

Research Paper

# DGK $\zeta$ Plays Crucial Roles in the Proliferation and Tumorigenicity of Human glioblastoma

Xuefeng Gu<sup>1,2,6,†</sup>, Guoqing Wan<sup>1,6,†</sup>, Nianhong Chen<sup>1,6,†</sup>, Jinhong Li<sup>3</sup>, Bing Chen<sup>4</sup>, Yeling Tang<sup>1</sup>, Wangxian Gu<sup>1</sup>, Cuiting Jin<sup>1</sup>, Jihong Meng<sup>1</sup>, Peng Zhang<sup>5</sup>, Li Liu<sup>2</sup>, Zhifang Yang<sup>2,✉</sup>, Changlian Lu<sup>1,6,✉</sup>

1. Collaborative Research Center, Shanghai University of Medicine & Health Sciences, Shanghai, PR China
2. College of Fundamental Medicine, Shanghai University of Medicine & Health Sciences, Shanghai, PR China
3. Department of Neurosurgery, Jiangmen Central Hospital, Jiangmen, Guangdong, PR China
4. Department of Neurosurgery, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong, PR China
5. College of Clinical Medicine, Shanghai University of Medicine & Health Sciences, Shanghai, PR China
6. Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine & Health Sciences, Shanghai, PR China

† contributed equally to this work

✉ Corresponding authors: 1. Changlian Lu, Collaborative Research Center, Shanghai University of Medicine & Health Sciences, Shanghai 201318, PR China. Tel.: +86-21-65883218; E-mail: lvcl@sumhs.edu.cn. 2. Zhifang Yang, College of Fundamental Medicine, Shanghai University of Medicine & Health Sciences, Shanghai 201318, PR China. Tel.: +86-21-65882635; E-mail: yangzf@sumhs.edu.cn

© The author(s). This is an open access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>). See <http://ivyspring.com/terms> for full terms and conditions.

Received: 2019.03.25; Accepted: 2019.06.23; Published: 2019.07.20

## Abstract

Glioblastoma is one of the most malignant brain cancers in adults, and it is a fatal disease because of its untimely pathogenetic location detection, infiltrative growth, and unfavorable prognosis. Unfortunately, multimodal treatment with maximal safe resection, chemotherapy and radiation has not increased the survival rate of patients with glioblastoma. Gene- and molecular-targeted therapy is considered to be a promising anticancer strategy for glioblastoma. The identification of novel potential targets in glioblastoma is of high importance. In this study, we found that both the mRNA and protein levels of diacylglycerol kinase  $\zeta$  (DGK $\zeta$ ) were significantly higher in glioblastoma tissues than in precancerous lesions. The silencing of DGK $\zeta$  by lentivirus-delivered shRNA reduced glioblastoma cell proliferation and induced G0/G1 phase arrest. Moreover, knockdown of DGK $\zeta$  expression in U251 cells markedly reduced *in vitro* colony formation and *in vivo* tumorigenic capability. Further study showed that DGK $\zeta$  inhibition resulted in decreases in cyclin D1, p-AKT and p-mTOR. Moreover, the rescue or overexpression of DGK $\zeta$  in glioblastoma cells demonstrated the oncogenic function of DGK $\zeta$ . In conclusion, these studies suggest that the suppression of DGK $\zeta$  may inhibit the tumor growth of glioblastoma cells with high DGK $\zeta$  expression. Thus, DGK $\zeta$  might be a potential therapeutic target in malignant glioblastoma.

Key words: Diacylglycerol kinase zeta (DGK $\zeta$ ), glioblastoma, phosphorylation, cell proliferation, tumorigenicity

## Introduction

Glioblastoma multiforme (GBM) is reported to be the most malignant brain tumor. It represents approximately 70% of glial tumors in the central nervous system of humans. Generally, the peak incidence of GBM occurs around 30-40 years of age [1]. GBM is the second greatest cause of mortality in patients younger than 34 years of age. GBM is deadly due to its untimely pathogenetic location detection, infiltrative growth and high recurrence rate. Despite advanced improvements in surgery skill,

chemotherapeutic drugs, and stereotaxic radiotherapy, the median overall survival time varies between 12 and 15 months [2]. Therefore, it is very important to find new therapeutic methods to suppress GBM growth and progression.

The functional study of genes or proteins that influence the occurrence and development of GBM will help us understand the molecular mechanisms of the pathogenesis of this fatal disease and identify effective therapeutic methods. Lipids are a component

of the cell membrane and play an essential role in many cellular events; lipid levels are regulated by lipid-metabolizing enzymes, such as phospholipases and lipid kinases [3]. Diacylglycerol kinase (DGK) is a lipid kinase that catalyzes the phosphorylation of diacylglycerol (DAG) to produce phosphatidic acid (PA), which uses ATP as a phosphate donor [4-6]. Both DAG and PA serve as second messengers in cell signaling. DAG regulates a variety of important signal transducers, including protein kinase C (PKC) [7] and RAS guanyl releasing protein (RasGRP) [8, 9], while PA plays a key role in regulating signaling proteins such as aPKC, c-Raf, chimaerin [10-12], mTOR [13, 14] and PIP5K [15]. Thus, DGK acts as a molecular hub for many signaling pathways through regulating intracellular DAG and PA levels.

To date, ten DGK isozymes have been reported in mammals, including  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\eta$ ,  $\kappa$ ,  $\epsilon$ ,  $\zeta$ ,  $\iota$  and  $\theta$ . These isozymes are divided into five groups (I, II, III, IV and VI) according to their structural characteristics [16]. They exhibit obviously different tissue distributions, expression patterns and biological functions. There is growing evidence that DGKs play an important role in human malignant tumors. For example, several studies showed aberrant expression of DGK $\alpha$  in hepatocellular carcinoma (HCC), mammary carcinoma, melanoma and GBM [17-19], and it is thought to serve as a potential target for drug design [19]. The important crystal structure and properties of Ca<sup>2+</sup>-bound DGK $\alpha$ -EF were reported by Takahashi [3]. Moreover, increased expression of DGK $\eta$  was found in malignant lung carcinoma with *EGFR* or *KRAS* mutations, and silencing DGK $\eta$  suppressed tumor cell growth [20]. DGK $\gamma$  plays a tumor suppressor role in HCC and colon cancer by inhibiting cell proliferation and cell migration [21, 22]. It should be noted that diacylglycerol kinases  $\zeta$  (DGK $\zeta$ ) is characterized as type IV due to its myristoylated alanine-rich C-kinase substrate (MARCKS), ankyrin, and PDZ binding domains [23, 24]. Similar to other DGKs, DGK $\zeta$  is also reported to be abnormally expressed in human colorectal cancer cells, and it is indispensable for the proliferation of cancer cells. In addition, DGK $\zeta$  knockout attenuated the invasiveness of MDA-MB-231 mammary carcinoma and PC-3 prostate tumor cells [25]. Interestingly, Topham et al. [7] found that the MARCKS domain of DGK $\zeta$  could regulate the nuclear localization of DGK $\zeta$ . This finding suggests that DGK $\zeta$  might play different roles in different cellular events. Torres-Ayuso P et al. [13] reported that DGK $\zeta$  regulates mTORC1 and lipometabolism in human colorectal tumor cells through SREBP-1. Moreover, Tanaka T et al. [26] found that DGK $\zeta$  is involved in regulating the crosstalk between NF- $\kappa$ B and p53, two

major signaling pathways in cell survival and death. Collectively, these findings suggest that regulation of DGK $\zeta$  expression might be an effective way to inhibit the proliferation of metastatic tumor cells, including glioblastoma cells. It will be very important to explore this possibility as there are no studies of DGK $\zeta$  expression patterns and potential roles in patients with glioblastoma.

In this work, we have presented evidence that DGK $\zeta$  mRNA and protein levels were significantly higher in tumor tissues from glioblastoma patients than in precancerous lesions. Silencing DGK $\zeta$  by lentivirus-delivered shRNA reduced glioblastoma cell proliferation and induced G0/G1 phase arrest. The rescue and overexpression of DGK $\zeta$  in glioblastoma cells demonstrated the function of DGK $\zeta$  as an oncogene. Moreover, knockdown of DGK $\zeta$  expression in U251 cells markedly reduced colony formation, endothelial cell tube formation *in vitro* and tumorigenic capability *in vivo*. Furthermore, western blots showed that the possible downstream molecules of DGK $\zeta$  were phosphorylated mTOR and AKT. In conclusion, these studies suggest that the suppression of DGK $\zeta$  may inhibit tumor development in glioblastoma cells with high DGK $\zeta$  expression. DGK $\zeta$  appears to be a potential therapeutic target in malignant glioblastoma.

## Materials & Methods

### Tissue Samples

Human glioblastoma tissues and corresponding para-carcinoma tissues were obtained from patients who were diagnosed at the Department of Neurosurgery, Affiliated Hospital of Guangdong Medical University (Zhanjiang, China), from 2014 to 2016. The pathological-grade tumors were confirmed according to the WHO 2000 criteria. Normal brain specimens were from victims of automobile accidents without tumors. All patients enrolled in this work gave written informed consent. The protocol was approved by the Ethics Committee of Guangdong Medical University. The clinical samples were washed with normal saline and then immediately frozen in liquid nitrogen for RNA extraction or preserved in 10% formalin for immunohistochemical staining.

### Immunohistochemistry

Immunohistochemical staining for DGK $\zeta$  was performed on clinical specimens as previously described [27]. The slices were stained with a rabbit polyclonal antibody against human DGK $\zeta$  purchased from LSBio (Seattle WA 98121, USA). Immunoperoxidase staining was performed using an ABC Peroxidase Standard Staining Kit (Pierce, Rockford, IL, USA) according to the manufacturer's

instructions.

### Cell Culture

U87 MG, HS683, SHG-44, U373, LN229, and U251 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H4 and HEB cells were purchased from Bena Culture Collection (Kunshan, China). According to the supplier's recommendations, all cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, MD, USA) supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

### DGKζ silencing and overexpression

We created lentiviral constructs that inducibly express DGKζ-targeting shRNAs (GeneBank Accession: NM\_003646) or control shRNA. The shRNA sequences were as follows: DGKζ-shRNA1, 5'-TACTCTGAAAGCAAGCAAGAA-3'; DGKζ-shRNA2, 5'-AGTGGTGTGTGATGGAATGGACT-3'; and scrambled, 5'-TTCTCCGAACGTGTCACGT-3'. Lentiviruses were packaged and purified to yield Lv-DGKζ-shRNA1, Lv-DGKζ-shRNA2, and Lv-scr-shRNA, which were used to infect glioblastoma cells. DGKζ expression after targeted shRNA lentivirus infection was examined by qRT-PCR or western blot analysis on the fourth day.

DGKζ overexpression (DGKζ OE) and negative control lentiviral vectors were obtained from GeneChem Co., Ltd. (Shanghai, China). After infecting U251 cells for 4 days, the expression of DGKζ was examined by western blot analysis.

### RNA extraction and qRT-PCR

TRIzol reagent (Invitrogen, Karlsruhe, Germany) was used for total RNA isolation. Reverse transcription PCR was performed using a one-step RNA PCR kit (TaKaRa, China). SYBR-Green Master PCR Mix (Applied Biosystems, Life Technologies, USA) was used for qRT-PCR. All qRT-PCR primer pairs were purchased from SABiosciences. Data collection was performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems, Life Technologies, USA). β-actin was selected as endogenous control to eliminate errors in reverse transcription PCR and to standardize the measurement of RNA expression levels. The relative quantification value for each target gene was compared with the control (β-actin), which was expressed as  $2^{-(C_{test}-C_{control})}$  ( $C_{test}$  and  $C_{control}$  are the mean cycle threshold (Ct) value differences after normalization to β-actin). The relative expression levels of the samples were determined by semi-log plot.

### Western blot analysis

Total protein was extracted from glioblastoma cells using RIPA buffer (Thermo Fisher Scientific, Waltham, MA). The protein concentrations were examined using a BCA Protein Quantification Kit (Yeasen, Shanghai, China). Samples containing 30 µg protein were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with solution containing 5% nonfat dry milk and then incubated overnight at 4 °C with specific primary antibodies, which included DGKζ (LS-C101153), anti-cyclin D1, anti-mTOR, anti-phospho-mTOR (Ser2448), anti-AKT and anti-phospho-AKT(Ser473) (1:1000, CST, Beverly, MA) antibodies, followed by incubation with a secondary antibody (goat anti-rabbit IgG (HRP) from Abcam, ab6721). After the membranes were washed, the blots were developed using an ECL kit (Yeasen, Shanghai, China).

### Edu assay

Infected cells were cultured in 96-well plates and incubated with 50 mmol/L EdU (RiboBio) for 2 hours. Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After permeabilization with 0.5% Triton-X-100, the cells were incubated with fluorescent staining solution for 30 minutes. Then, the DNA in the cells was stained with Hoechst 33342 for 30 minutes. Cells were then observed with a confocal fluorescence microscope (Zeiss, Thornwood, NY).

### Flow cytometry analysis of the cell cycle

U251 cells were cultured in 6-well plates and treated for 48 hours. Subsequently, the transfected cells were collected after washing twice with PBS and then fixed in 70% ice-cold ethanol at -20 °C overnight. The next day, the fixed cells were stained with 50 µg/mL PI (propidium iodide, ABCONE, Shanghai, China) containing 50 µg/mL RNase A (DNase-free) for 15 minutes at room temperature. Flow cytometric analysis was performed using a flow cytometer (FACSCalibur, Becton Dickinson) and FlowJo software (Tree Star).

### Colony formation assay

For the colony formation assay, U251 and U87 MG cells (500 per well) were seeded in a 6-well plate at 24 h post-transfection and cultured in medium containing 10% FBS for 10 days. When the colonies reached more than 50 cells, they were fixed with 10% formaldehyde for 15 minutes and stained with an 8% crystal violet solution for 10 minutes; then, the colonies were imaged using a digital camera. Each experiment was repeated three times.

### Tube formation assay

A 96-well plate was coated with growth factor-reduced Matrigel (BD Biosciences, USA); then,  $2 \times 10^5$  HUVECs/ml in complete medium and 100  $\mu$ l of U251 medium were added to each well. One hundred microliters of 60% diluted tumor conditioned medium from cells treated with DGK $\zeta$  shRNA1 or shRNA2 was added to each well, and tube formation was monitored with an inverted microscope after 6 hours of incubation. The total tube length in each well was measured and calculated using ImageJ. Experiments were performed in triplicate.

### Tumorigenesis assay

Female nude athymic mice (7 weeks old, weighing approximately 18 g) were purchased from Shanghai Sippr BK Laboratory Animals Ltd. (Shanghai, China). The mice were maintained in a specific pathogen-free (SPF) animal facility. All animals received humane care according to the National Research Council's guidelines. U251 cells infected with Scr-shRNA or DGK $\zeta$ -shRNA1 lentivirus were suspended in PBS at a final concentration of  $5 \times 10^7$  cells/ml. A 100- $\mu$ l aliquot of the cell suspension (approximately  $5 \times 10^6$  U251 cells) was injected into the axilla region of nude mice in the treatment and control groups. The mice were sacrificed 28 days post-injection, and tumor growth was examined. The tumor sizes and volumes were determined through external measurements and calculated by the formula:  $V = [L \times W^2] \times 0.5$  ( $V$  = volume,  $L$  = length and  $W$  = width). The data were analyzed using Student's t-test, and differences were considered significant at  $P < 0.05$ .

### Statistical methods

GraphPad Prism 6.0 software (GraphPad Software, CA, USA) was used for data analysis. The data are expressed as the mean  $\pm$  standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was utilized to analyze the differences between the two groups.  $P < 0.05$  was considered statistically significant.

## Results

### DGK $\zeta$ expression was upregulated in glioblastoma.

DGK $\zeta$  mRNA expression was first examined in glioma and corresponding para-carcinoma tissues from 44 clinical patients diagnosed with gliomas. The qRT-PCR results showed that DGK $\zeta$  mRNA expression was obviously higher in glioma tissues

than in paired normal tissues from most (39 of 44) patients (Fig. 1A and B). Subsequently, immunohistochemical staining was performed to confirm the protein expression of DGK $\zeta$  in the glioma tissues. DGK $\zeta$  levels appeared to be higher in malignant glioma tissues than in precancerous tissues from glioma patients (Fig. 1C and D). However, there was no significant correlation between DGK $\zeta$  and glioma tumor grades because of the limited sample size (Table 1). To further detect the correlation between DGK $\zeta$  expression and glioma, we used eight cell lines, HEB, LN229, HS683, SHG-44, H4, U373, U251 and U87 MG, to examine DGK $\zeta$  mRNA expression by qRT-PCR analyses. The qRT-PCR results showed that DGK $\zeta$  mRNA levels were higher in 4 cell lines than in normal brain tissue samples; U251 and U87 MG cells had the highest expression levels (Fig. 1E). In conclusion, our results suggested that DGK $\zeta$  expression was upregulated in gliomas.

**Table 1.** Associations between DGK $\zeta$  expression and the clinicopathological characteristics of 44 glioma patients

Clinicopathological characteristics	n	< 0	0-1	1-2	> 2	P value	
Age	$\leq 60$	36	3	2	16	15	0.303
	$> 60$	8	2	1	4	1	
Sex	male	26	4	2	11	9	0.761
	female	18	1	1	9	7	
Grade	I-II	15	3	2	5	5	0.295
	III-IV	29	2	1	15	11	

P values were detected by the chi-square test.

### Overexpression of DGK $\zeta$ in U373 and H4 glioma cells enhanced cell proliferation

Gain- or loss-of-function studies were performed to investigate the potential role of DGK $\zeta$  in glioma cells. First, we overexpressed DGK $\zeta$  in U373 and H4 glioma cells with low endogenous DGK $\zeta$  expression (Fig. 1C, 2A and 2D). The effect of DGK $\zeta$  on glioma cell proliferation was measured using the CCK8 assay. A significant increase in the cell growth curve after transfection with DGK $\zeta$  was found in both U373 and H4 cells (Fig. 2B and E). This result indicated that cell proliferation was enhanced due to the upregulation of DGK $\zeta$ . To further confirm this result, we carried out EdU proliferation assays. Consistent with the CCK8 analysis results, both U373 and H4 cells showed higher EdU-positive staining after overexpression of DGK $\zeta$  (Fig. 2C and F). These results suggest that DGK plays a role in promoting glioma cell proliferation.

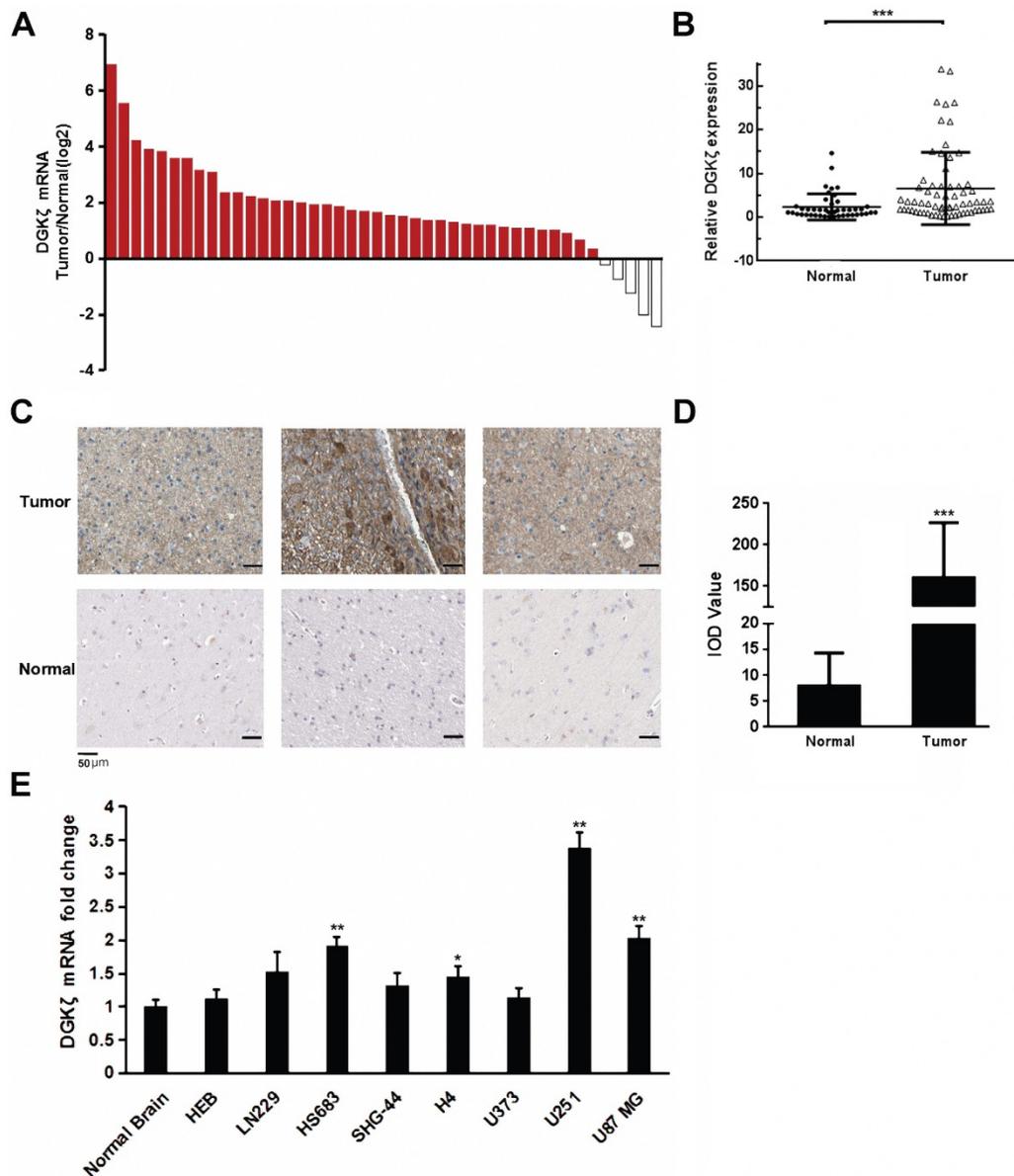
### Knockdown of DGKζ in U251 and U87 MG glioma cells inhibited cell proliferation, while rescue of DGKζ increased cell proliferation

Subsequently, high DGKζ expression in the U87 MG and U251 cell lines was knocked down (Fig. 3A and D). Five days after DGKζ-shRNA knockdown in the U87 MG and U251 cell lines, CCK8 analysis was performed, and the results showed cell growth inhibition compared with that in Scr-shRNA-infected cells. The viability of DGKζ-shRNA-infected cells was obviously lower ( $P < 0.01$ ) than that of Scr-shRNA-infected control cells (Fig. 3B and E).

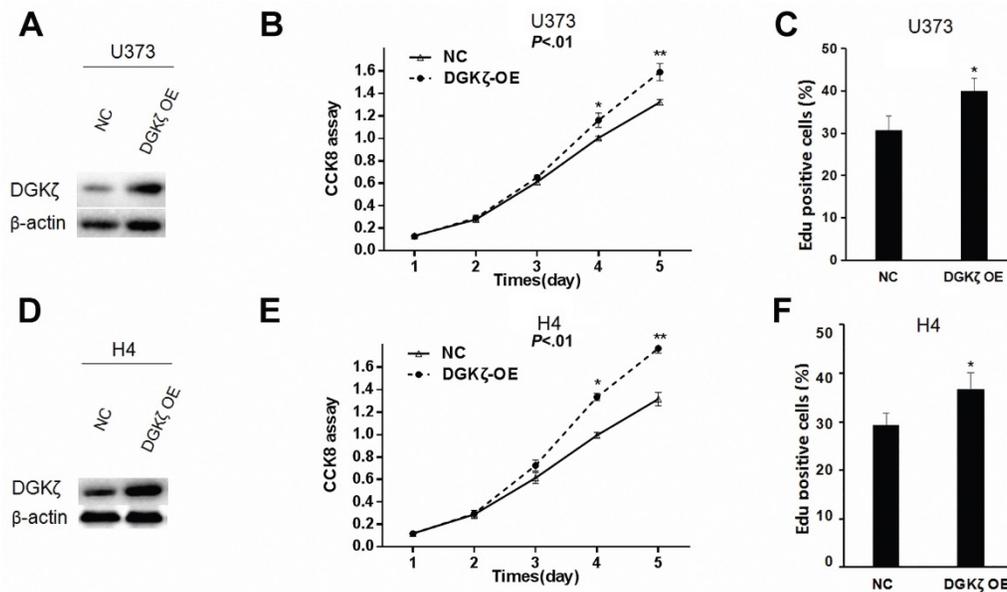
An EdU proliferation assay was employed as an alternative method to measure cell proliferation.

Consistent with the CCK8 analysis results, both U251 and U87 MG cells showed lower EdU-positive staining levels after transfection with DGKζ shRNA than after transfection with control shRNA (Fig. 3C and F).

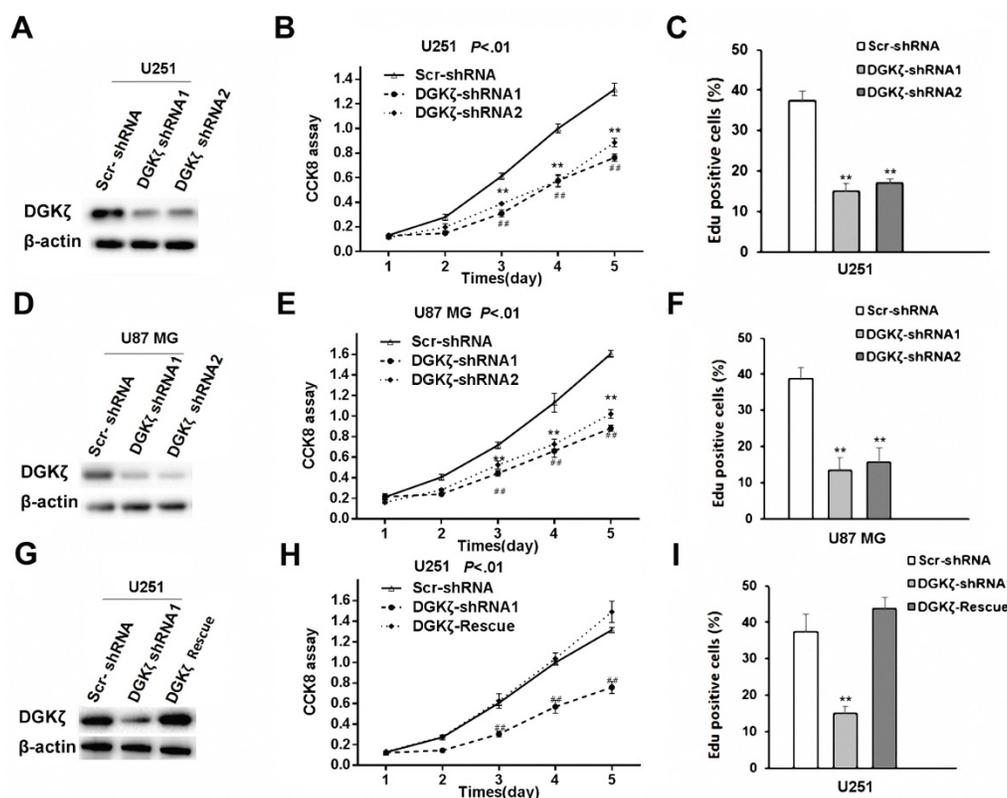
A rescue experiment was used to confirm the specificity of the effects of DGKζ knockdown in glioma cells. DGKζ expression was enhanced after transfecting the DGKζ vector in DGKζ-shRNA1-infected U251 cells (Fig. 3G). Furthermore, enhancing DGKζ expression rescued the decrease in cell viability induced by RNAi-mediated DGKζ depletion (Fig. 3H and I).



**Figure 1. DGKζ is highly expressed in glioma cells.** (A, B) DGKζ mRNA expression was obviously higher in glioma tissues than in paired normal tissues in most patients (39/44). (C, D) Representative immunohistochemical staining for DGKζ expression in human glioma tissues (upper) and precancerous tissues (lower). The average IOD value was obtained by analyzing five fields per slide with Image-Pro Plus software (v. 6.0) and recorded in histograms. (E) DGKζ mRNA levels in normal brain tissues and glioma cell lines detected by qRT-PCR analysis. The results represent at least three separate experiments. Error bars:  $\pm$  S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 2. DGKζ promotes glioma cell proliferation *in vitro*.** (A) U373 cells were infected with lentivirus carrying DGKζ (DGKζ OE) or negative control (NC). The cell lysates were immunoblotted with a DGKζ antibody, and β-actin was used as a loading control. (B) *In vitro* growth of U373-NC and U373-DGKζ-OE cells was measured using CCK8 assays. (C) *In vitro* growth of U373-NC and U373-DGKζ-OE cells was measured using Edu assays. (D) H4 cells were infected with lentivirus carrying DGKζ (DGKζ OE) or negative control (NC). The cell lysates were immunoblotted with a DGKζ antibody, and actin was used as a loading control. (E) *In vitro* growth of H4-NC and H4-DGKζ-OE cells was measured using CCK8 assays. (F) *In vitro* growth of H4-NC and H4-DGKζ-OE cells was measured using Edu assays. Error bars: ± S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



**Figure 3. Inhibition of DGKζ remarkably suppresses cell viability in glioma cells with high DGKζ expression.** (A) U-251 cells were transfected with DGKζ shRNA1 or DGKζ shRNA2 and scrambled shRNA as a control (Scr shRNA). DGKζ levels were detected by immunostaining with a DGKζ antibody, and actin was used as a loading control. (B) *In vitro* growth of U-251/Scr shRNA and U-251/ DGKζ shRNA1 or shRNA2 cells was measured using CCK8 assays. (C) *In vitro* growth of U-251/Scr shRNA and U-251/ DGKζ shRNA cells was measured using Edu assays. (D) U87 MG cells were transfected with DGKζ shRNA1 or DGKζ shRNA2 and scrambled shRNA as a control (Scr shRNA). DGKζ levels were detected by immunostaining with a DGKζ antibody, and actin was used as a loading control. (E) *In vitro* growth of U87 MG/Scr shRNA and U87 MG/DGKζ shRNA1 or shRNA2 cells was measured using CCK8 assays. (F) *In vitro* growth of U87 MG Scr shRNA and U87 MG/DGKζ shRNA cells was measured using Edu assays. (G) DGKζ protein expression in U251 cells infected with a vector expressing DGKζ after DGKζ-shRNA1-mediated DGKζ knockdown. (H) Cell viability curves of the three U251 cell groups over 5 days were evaluated by CCK8 assay. (I) *In vitro* growth of U251 Scr shRNA, U251/DGKζ shRNA1 and U251-DGKζ-rescue cells was measured using Edu assays. Error bars: ± S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

### Inhibition of DGKζ significantly augments the tumor growth of DGKζ-expressing glioma cells

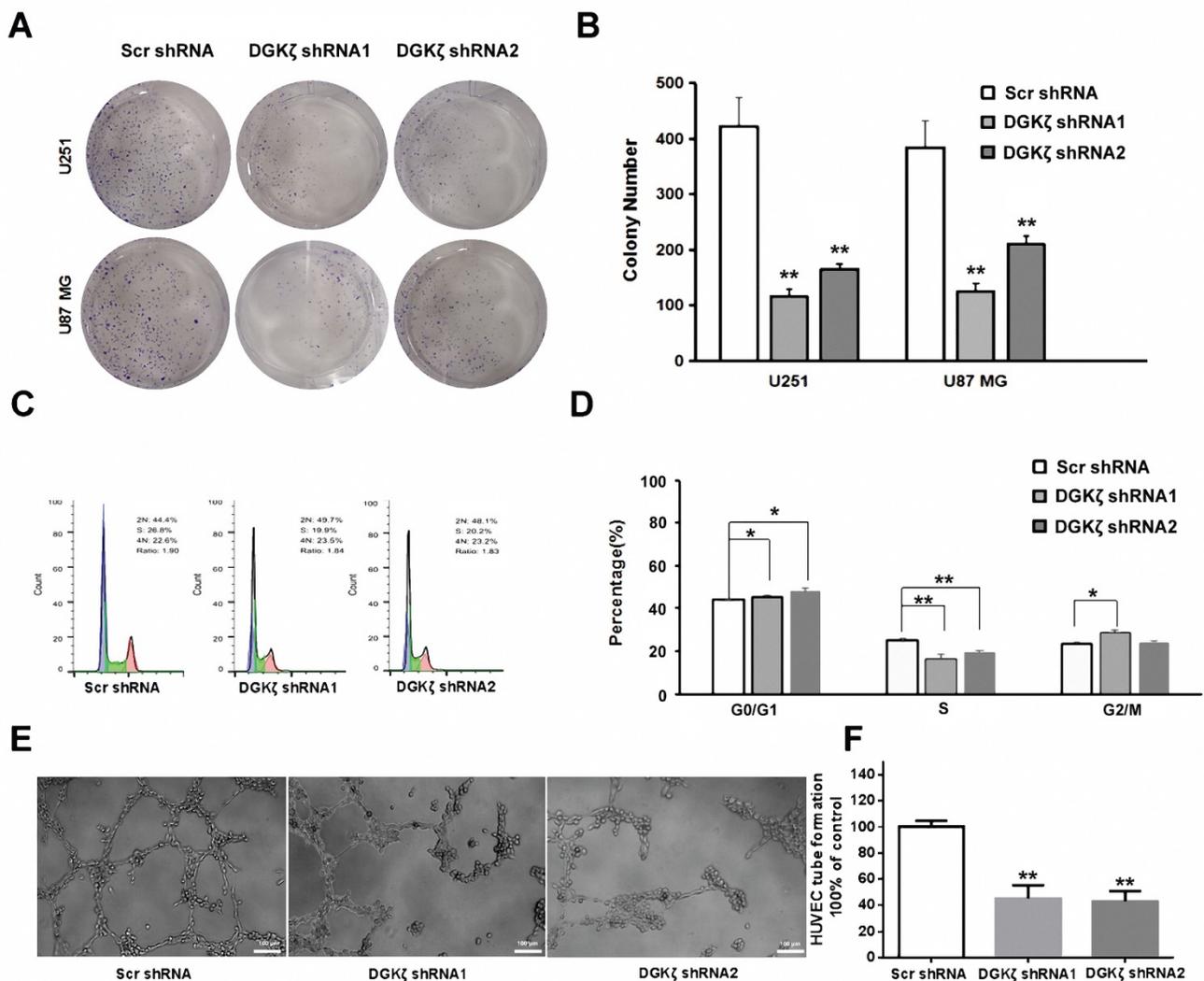
To further investigate the effects of DGKζ inhibition in glioma cells, colony formation, flow cytometry analysis of the cell cycle, and tube formation assays were performed *in vitro*. Transfection of U251 and U87 MG cells with DGKζ shRNA resulted in a significant decrease in the cell numbers of each colony compared with nonsense shRNA transfection. The number of colonies with more than 50% cells was also decreased in DGKζ shRNA-transfected cells (Fig. 4A and B).

After U251 cell PI staining, flow cytometry analysis showed that DGKζ inhibition led to a significant increase in G0/G1 phase arrest but a

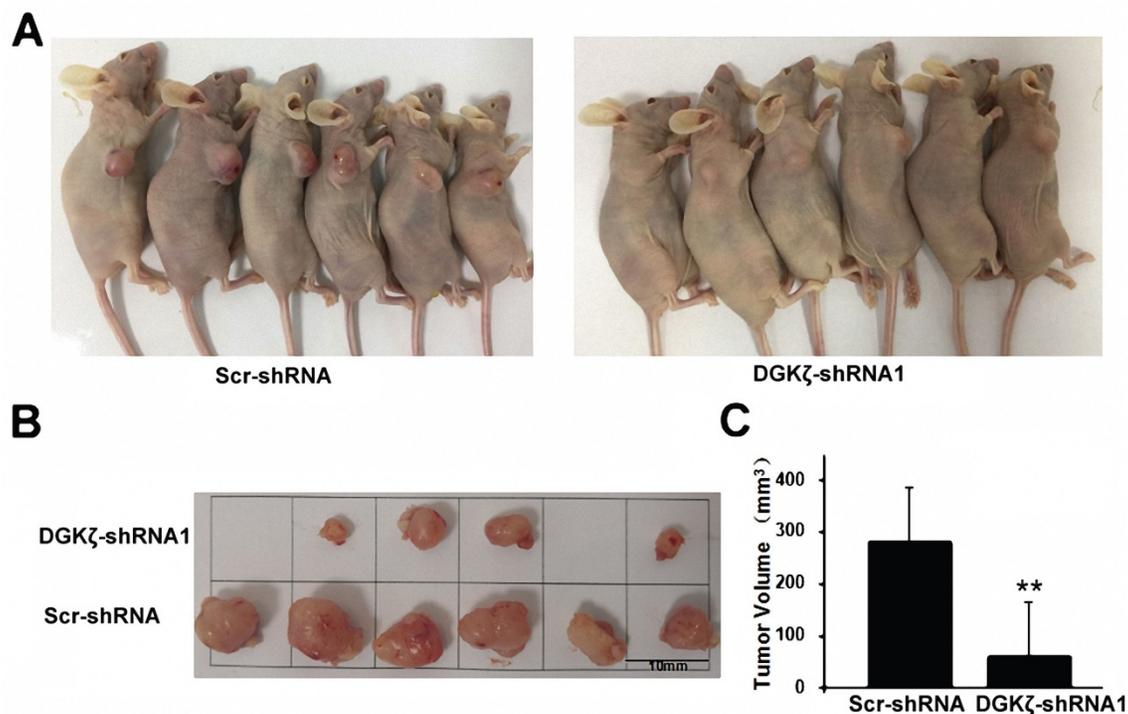
decrease in the number of S phase cells (Fig. 4C and D). These data demonstrated that DGKζ inhibition results in U251 cells in the quiescent phase.

Moreover, a tube formation assay revealed that knockdown of DGKζ could inhibit the formation of extravascular blood vessels (Fig. 4E and F).

We next examined the *in vivo* effect of DGKζ using a subcutaneous xenograft nude mouse model. Significant inhibition of tumor growth relative to the control group (82.8%;  $P < 0.01$ ) was recorded in the DGKζ knockdown group (Fig. 5). Considering all the experiments together, the most significant result obtained was that the inhibition of DGKζ expression suppressed the tumor growth of glioma cells with high DGKζ expression.



**Figure 4. Inhibition of DGKζ prominently suppresses tumor development in glioma cells.** (A, B) Colony formation assay of cells from the three groups. Cells were seeded at 500 cells/well and allowed to form colonies for 10 days. The colonies were stained with crystal violet and observed. (C, D) Cell cycle analysis of cells from the three groups as determined by PI staining and FACS analysis (left). The percentages of cells in each phase represent the mean ± S.D. of three independent experiments (right). \*  $P < 0.05$ , \*\*  $P < 0.01$ . (E, F) Representative images from the HUVEC tube-formation assay. Quantified data correspond to the mean ± S.D. of three independent experiments (right). \*\*  $P < 0.01$ .

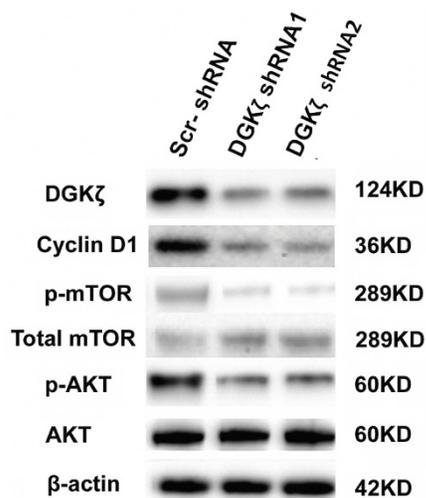


**Figure 5. DGKζ inhibits glioma cell proliferation in vivo.** (A) Scr-shRNA and DGKζ-shRNA groups of nude mice were injected in the axilla region with the corresponding infected U251 cells (10<sup>6</sup> cells per mouse). After 4 weeks, the mice were sacrificed, and their tumors were examined (n=6). (B). (C) Quantified data correspond to the mean ± S.D. of two groups (n=6). \*\* P < 0.01.

### DGKζ silencing caused the downregulation of cyclin D1, AKT phosphorylation and mTOR phosphorylation

We next investigated the mechanism by which DGKζ inhibition suppressed glioma growth *in vitro* and *in vivo*. Because the role of DGKζ in carcinogenesis as well as in tumor progression has been poorly documented, only a few studies have reported that DGKζ has an anti-apoptotic function that is dependent on the mTOR pathway [28-31]. Moreover, the flow cytometry results showed that U251 glioma cells were arrested at the G0/G1 phase. PI3K/AKT/mTOR is an important intracellular signal transduction pathway involved in the cell cycle; thus, AKT/mTOR-associated signaling pathways were examined first. Western blot analysis showed that the phosphorylation levels of mTOR and AKT were lower in DGKζ-shRNA-infected cells than in control cells (Fig. 6), while there was no evident change in total AKT and mTOR, indicating that DGKζ silencing inactivated the AKT/mTOR pathway in glioma cells through a phosphorylation/dephosphorylation mechanism. We also found the downregulation of cyclin D1 in DGKζ knockdown cells. The main function of cyclin D1 is to promote cell proliferation and G1/S phase transition by binding and activating cyclin-dependent kinase CDK4, phosphorylating cyclin inhibitor protein (Rb) in the G1 phase,

dissociating the phosphorylated Rb protein from its binding E2F transcription factor, and initiating the transcription of genes in the cell cycle. The molecular mechanisms of how these genes work downstream of DGKζ require future evaluation.



**Figure 6. DGKζ silencing decreases cyclin D1, phosphorylated AKT and phosphorylated mTOR levels in U251 cells.** U251 cells were transfected with lentiviruses expressing the shRNAs. DGKζ, cyclin D1, phosphorylated mTOR and phosphorylated AKT protein levels were assayed by western blots. Western blot analyses indicated that DGKζ silencing decreased cyclin D1 and phosphorylated mTOR and phosphorylated AKT protein levels. One representative result is shown.

## Discussion

Gliomas can occur at any age, regardless of sex and race. Based on their histopathology, gliomas can be subdivided into grades I-IV according to the World Health Organization (WHO) classification for CNS tumors [32]. Grade III and IV gliomas are more aggressive and difficult to cure due to the frequent dysfunction of tumor suppressors and oncogenes. Despite the combination of surgery, radiotherapy, and chemotherapy, the prognosis for patients remains very poor, and less than 5% of patients with malignant glioma survive 5 years [33]. Presently, the only chemotherapeutic drug is temozolomide; Everolimus is a molecule-targeted drug for subependymal giant cell astrocytoma (SEGA) associated with tuberous sclerosis (TS) that cannot be treated surgically, and Avastin is a monoclonal antibody (mAb) drug that is the only second-line treatment for malignant glioma approved by the FDA [34]. As we better understand the molecular mechanisms underlying glioma development, molecular targeted therapy has been proposed as a promising treatment for glioma. For instance, BRAF [35], EGFR [36], EZH2 [37], MET [38] and mTOR [39] have been reported as potential protein targets for glioma treatment. However, because of the complexity of the occurrence and development of glioma, none of these targets can completely cure glioma; thus, more novel therapeutic targets are urgently needed.

In the present study, we demonstrated that DGK $\zeta$  mRNA and protein levels were significantly higher in tumor tissues from glioblastoma patients than in precancerous lesions. DGK $\zeta$  knockdown by lentivirus-delivered shRNA reduced glioblastoma cell proliferation and induced G0/G1 phase arrest. In addition, the rescue or overexpression of DGK $\zeta$  in glioblastoma cells further validated the function of DGK $\zeta$  as an oncogene. Moreover, knockdown of DGK $\zeta$  expression in U251 cells markedly reduced colony formation, endothelial cell tube formation *in vitro* and tumorigenic capability *in vivo*. At the molecular level, western blots showed that the possible downstream molecules of DGK $\zeta$  were phosphorylated mTOR and AKT. In conclusion, these studies suggest that the suppression of DGK $\zeta$  could inhibit the tumor development of glioblastoma cells with high DGK $\zeta$  expression, thus highlighting DGK $\zeta$  as a potential therapeutic target in malignant glioblastoma.

The underlying mechanism of DGK $\zeta$  inhibition of glioma cell growth was explored in this work. DAG and PA are important membrane components and biosynthetic precursors of phospholipids. DAG conversion into PA by DGK is the first step of

replenishing PIP2 in the so-called "PIP2 cycle", a reaction that provides an important lipid substrate, not only for the subsequent hydrolysis by phospholipase C (PLC) but also for the production of PIP3 by the action of PI3 kinase (PI3K). This information indicated that DGK $\zeta$  might play important roles in the signaling pathways related to PIP2 or PI3K. Moreover, the PI3K/AKT/mTOR pathway has been regarded as an important signaling pathway that regulates many intracellular upstream signaling pathways that influence cell growth, metabolism, survival polarity, angiogenesis, and vesicle trafficking. In our study, AKT/mTOR was markedly inhibited by DGK $\zeta$  knockdown in U251 cells, indicating the interesting role of DGK $\zeta$  in glioma. Moreover, our results demonstrated that DGK $\zeta$  inhibition arrested the cell cycle at the G0/G1 phases. The AKT/GSK3 $\beta$  pathway induces cell cycle progression by enhancing cyclin D1 expression. The reason for this finding might be that these cells were screened during the process of tumor development, and some compensatory DGK $\zeta$ -independent pathway was already activated in these screened cells to support cell survival and proliferation. However, the exact mechanisms require further investigation.

## Abbreviations

DGK $\zeta$ , diacylglycerol kinase zeta; shRNA, short hairpin RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Scr-shRNA, scrambled shRNA; qRT-PCR, quantitative real-time PCR; CCK8, Cell Counting Kit 8; PI, propidium iodide; PIP2 cycle, phosphatidylinositol-4,5-bisphosphate cycle; PI3K, phosphatidylinositol 3-kinase; AKT, V-akt murine thymoma viral oncogene homolog; mTOR, mammalian target of rapamycin.

## Acknowledgments

This work was supported by the following funding: the Special Program for Collaborative Innovation, and Shanghai Municipal Education Commission (Class II Plateau Disciplinary Construction Program for Medical Technology of SUMHS, 2018-2020); the Seed fund of SUMHS; the Natural Science Foundation of Guangdong Province (2016A030313680).

The authors thank Vitaly Ryu for critical reading and editing of the manuscript.

## Competing Interests

The authors have declared that no competing interest exists.

## References

- Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med.* 2008; 359: 492-507.
- Koshy M, Villano JL, Dolecek TA, Howard A, Mahmood U, Chmura SJ, et al. Improved survival time trends for glioblastoma using the SEER 17 population-based registries. *J Neurooncol.* 2012; 107: 207-212.
- Takahashi D, Suzuki K, Sakamoto T, Iwamoto T, Murata T, Sakane F. Crystal structure and calcium-induced conformational changes of diacylglycerol kinase  $\alpha$  EF-hand domains. *Protein Sci.* 2019; 28: 694-706.
- Baldanzi G. Inhibition of diacylglycerol kinases as a physiological way to promote diacylglycerol signaling. *Adv Biol Regul.* 2014; 55: 39-49.
- Kanoh H, Yamada K, Sakane F. Diacylglycerol kinase: a key modulator of signal transduction? *Trends Biochem Sci.* 1990; 15: 47-50.
- Han GS, O'Hara L, Siniouoglou S, Carman GM. Characterization of the yeast DGK1-encoded CTP-dependent diacylglycerol kinase. *J Biol Chem.* 2008; 283: 20443-20453.
- Topham MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ, Prescott SM. Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. *Nature.* 1998; 394: 697-700.
- Brose N, Rosenmund C. Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J Cell Sci.* 2002; 115: 4399-4411.
- Kazanietz MG. Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol Pharmacol.* 2002; 61: 759-767.
- English D. Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signalling. *Cell Signal.* 1996; 8: 341-347.
- Exton JH. Phosphatidylcholine breakdown and signal transduction. *Biochim Biophys Acta.* 1994; 1212: 26-42.
- Hodgkin MN, Pettitt TR, Martin A, Michell RH, Pemberton AJ, Wakelam MJ. Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem Sci.* 1998; 23: 200-204.
- Torres-Ayuso P, Tello-Lafos M, Merida I, Avila-Flores A. Diacylglycerol kinase-zeta regulates mTORC1 and lipogenic metabolism in cancer cells through SREBP-1. *Oncogenesis.* 2015; 4: e164.
- Fang Y, Vilella-Bach M, Bachmann R, Flanagan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science.* 2001; 294: 1942-1945.
- Brown JR, Auger KR. Phylogenomics of phosphoinositide lipid kinases: perspectives on the evolution of second messenger signaling and drug discovery. *BMC Evol Biol.* 2011; 11: 4.
- Sakai H, Murakami C, Matsumoto KI, Urano T, Sakane F. Diacylglycerol kinase  $\delta$  controls down-regulation of cyclin D1 for C2C12 myogenic differentiation. *Biochimie.* 2018; 151: 45-53.
- Torres-Ayuso P, Daza-Martin M, Martin-Perez J, Avila-Flores A, Merida I. Diacylglycerol kinase  $\alpha$  promotes 3D cancer cell growth and limits drug sensitivity through functional interaction with Src. *Oncotarget.* 2014; 5: 9710-9726.
- Takeishi K, Taketomi A, Shirabe K, Toshima T, Motomura T, Ikegami T, et al. Diacylglycerol kinase  $\alpha$  enhances hepatocellular carcinoma progression by activation of Ras-Raf-MEK-ERK pathway. *J Hepatol.* 2012; 57: 77-83.
- Dominguez CL, Floyd DH, Xiao A, Mullins GR, Kefas BA, Xin W, et al. Diacylglycerol kinase  $\alpha$  is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. *Cancer Discov.* 2013; 3: 782-797.
- Nakano T, Irvani A, Kim M, Hozumi Y, Lohse M, Reichert E, et al. Diacylglycerol kinase  $\epsilon$  modulates oncogenic properties of lung cancer cells. *Clin Transl Oncol.* 2014; 16: 29-35.
- Guo Z, Jia J, Yao M, Kang J, Wang Y, Yan X, et al. Diacylglycerol kinase  $\gamma$  predicts prognosis and functions as a tumor suppressor by negatively regulating glucose transporter 1 in hepatocellular carcinoma. *Exp Cell Res.* 2018; 373: 211-220.
- Kai M, Yamamoto E, Sato A, Yamano HO, Niinuma T, Kitajima H, et al. Epigenetic silencing of diacylglycerol kinase  $\gamma$  in colorectal cancer. *Mol Carcinog.* 2017; 56: 1743-1752.
- Mérida I, Torres-Ayuso P, Ávila-Flores A, Arranz-Nicolás J, Andrada E, et al. Diacylglycerol kinases in cancer. *Adv Biol Regul.* 2017; 63: 22-31.
- Mérida I, Andrada E, Gharbi SI, Ávila-Flores A. Redundant and specialized roles for diacylglycerol kinases  $\alpha$  and  $\zeta$  in the control of T cell functions. *Sci Signal.* 2015; 8: re6.
- Cai K, Mulatz K, Ard R, Nguyen T, Gee SH. Increased diacylglycerol kinase zeta expression in human metastatic colon cancer cells augments Rho GTPase activity and contributes to enhanced invasion. *BMC Cancer.* 2014; 14: 208.
- Tanaka T, Okada M, Hozumi Y, Tachibana K, Kitanaka C, Hamamoto Y, et al. Cytoplasmic localization of DGK $\zeta$  exerts a protective effect against p53-mediated cytotoxicity. *J Cell Sci.* 2013; 126: 2785-2797.
- Gu X, Wang C, Wang X, Ma G, Li Y, Cui L, et al. Efficient inhibition of human glioma development by RNA interference-mediated silencing of PAK5. *Int J Biol Sci.* 2015; 11: 230-237.
- Sabates-Bellver J, Van der Flier LG, de Palo M, Cattaneo E, Maake C, Rehrauer H. Transcriptome profile of human colorectal adenomas. *Mol Cancer Res.* 2007; 5: 1263-1275.
- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012; 2: 401-404.
- Provenzani A, Fronza R, Loreni F, Pascale A, Amadio M, Quattrone A. Global alterations in mRNA polysomal recruitment in a cell model of colorectal cancer progression to metastasis. *Carcinogenesis.* 2006; 27: 1323-1333.
- Ducker GS, Atreya CE, Simko JP, Hom YK, Matli MR, Benes CH. Incomplete inhibition of phosphorylation of 4E-BP1 as a mechanism of primary resistance to ATP-competitive mTOR inhibitors. *Oncogene.* 2014; 33: 1590-1600.
- Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 world health organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* 2016; 131: 803-820.
- Pan Y, Yuan F, Li Y, Wang G, Lin Z, Chen L. Bromodomain PHD finger transcription factor promotes glioma progression and indicates poor prognosis. *Oncol Rep.* 2019; 41: 246-256.
- [Internet] Roche. FDA grants Roche's Avastin full approval for most aggressive form of brain cancer. <https://www.roche.com/investors/updates/inv-update-2017-12-06.htm>
- Karsy M, Guan J, Cohen AL, Jensen RL, Colman H. New Molecular Considerations for Glioma: IDH, ATRX, BRAF, TERT, H3 K27M. *Curr Neurol Neurosci Rep.* 2017; 17: 19.
- Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature.* 1985; 313: 144-147.
- Mohammad F, Weissmann S, Leblanc B, Pandey DP, Højfeldt JW, Comet I, et al. EZH2 is a potential therapeutic target for H3K27M-mutant pediatric gliomas. *Nat Med.* 2017; 23: 483-492.
- Kwak Y, Kim SI, Park CK, Paek SH, Lee ST, Park SH. C-MET overexpression and amplification in gliomas. *Int J Clin Exp Pathol.* 2015; 8: 14932-14938.
- Foltyn M, Luger AL, Lorenz NI, Sauer B, Mittelbronn M, Harter PN, et al. The physiological mTOR complex 1 inhibitor DDIT4 mediates therapy resistance in glioblastoma. *Br J Cancer.* 2019; 120: 481-487.