulated miRNome in glioblastoma: identification of miR-326 as a tumour suppressor miRNA. Mol. Cancer 15, 74

Omuro A. and DeAngelis L.M. (2013). Glioblastoma and other malignant gliomas: a clinical review. JAMA 310, 1842-1850.

Pamudurti N.R., Bartok O., Jens M., Ashwal-Fluss R., Stottmeister C., Ruhe L., Hanan M., Wyler E., Perez-Hernandez D., Ramberger E., Shenzis S., Samson M., Dittmar G., Landthaler M., Chekulaeva M., Rajewsky N. and Kadener S. (2017). Translation of CircRNAs. Mol. Cell 66, 9-21 e27.

Qi L., Wang W., Zhao G., Jiang H., Zhang Y., Zhao D., Jin H., Yu H. and Xu H. (2021). Circular RNA circitga7 accelerates glioma progression via miR-34a-5p/VEGFA axis. Aging (Albany NY) 13, 13138-13152.

Stella M., Falzone L., Caponnetto A., Gattuso G., Barbagallo C., Battaglia R., Mirabella F., Broggi G., Altieri R., Certo F., Caltabiano R., Barbagallo G.M.V., Musumeci P., Ragusa M., Di Pietro C., Libra M., Purrello M. and Barbagallo D. (2021). Serum extracellular vesicle-derived circHIPK3 and circSMARCA5 are two novel diagnostic Biomarkers for glioblastoma multiforme. Pharmaceuticals (Basel) 14, 618.

Sun J., Li B., Shu C., Ma Q. and Wang J. (2020). Functions and clinical significance of circular RNAs in glioma. Mol. Cancer 19, 34.

Wang S., Lu S., Geng S., Ma S., Liang Z. and Jiao B. (2013). Expression and clinical significance of microRNA-326 in human glioma miR-326 expression in glioma. Med. Oncol. 30, 373.

Wang B., Li B. and Si T. (2020). Knockdown of circ0082374 inhibits cell viability, migration, invasion and glycolysis in glioma cells by miR-326/SIRT1. Brain Res. 1748, 147108.

Weller M., Wick W., Aldape K., Brada M., Berger M., Pfister S.M., Nishikawa R., Rosenthal M., Wen P.Y., Stupp R. and Reifenberger G. (2015). Glioma. Nat. Rev. Dis. Primers 1, 15017.

Wu X.J., Xiao S.H., Zhang M.L., Yang L.X., Zhong J., Li B., Li F., Xia X., Li X., Zhou H., Liu D., Huang N., Yang X., Xiao F. and Zhang N. (2021). A novel protein encoded by circular SMO RNA is essential for Hedgehog signaling activation and glioblastoma tumorigenicity. Genome Biol. 22, 33.

Xiong Z., Zhou C., Wang L., Zhu R., Zhong L., Wan D. and Wang Q. (2019). Circular RNA SMO sponges miR-338-3p to promote the growth of glioma by enhancing the expression of SMO. Aging (Albany NY) 11, 12345-12360.

Yao T., Chen Q.Q., Fu L.Y. and Guo J.M. (2017). Circular RNAs: Biogenesis, properties, roles, and their relationships with liver diseases. Hepatol. Res. 47, 497-504.

Zhang X., Yang H., Zhao L., Li G. and Duan Y. (2019). Circular RNA PRKCI promotes glioma cell progression by inhibiting microRNA-545. Cell Death Dis. 10, 616.

Zheng J., Liu X., Xue Y., Gong W., Ma J., Xi Z., Que Z. and Liu Y. (2017). TTBK2 circular RNA promotes glioma malignancy by regulating miR-217/HNF1beta/Derlin-1 pathway. J. Hematol. Oncol. 10, 52.

Zhou J., Xu T., Yan Y., Qin R., Wang H., Zhang X., Huang Y., Wang Y., Lu Y. Fu D. and Chen J. (2013). MicroRNA-326 functions as a tumor suppressor in glioma by targeting the Nin one binding protein (NOB1). PLoS One 8, e68469.

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