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DGCR5 suppresses the EMT of pediatric primary glioblastoma multiforme cell and serves as a prognostic biomarker

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Abstract. – OBJECTIVE: Pediatric primary glioblastoma multiforme (GBM) is a common brain tumor among childhood. Nevertheless, the underlying mechanism of glioblastoma progresses remains to be illuminated. The current study explores the potential functions of long noncoding RNA (lncRNA) DGCR5 in the aggressiveness of GBM.

PATIENTS AND METHODS: The expression of DGCR5 was evaluated by quantitative real-time PCR (qRT-PCR). Immunoblotting was carried out to assess the protein expressions of N-cadherin and E-cadherin. Cell Counting Kit-8 (CCK-8), wound healing, transwell assay, and flow cytometry were applied to explore the roles of DGCR5 in glioblastoma cell malignant biological behaviors.

RESULTS: LncRNA DGCR5 was down expressed in glioblastoma. Transfection of DGCR5 markedly repressed cell viability and colony formation ability of U87 and U251 cells. Additional, DGCR5 overexpression caused cell cycle arrest and increased cell apoptosis. Moreover, upregulation of DGCR5 suppressed the growth and promoted cell apoptosis of U87 cell *in vivo*. Finally, overexpression of DGCR5 impaired the migration, invasiveness, and reversed epithelial-to-mesenchymal transition (EMT) process of glioblastoma cell.

CONCLUSIONS: LncRNA DGCR5 inhibited the proliferation, aggressiveness phenotypes, and EMT of glioblastoma cell.

Key Words:
DGCR5, Glioblastoma, EMT, Metastasis, Apoptosis.

Introduction

Glioblastoma multiforme (GBM) is still one of the most common and fatal brain cancer types[1]. Although the recent advance in the comprehension of its pathogenesis, GBM remains an incurable disease[2]. The five-year survival rate of patient with GBM is only 10%[3-5]. Efforts to develop novel treatment options for GBM, recent investigations have focused on the molecular phenotyping of GBM subtypes to explore new biomarkers for GBM[6-9].

Recently, long noncoding RNAs (lncRNAs) have been identified as critical regulators in various biological processes among cancers. For example, lncRNA MELTF Antisense RNA 1 (MFI2-AS1) promotes colon cancer cell growth, migration, and invasion *via* regulating miR-574-5p/MYCBP axis[10]. LncRNA UICLM promotes the liver metastasis of colorectal cancer by acting as a competing endogenous RNA (ceRNA) for microRNA-215 (miR-215) to regulate zinc finger E-Box binding homeobox 2 (ZEB2) expression[11]. In papillary thyroid carcinoma (PTC), lncRNA small nucleolar RNA host gene 12 (SNHG12) facilitates the cell growth and metastasis of PTC cell by modulating Wnt/beta-catenin signaling pathway[12]. LncRNA linc00511 increases the proliferation and migration of osteosarcoma cell by sponging miR-765[13].

Increasing evidence has demonstrated the crucial function of lncRNAs during the progression of glioblastoma. Cell growth and metastatic-related traits of glioblastoma cell have been proved to be regulated by several lncRNAs, such as Linc00152, Linc01446, HIF1A Antisense RNA 2 (HIF1A-AS2), and Linc01426[14-17]. Nevertheless, other important lncRNAs which are significantly associated with glioblastoma remain to be investigated. LncRNA DiGeorge syndrome critical region gene 5 (DGCR5) is a suppressor in pancreatic ductal adenocarcinoma, hepatocellular carcinoma (HCC), and human lung cancer. In

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papillary thyroid carcinoma (PTC), DGCR5 is remarkably downregulated in PTC tissues and lncRNA DGCR5 suppresses the progression of PTC by sponging miR-2861[18]. In addition, DGCR5 is involved into the regulation of growth and metastasis of lung cancer by regulating the level of miR-1180[19]. Downregulation of lncRNA DGCR5 also facilities the aggressive phenotypes of cervical carcinoma cell by activating the Wnt signaling pathway[3]. Nevertheless, the potential role of lncRNA DGCR5 in glioblastoma remains not well known.

We proved that lncRNA DGCR5 was down expressed in human glioblastoma. Low level of DGCR5 was related with poor clinical outcome of patient with glioblastoma. Overexpression of DGCR5 markedly suppressed the growth and metastatic-related traits of glioblastoma cell. In addition, the EMT process was inhibited by lncRNA DGCR5, Altogether, these findings imply that DGCR5 suppresses the EMT of glioblastoma and serves as a prognostic indicator.

Patients and Methods

Glioblastoma Tissues

Surgically resected paired human glioblastoma tissue and corresponding normal brain tissue were obtained from patient with primary glioblastoma from Xi'an No. 9 Hospital. The histological grade of all glioblastoma tissues was classified by experienced pathologists using WHO criteria. All enrolled patients did not receive any treatment. All glioblastoma samples were preserved in liquid nitrogen and then stored at -80°C. The clinicopathological features of these patients with glioblastoma were summarized in Table I. The research was approved by the Ethical Committee in Xi'an No.9 Hospital (Xi'an, China) and the informed consents were obtained from patients before this study.

Cell Lines

Human glioblastoma cells (LN229, A172, U251, and U87) were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The normal human astrocytes (NHAs) were bought from ScienCell Research Laboratories (ScienCell; Invitrogen, Carlsbad, CA, USA). The cells were maintained using Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 microg/mL streptomycin and 100 UI/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and cultured at 37°C with 5% CO_2.

Cell Viability

U87 or U251 cell were plated into 96-well plates. The proliferative rate was monitored for five days. 10 µl of cell counting kit-8 (CCK-8) solutions (Beyotime, Nantong, Jiangsu, China) were added into well at each monitored time point. After further incubation for 4 hours, the absorbance value was assessed at 450 nm using a microplate reader.

Table I. Relationship between the expression of lncRNA DGCR5 and clinicopathological parameters in glioblastoma.

Parameter	lncRNA DGCR5		*p*-value
	High (n)	Low (n)	
Age (years)			0.271
< 50	20	16	
> 50	0	0	
Gender			0.609
Male	8	11	
Female	7	10	
Extent of resection			0.122
< 98%	11	7	
≥ 98%	6	12	
WHO grade			0.003
I-II	5	16	
III-IV	4	11	
KPS score			0.023
< 80	6	10	
≥ 80	8	12	

Colony Formation

U87 and U251 were seeded into a six well plate and were maintained using fresh medium for two weeks. Then, cell colonies were stained using crystal violet (1%) for 15 min. The cell colony (with over 50 cells) was recorded in each group.

Soft Agar Assay

U87 or U251 cells were suspended in 1 ml of 0.3% agar and seeded into 60 mm dishes that were overlayed with 0.6% agar. Culture medium in each well was changed every 3 days. After 14 days, cell colonies (over 50 cells) were stained using 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA).

Migration Assay

U87 and U251 cells were cultured in six well plates overnight. Then, an artificial wound was made using sterile pipette tips. Cells were cultured at 37°C for 24 h. The percentage of wound closure was quantified.

Invasion Assay

The polycarbonate membrane in Boyden chambers (8 μm pores, transwell, Costar, Cambridge, MA, USA) was coated with Matrigel (BD Biosciences; Franklin Lakes, NJ, USA). 100 μl of cells (1×10^6) was added to the upper chamber and 600 μl DMEM containing 20% FBS was added to the lower chamber of Boyden. After 18 hours, the invaded cells were fixed and stained using crystal violet for 15 min. Finally, the number of invaded cells was counted.

Cell Cycle Analysis

U87 cells and U251 cells were fixed using 70% ethanol for 15 min. After RNA degradation using 20 mg/ml of RNase (Sigma-Aldrich, St. Louis, MO, USA), cells were stained with propidium iodide (PI) solution (20 mg/ml; Bioworld, Nanjing, Jiangsu, China). Finally, the cell proportion was detected using FACSCalibur flow cytometry (BD Biosciences; Franklin Lakes, NJ, USA).

Cell Apoptosis

U87 and U251 cells (2×10^6) were seeded in 6-well plates for 24 hours. Then, cells were collected and incubated with Annexin V-FITC (5 μl) and of PI solution (5 μl; Bioworld, Nanjing, Jiangsu, China) for 15 min. Subsequently, cells were suspended in 400 μl of binding buffer. Finally, cell apoptosis was then analyzed using flow cytometry.

Quantitative Real-Time PCR (qRT-PCR) Assay

RNAs were extracted using TRIzol Reagent (Thermo Fisher Scientific; Waltham, MA, CA, USA). The RNAs were transcribed into cDNAs using the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Otsu, Shiga, Japan). qRT-PCR assay was carried out using the SYBR Premix Ex Taq Kit (TaKaRa, Otsu, Shiga, Japan) in an ABI PRISM 7500 Real-Time System. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included as internal control. The fold change was calculated using relative quantification $2^{-\Delta\Delta Ct}$. The primers sequences were shown in Table II.

Table II. Primers for qRT-PCR assay.

Primer	Primer Sequence (5′-3′)	
DGCR5	Forward	5’-CCAAGCCTGTCTGTGTGTTC-3’
	Reverse	5’-GGGAGACACAGACCACAAGA-3’
GAPDH	Forward	5’-TATGATGATATCAAGAGGGTAGT-3’
	Reverse	5’-TGTATCCAAACTCATTGTCATAC-3’
ZEB1	Forward	5’-GATGATGAATGCGAGTCAGATGC-3’
	Reverse	5’-ACAGCAGTGTCTTGTTGTTGT-3’
Vimentin	Forward	5’-CGTCCACACGCACCTACAG-3’
	Reverse	5’-GGGGGATGAGGAATAGAGGCT-3’
Fibronection	Forward	5’-GAGAATAAGCTGTACCATCGCAA-3’
	Reverse	5’-CGACCACATAGGAAGTCCCAG-3’
ZO-1	Forward	5’-GTGTTGTGGATACCTTGT-3’
	Reverse	5’-GATGATGCCTCGTTCTAC-3’
α-catenin	Forward	5’-CTTGAGGAAGTTCGCAAAGAAAG-3’
	Reverse	5’-GCAAGAGGCACATGACATCAAT-3’
β-catenin	Forward	5’-CATCTACACAGTTTGATGCTGCT-3’
	Reverse	5’-GCAGTTTTGTCAGTTCAGGGA-3’

Immunoblotting

Total proteins in cells were extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer. 30 μg proteins were separated by 10% sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. After blocking, the PVDF membrane was incubated with N-cadherin, E-cadherin or GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then, PVDF membrane was incubated with secondary antibody (Bioworld, Nanjing, Jiangsu, China), and the immunoreactivity was detected using enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA).

Xenograft Model of Glioblastoma

Control vector or DGCR5 transfected U87 cells (5×10^6) were inoculated into athymic BALB/c nude mice. Tumor dimension was measured once a week. Tumor volume was calculated as length × width^2/2. After four weeks, the mice were sacrificed and tumor tissues were weighed. Tumor tissue was paraffin embedded and cut into 4 μm slide. The slide was subject for immunohistochemistry (IHC) assay. Animal experiments were approved by the Committee for Animal Research of Xi'an No. 9 Hospital. The animal experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).

Statistical Analysis

Data were expressed as Mean ± SD. Comparisons between groups were analyzed using Student's *t*-test. The survival rate was calculated by Kaplan-Meier method and was analyzed by log-rank test. $p<0.05$ was considered as statistically significant.

Results

LncRNA DGCR5 Is Down Expressed in Glioblastoma

First, we assessed the expression of DGCR5 in glioblastoma tissues in The Cancer Genome Atlas (TCGA) database. As shown in Figure 1A, DGCR5 was significantly down-regulated in glioblastoma tissues compared to that in non-tumor tissue samples. Moreover, we measured the expressions of DGCR5 in 36 pairs of glioblastoma and non-malignant tissues. Unsurprisingly, the level of DGCR5 was lower in glioblastoma tissues than that in non-malignant tissues (Figure 1B). Furthermore, the level of DGCR5 in tissues with advanced stage was also significantly lower than that in early stage tissue (Figure 1C). The lower level of DGCR5 was also observed in glioblastoma cell lines, when compared to that in the normal cell (Figure 1D). According to the mean value of DGCR5 expression, glioblastoma tissues were divided into two groups (high expression of DGCR5 and low expression of DGCR5). Subsequently, we observed that the low expression of DGCR5 was related with poor overall survival (Figure 1E). Such results indicate that DGCR5 acts as a suppressive lncRNA in the progression of glioblastoma.

LncRNA DGCR5 Inhibits the Colony Formation of U87 and U251

To illuminate the role of DGCR5 in glioblastoma, U87 and U251 cell was transfected with control vector or DGCR5 (Figure 2A). Then, cell viability was detected in glioblastoma cell lines after transfected with DGCR5. As shown in Figure 2B, the cell viability in DGCR5-transfected cell was significantly inhibited. Likewise, transfection of DGCR5 decreased the colony formation abilities of U87 and U251 cell (Figure 2C-2D). All these results indicate that overexpression of DGCR5 inhibits cell viability and colony formation of glioblastoma cells.

Transfection of DGCR5 Arrested Cell Cycle and Induces Cell Apoptosis in Glioblastoma

The inhibitory impacts of DGCR5 on glioblastoma cell were then determined on cell apoptosis. In cell cycle assay, we observed that lncRNA DGCR5 caused cell cycle arrested (Figure 3A). The cell proportion in S phase was nearly 59% in control U87 cell, whereas was decreased nearly 20% in DGCR5-transfected U87 cell. Accordingly, the cell proportion in the G0/G1 phase in U87 cell was increased by approximately 14% after transfection of DGCR5. Comparable cell cycle arrest at the G0/G1 phase was also found in U251 cell. Then, the apoptotic rate of U87 and U251 cell was determined. While the apoptotic rate of control U87 and U251 cell was approximately 10%, while the apoptotic rate was up to 45% in DGCR5-transfected U87 cell and 41% in DGCR5-transfected U251

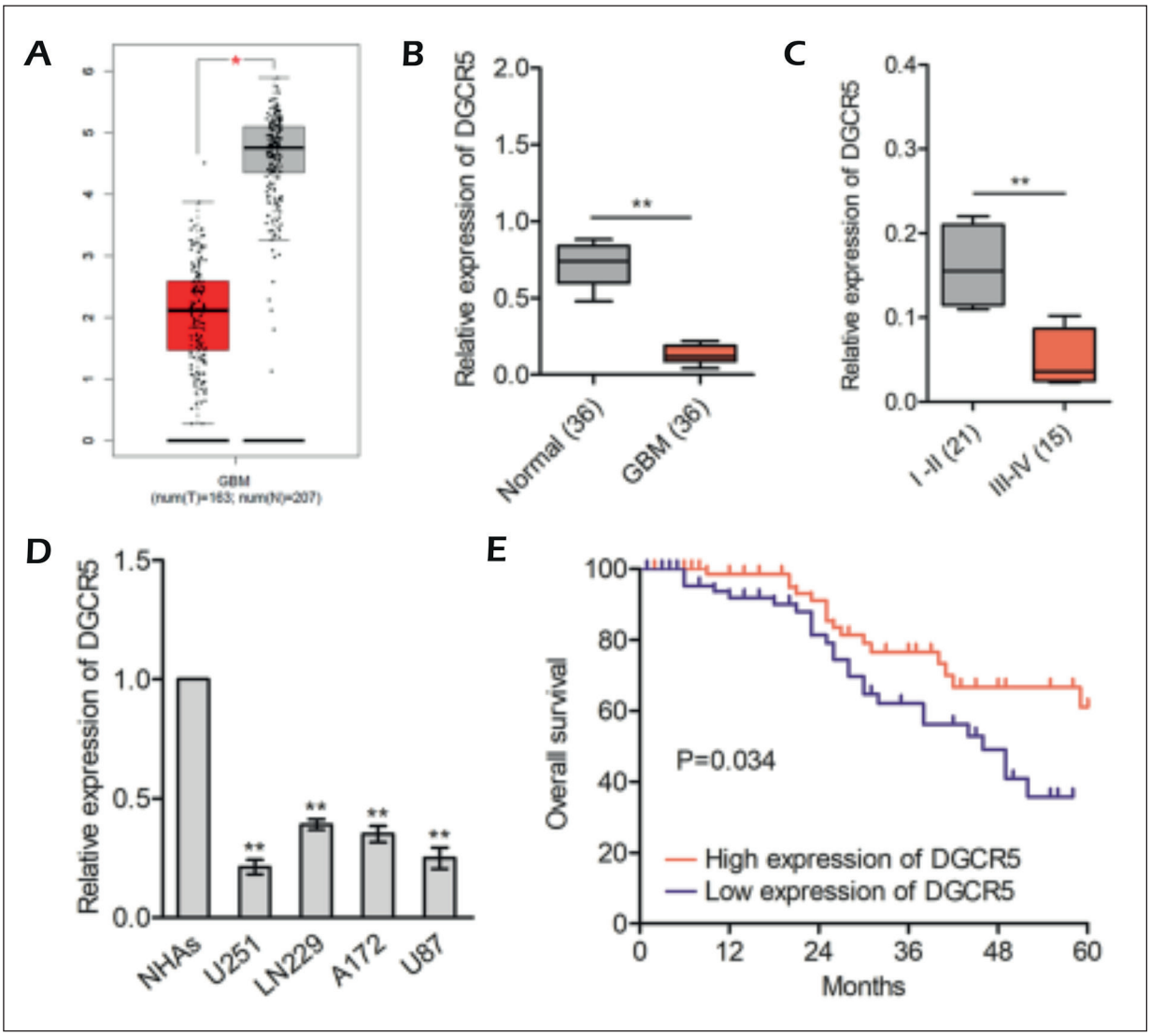

Figure 1. Highly expressed DGCR5 predicts poor prognosis for patients with GBM. **A,** Expression pattern of SNHG3 in GBM tissues and normal tissues of TCGA database was identified and analyzed. **B,** QRT-PCR detected the level of DGCR5 in 36 pairs of GBM tissues and non-malignant tissues. $^{**}p<0.01$ *vs.* Normal. **C,** Expressions of DGCR5 in GBM tissues which was in different tumor stages were examined with qRT-PCR. $^{**}p<0.01$ *vs.* I-II. **D,** Expressions of DGCR5 in GBM cells were determined by qRT-PCR. $^{**}p<0.01$ *vs.* NHAs. **E,** Kaplan-Meier was analyzed by log-rank test to analyze the correlation between the expression of DGCR5 and patients' overall survival.

cell (Figure 3B). These findings indicate that upregulation of DGCR5 induces cell apoptosis in glioblastoma.

LncRNA DGCR5 Inhibits the Tumor Growth of Glioblastoma In Vivo

DGCR5 transfected U87 cells were inoculated into nude mice to establish a xenograft model of glioblastoma cell. 28 days after cell inoculation, tumor tissues were resected and we observed that mice injected with DGCR5 transfected cells had smaller size compared to control group (Figure 4A). Meanwhile, the weight of tumor tissue from DGCR5 group was markedly less than that in control (Figure 4B). Finally, the Immunohistochemical (IHC) analysis suggested that the tumor tissue from DGCR5-transfected group had less Ki-67 positive staining (Figure 4C). Meanwhile, neoplasia from DGCR5-transfected group exhibited more TUNEL-positive staining (Figures 4D). All these observations suggest that DGCR5 inhibits tumor growth in glioblastoma.

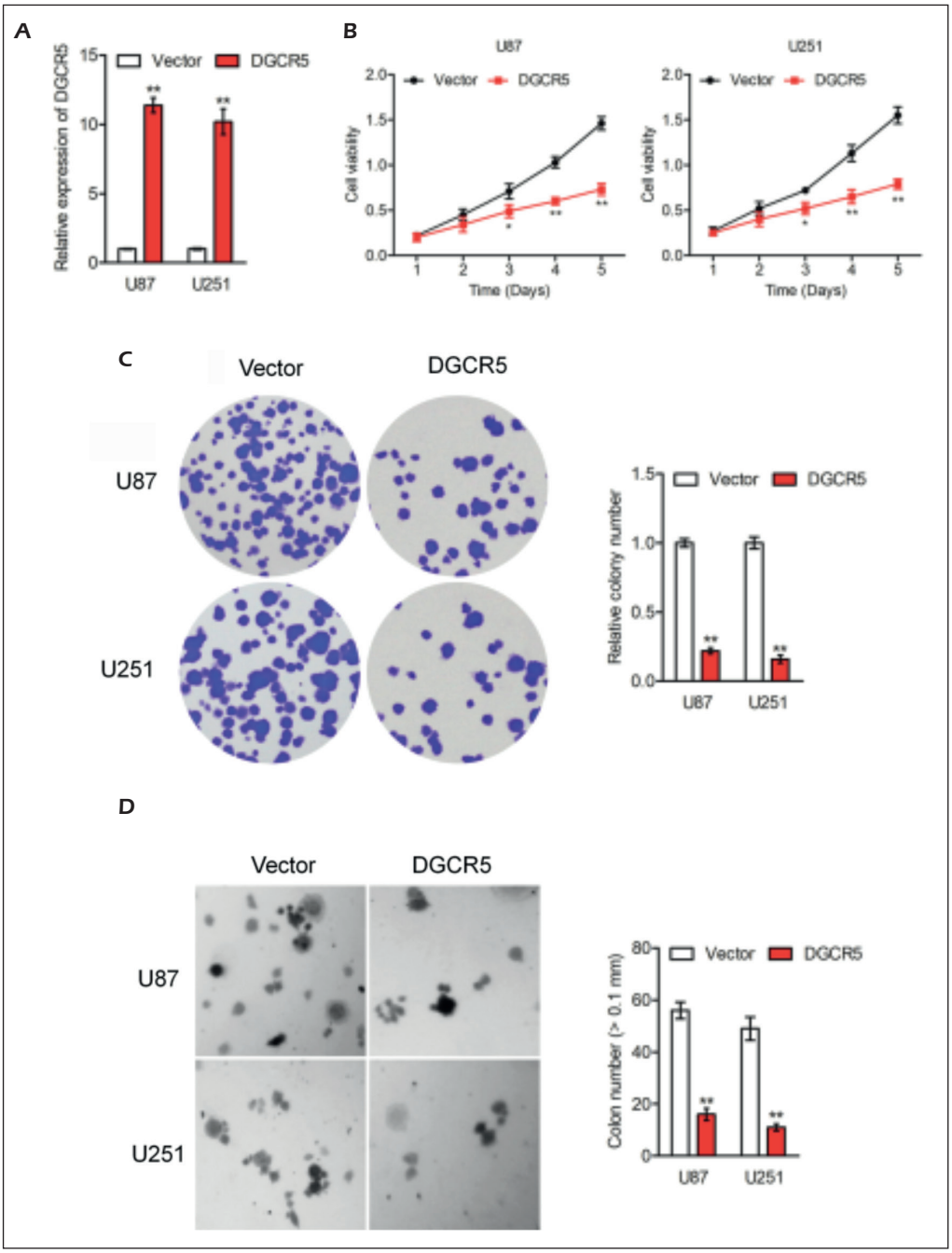

Figure 2. LncRNA DGCR5 inhibits cell viability and colony formation capacity in U87 and U251 cells. **A,** Levels of DGCR5 in U87 and U251 cell that was transfected with empty vector or DGCR5 were detected by qRT-PCR assay. **B,** After transfection of DGCR5 in U87 and U251 cells, cell proliferative rates were monitored in a consecutive of 5 days. **C,** Control and DGCR5-transfected cells were subject to colony formation assay in U87 and U251 cells. Formed colonies were stained with crystal violet, and all colonies in each group were manually counted. **D,** DGCR5 suppressed the colony formation of U87 and U251 cell in soft agar. $^{**}p<0.01$ *vs.* Vector.

Overregulation of DGCR5 Inhibits Cell Migration in Glioblastoma

The migration capacity and invasiveness of glioblastoma cell were analyzed using wound healing and transwell assay. We found that the migration of both U87 and U251 cells was markedly inhibited by DGCR5 (Figure 5A). The wound closure was decreased by 46.7% in U87 cell and 45% in U251 cell. Meanwhile, the invasion test indicated that DGCR5 transfection repressed the

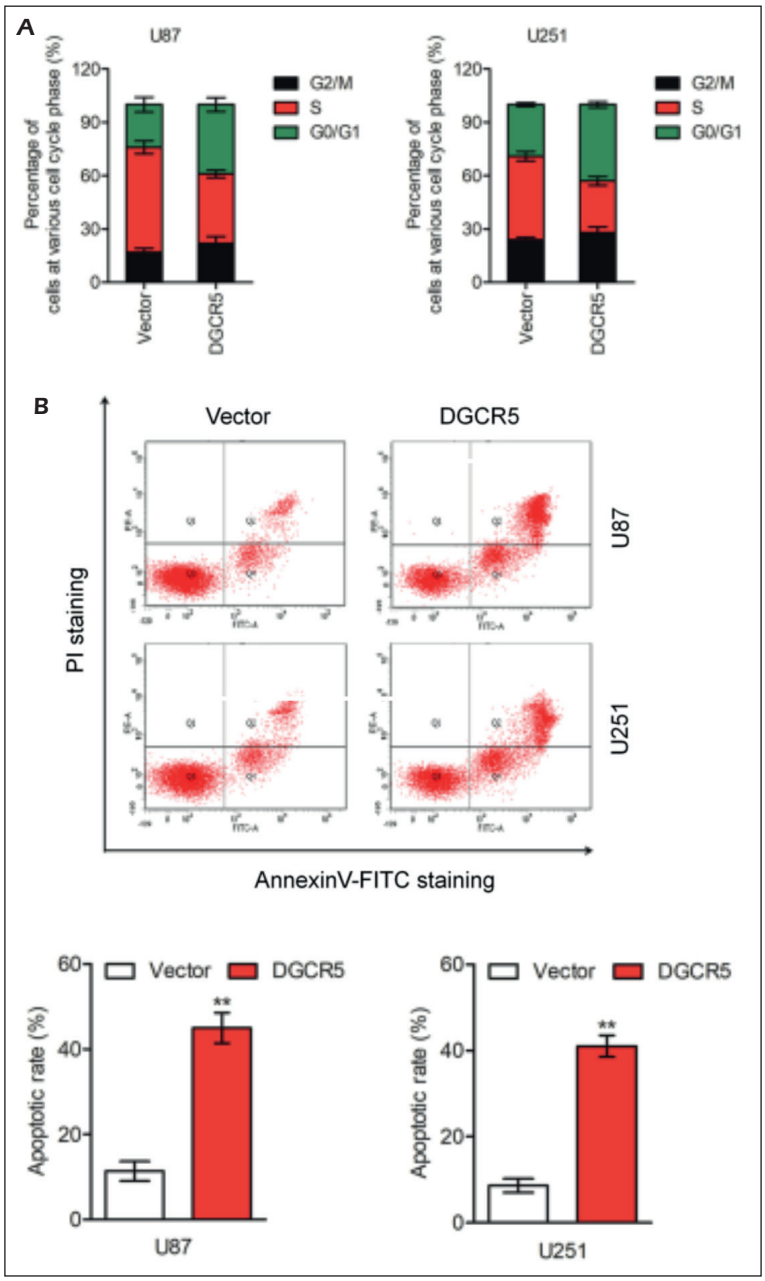

Figure 3. DGCR5 induces cell apoptosis in glioblastoma. **A**, Cell cycle progression was analyzed in both U87 cells and U251 cells that were transfected with vector or DGCR5. **B,** Cell survival was determined in both cell lines with or without DGCR5 transfection. $^{**}p<0.01$ *vs.* Vector.

invasion of glioblastoma cell (Figure 5B). All the results indicate that overregulation of DGCR5 suppresses the migration and invasion of glioblastoma cell.

DGCR5 Inhibits the EMT Process of Glioblastoma

Finally, the epithelial marker (E-cadherin) and mesenchymal marker (N-cadherin) in glioblastoma cell were detected using Western blotting assay. As shown in Figure 6A, DGCR5 increased the protein of E-cadherin and reduced the level of N-cadherin in both U251 and U87 cell. Consistently, qRT-PCR analysis also suggested that the mRNA levels of epithelial markers (α-catenin, ZO-1 and β-catenin) were increased and the levels of mesenchymal markers (Vimentin, ZEB1 and Fibronection) were decreased by DGCR5 overexpression (Figure 6B). All these results suggest that upregulation of DGCR5 reverses the EMT process in glioblastoma cell.

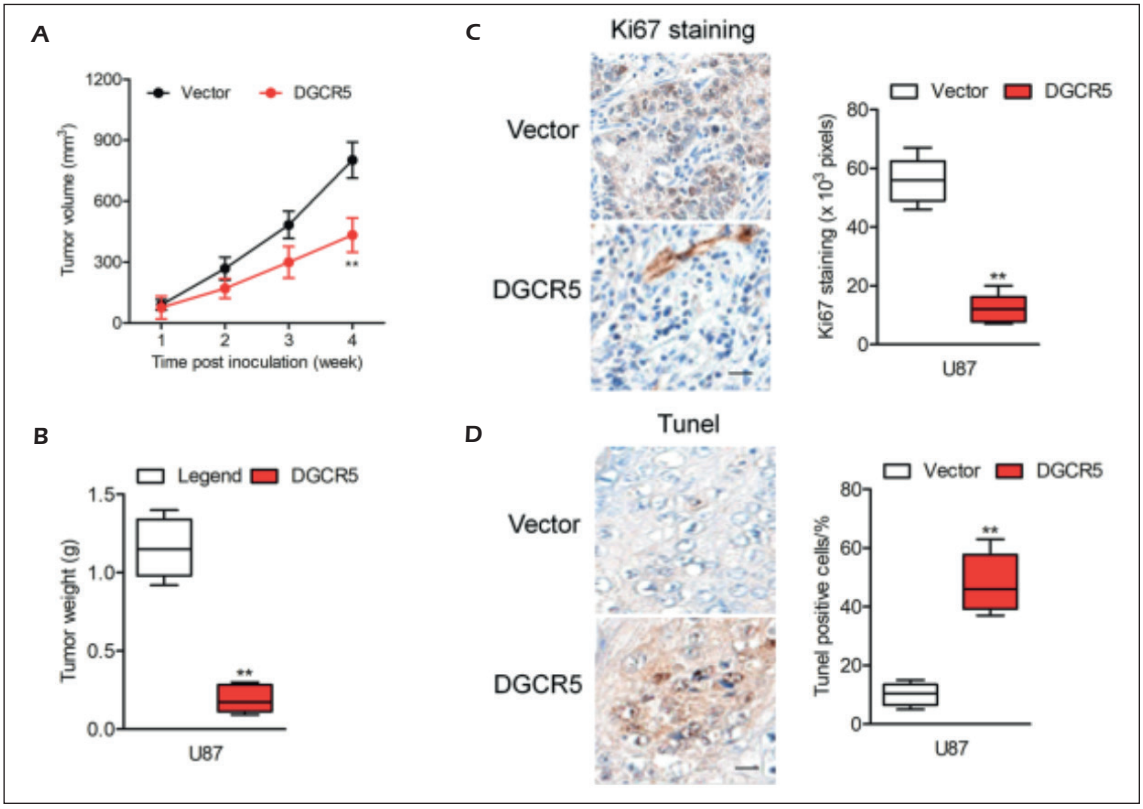

Figure 4. DGCR5 inhibits tumor growth in glioblastoma *in vivo*. **A,** U87 cells were transfected with the empty (control group) or DGCR5 prior to inoculation into nude mice. During the 4-week monitoring, tumor dimensions were measured and tumor volume was calculated for each group of mice. **B,** Four weeks after inoculation, neoplasia were resected and weighed. **C**, Tumor tissues were subject to immunohistochemistry analysis of Ki-67. **D,** Tumor tissues were subject to immunohistochemistry analysis of TUNEL staining. ***p*<0.01 *vs.* Vector.

Discussion

Glioblastoma remains the major health problem which threating children's lives worldwide. Furthermore, traditional therapeutic options for glioblastoma are related with several side effects. Hence, exploration of molecular biomarkers during glioblastoma development is necessary to find potential therapeutic target for patients with glioblastoma. Previous investigations have indicated that high level of lncRNA DGCR5 is correlated with the better overall survival and DGCR5 impairs the progression of bladder cancer *via* modulating P21 expression[20]. In hepatocellular carcinoma (HCC), DGCR5 restrains the progression of HCC by modulating miR-346/KLF14 axis[21]. In addition, downregulation of DGCR5 increases the radio-sensitivity of laryngeal carcinoma cell by regulating miR-195[22]. Nevertheless, limited knowledge is available about whether lncRNA DGCR5 affected the biological aggressiveness of glioblastoma cell.

The current research provided evidence that DGCR5 plays suppressive functions in glioblastoma. First, we proved that DGCR5 is markedly down expressed in glioblastoma tissues and cells. Then, the colony formation and cell viability of glioblastoma cell U87 and U251 were markedly suppressed after transfection of DGCR5 in glioblastoma cell lines. *In vivo*, the growth of U87 cell was also impaired in xenografted model of glioblastoma cell. Deregulation of cell cycle is one of the hallmarks of cancer cell proliferation and is associated with apoptosis. We furthermore proved that DGCR5 overexpression induced cell cycle arrest and increased cell apoptosis. Altogether, these results imply that DGCR5 inhibits cell growth in glioblastoma.

Metastasis is a hallmark of a more aggressive cancer. Cancer cell metastasis requires growth, angiogenesis, and invasion into local lymphatic or/and capillary network. The key aspects during cancer cell metastasis are acquisition of migration and invasion. In this study, we ob-

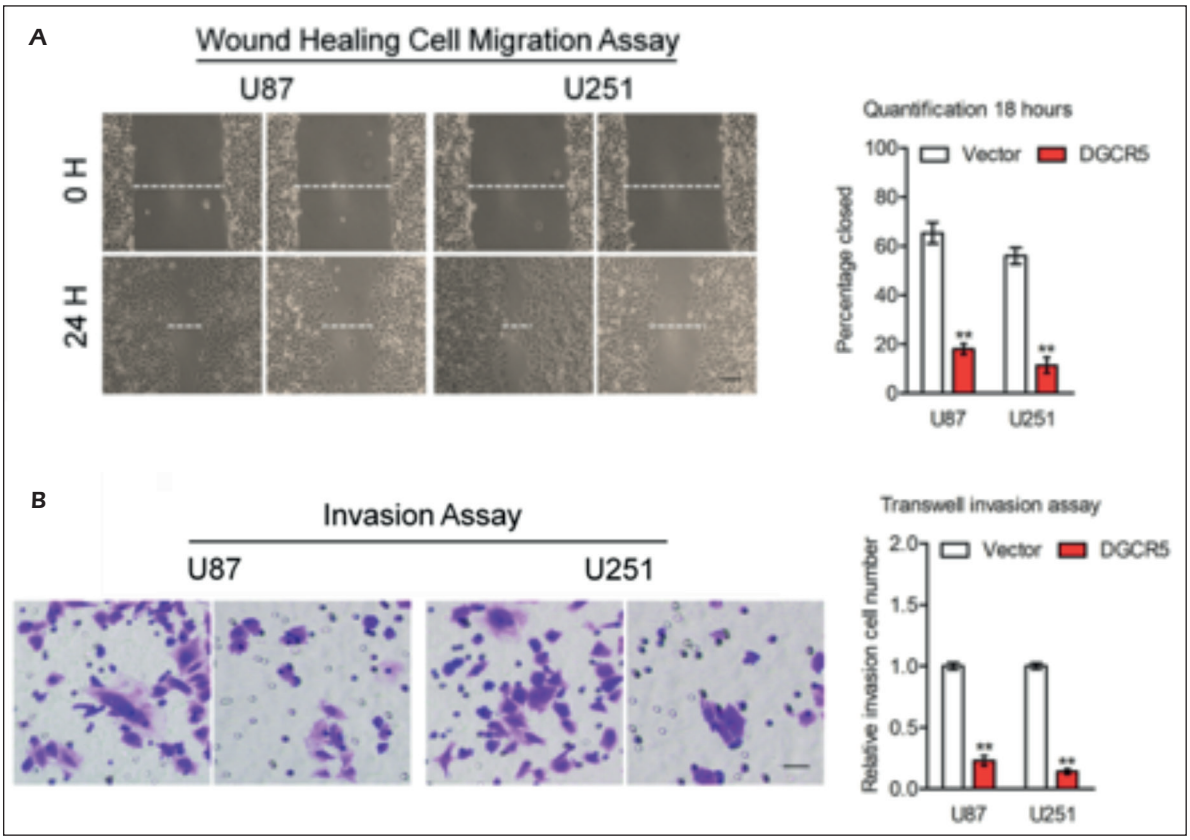

Figure 5. DGCR5 inhibits cell migration and invasion of glioblastoma cell. **A,** Control or DGCR5-transfected U87 and U251 cells were subject to wound-healing assay. Representative images showing the wound recovery were shown at 0 h and 24 h for both cell lines. The wound-recovered area which represented the cell migration capacity was calculated for each group of cells. **B,** Both U87 and U251 cells were subject to transwell invasion assay. Cells that invaded to the lower surface were stained with crystal violet. Invaded cells were counted and averaged from 5 randomly selected fields. $^{**}p<0.01$ *vs.* Vector.

served that overexpression of DGCR5 impaired the migration and invasion capacities of U87 and U251 cells as evidenced by wound closure and transwell invasion assay. These results are consistent with the previous report, in which DGCR5 overexpression inhibits the growth and invasion of papillary thyroid carcinoma cell[18].

The epithelial-mesenchymal transition (EMT) process is regulated by several cellular procedures, including increased levels of mesenchymal markers, N-cadherin and vimentin, reduced the expressions of epithelial marker, E-cadherin. Increasing investigations have demonstrated that EMT is allied to human tumorigenesis. In the current study, we proved that the protein expression of N-cadherin was decreased while the epithelial marker, E-cadherin was increased in cell transfected with DGCR5, indicating that the EMT process was reversed by DGCR5 overexpression. Therefore, DGCR5 overexpressing glioblastoma cells had less invasion and migration abilities.

Conclusions

In the present study, we identify lncRNA DGCR5 as a crucial regulator of cell viability and migrate and invasion abilities in glioblastoma. LncRNA DGCR5 is markedly down expressed in glioblastoma. Upregulation of DGCR5 impairs the growth and aggressiveness of glioblastoma cell. Finally, we demonstrate that lncRNA DGCR5 regulates mRNA and protein expression of classic epithelial-mesenchymal transition (EMT) markers. All these results indicate that lncRNA DGCR5 inhibits glioblastoma progression and serves as a prognostic biomarker.

Conflict of Interest

The Authors declare that they have no conflict of interests.

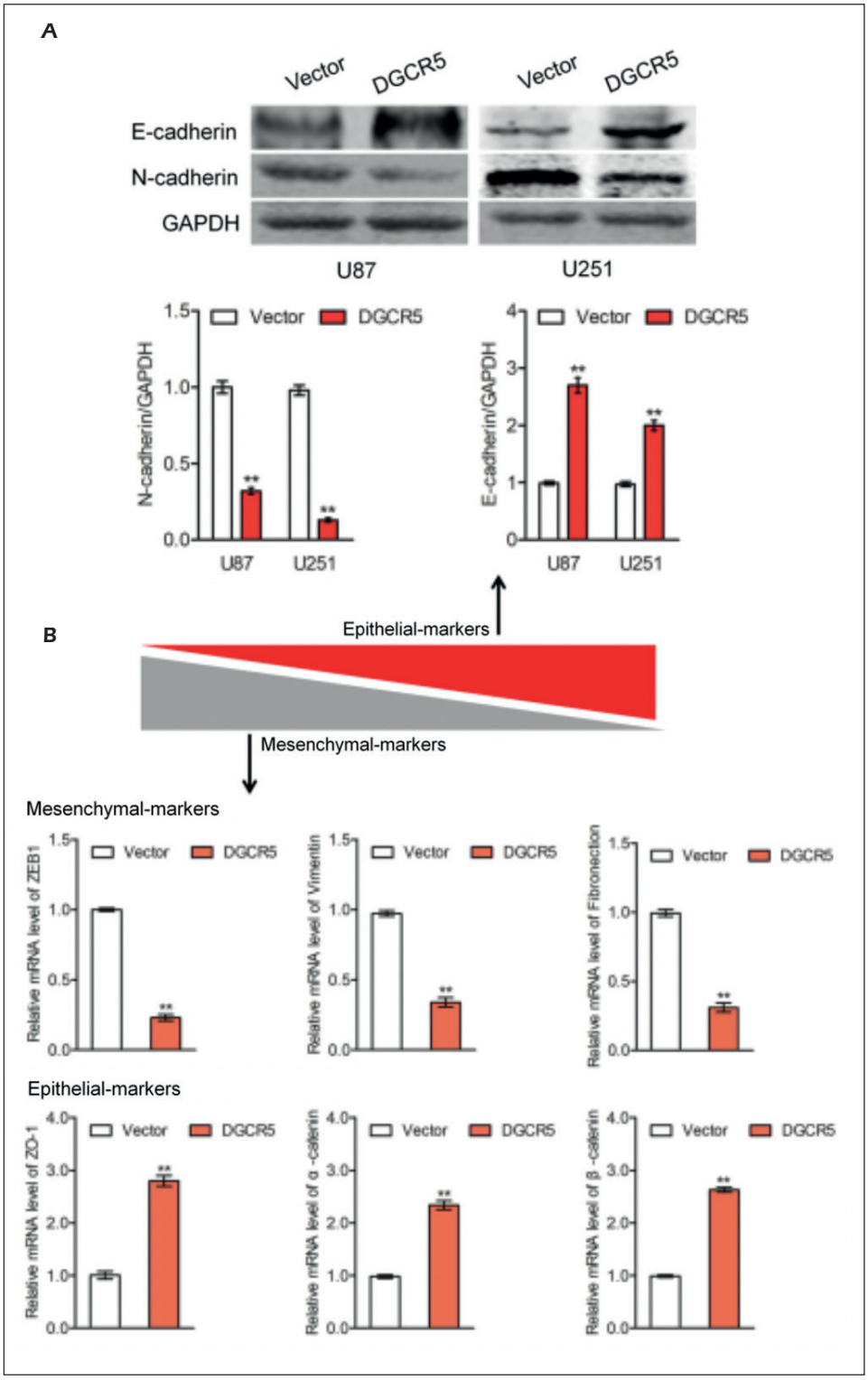

Figure 6. DGCR5 reverses the EMT process of glioblastoma cell. **A,** U87 and U251 ells were transfected with vector or DGCR5 and the expressions of N-cadherin and E-cadherin were detected using Western blotting assay. **B,** MRNA levels of epithelial markers (α-catenin, ZO-1 and β-catenin) and mesenchymal markers (Vimentin, ZEB1 and Fibronection) in U87 and U251 cells were measured by qRT-PCR analysis. **$p<0.01$ compared with control. $^{**}p<0.01$ *vs.* Vector.

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