ARTICLE

Oncogenic potential of PIK3CD in glioblastoma is exerted through cytoskeletal proteins PAK3 and PLEK2

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The Class I_A phosphoinositide-3-kinase catalytic isoforms p110α, p110β, and p110δ have been implicated to play vital but overlapping roles in various cancers, including glioblastoma (GBM). We have previously shown that *PIK3CD*, encoding p110δ, is highly expressed in multiple glioma cell lines and involved in glioma cell migration and invasion. Based on the RNA sequencing data from The Cancer Genome Atlas (TCGA) database, we found the level of *PIK3CD* expression is significantly higher in GBM than WHO grade II and III gliomas and is closely related to poor survival. To further dissect the oncogenic roles of *PIK3CD* in glioma progression, we employed CRISPR/Cas9 to completely abrogate its expression in the GBM cell line U87-MG and have successfully isolated two knockout clones with different gene modifications. As expected, the knockout clones exhibited significantly lower migration and invasion capabilities when compared with their parental cells. Interestingly, knockout of *PIK3CD* also dramatically reduced the colony formation ability of the knockout cells. Further study revealed that *PIK3CD* deficiency could negate tumorigenesis in nude mice. To determine the downstream effect of *PIK3CD* depletion, we performed RT^2 profiler PCR array of selected gene sets and found that knockout of *PIK3CD* impaired the activity of p-21 activated kinase 3 (*PAK3*) and pleckstrin 2 (*PLEK2*), molecules involved in cancer cell migration and proliferation. This explains why the glioma cells without the *PIK3CD* expression exhibited weaker oncogenic features. Further, RNAseq analysis of parent and knockout clones revealed that this interaction might happen through axonogenesis signaling pathway. Taken together, we demonstrated that *PIK3CD* could be a potential prognostic factor and therapeutic target for GBM patients.

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INTRODUCTION

Glioblastoma (GBM) is the most devastating form of brain cancer that accounts for over 45% of malignant central nervous system cancers[1]. Current treatment arsenal against GBM is surgery followed by radiotherapy with concomitant temozolomide chemotherapy. Despite the advancement in GBM research, the age adjusted 2-year survival rate is still less than 30%[2]. Multiple factors including a diverse array of signaling pathways play significant roles in GBM pathogenesis[3], and one of the most studied is the phosphoinositide 3-kinase (PI3K) pathway.

PI3K signaling is crucial in cell survival, apoptosis, proliferation, migration, and invasion in many cancers including GBM[3]. Mutations of PI3Ks and phosphatase and tensin homolog (PTEN), a negative regulator of PI3K signaling pathway, are common in GBM[4]. In fact, most mutations are present in the activators of PI3Ks, the receptor tyrosine kinases, and the PI3Ks themselves[5,6]. There are three classes of PI3Ks and among them Class I members are found altered in most GBM cases[7]. Class I PI3Ks are heterodimeric enzymes, with a 110 kDa catalytic subunit and an 85 kDa regulatory subunit, grouped into Class I_A and Class I_B. There are three isoforms of the catalytic subunit in Class I_A, namely *PIK3CA*, *PIK3CB*, and *PIK3CD*[8]. *PIK3CA*, coding for the p110α catalytic subunit, is frequently found mutated in GBM and is regarded the most important PI3K isoform in the pathogenesis of GBM[4]. However, knockdown of this isoform in different glioma cell lines failed to block their survival[9] and no noticeable effect on glioma cell growth was observed in a xenograft mouse model[10]. Mutation of *PIK3CB* is rare in GBM but knocking it down and/or selectively inhibiting its coding protein p110β reduces glioma cell viability and blocks growth in a mouse model[11]. *PIK3CD*, however, is not studied extensively in glioma pathogenesis but more often in hematological malignancies because of the predominant expression of its coding protein, p110δ, in leukocytes[12]. Furthermore, *PIK3CD* has recently been implicated in colorectal cancer by activating AKT/GSK-3β/β-catenin signaling[13]. However, the function of *PIK3CD* in GBM remains elusive. Using siRNA knockdown of *PIK3CD* expression, we previously reported that p110δ is responsible, at least in part, for glioma cell migration and invasion[14]. The importance of *PIK3CD* in GBM is further supported by the fact that expression of p110δ in glioma cells is related to their resistance to erlotinib[15]. However, the fundamental molecular mechanisms are yet to be disclosed.

To elucidate the underlying roles of p110δ in GBM progression, we employed the CRISPR/Cas9 technique to knockout *PIK3CD* in U87-MG glioma cells. We demonstrated that *PIK3CD* exerts its oncogenic role through cytoskeletal proteins PAK3 and PLEK2 in GBM. Further, based on the transcriptome analysis, we collectively showed that *PIK3CD* could control the oncogenic feature of GBM

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cells by regulating the axonogenesis signaling pathway. The information gained may help in understanding the roles of *PIK3CD* for GBM targeted therapy.

MATERIALS AND METHODS

Cells and animals

A normal human astrocyte cell line was purchased from ScienCell™ Research Laboratories. Two low-grade (WHO II) glioma cell lines SHG44[16] and CHG5[17] were obtained from Third Military Medical University, China. Glioblastoma cell line U251 was obtained from China Center for Type Culture Collection (CCTCC) and U87-MG was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in Minimum Essential Medium Alpha (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). The U87-MG and the derived knockout cell lines were authenticated using Short Tandem Repeat analysis by ATCC. Female BALB/c nude mice were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China).

Patient data analysis

We collected clinical data of patients with glioblastoma (GBM, $N = 596$) and lower-grade glioma (LGG, $N = 511$) from previously published TCGA studies[18]. Only samples with definite WHO classification were included for *PIK3CD* and other genes expression analysis. We downloaded the standardized, normalized, batch-corrected and platform-corrected mRNA expression for GBM and LGG patients. The mRNA expression was calculated as $\log_2(x + 1)$ for further analysis. Kaplan–Meier plots were generated to illustrate the relationship between patients' overall survival and gene expression levels of *PIK3CD*. For comparing the overall survival time of GBM and LGG, we used the Cox proportional-hazards (PH) regression to calculate the hazard ratio (HR), the 95% confidence interval (95% CI), and p values, considering age at initial pathologic diagnosis and gender as covariates.

Application of CRISPR/Cas9 gene editing to knockout PIK3CD

The following two guide RNA sequences were used as the insert oligonucleotides for human *PIK3CD*: #1 5′-caccgAGGTTGGCATTGCGGGACAC-3′/5′-aaacGTGTCCCGCAATGCCAACCTc-3′ and #2 5′-caccgGGAGGAGAATCAGAGCGTTG-3′/5′aaacCAACGCTCTGATTCTCCTCCc-3′, which target exon 3 of the *PIK3CD* gene. LentiCRISPRv2 (one vector system) was used for sgRNA construction and delivery. Lentivirus for transfection was packaged in HEK293T cells using Lipofectamine 2000. Twenty-four hours after transfection, medium was collected and added to the U87-MG glioma cells. After selection by puromycin for two days, single cells were isolated by serial dilution, followed by an expansion period to establish clonal cell lines. The protein expression level of p110δ was monitored by Western blotting. DNA and exome sequencing were carried out to determine the type of mutation. Two stably knockout *PIK3CD* U87-MG glioma cell clones, designated as SD2 and SD13, were obtained.

In vitro assays

Cell proliferation, cell cycle, migration, invasion assays, and quantitative real-time PCR were performed as previously described[14]. The primary antibodies used for Western blotting include: p110α, p110β, p110δ, and PLEK2 (Santa Cruz); Akt, p-Akt, and β-actin (Cell Signaling Technology), PAK3 (Boster Bio), Cyclin D1(Abam).

RT^2 profiler PCR array

Human epithelial to mesenchymal transition (EMT) (code #PAHS-090Z), human focal adhesions (code #PAHS-145Z) RT^2 PCR Arrays, and RT^2 Real-Time SYBR Green/ROX PCR Mix were purchased from Qiagen. PCR was performed using the ABI ViiA 7 (Applied Biosystems). Gene expression of knockout and control samples were analysed separately in different PCR array plates. For each plate, results were normalized on the median value of a set of housekeeping genes. Changes in gene expression between knockout and control samples were calculated using online data analysis software developed by Qiagen.

RNA sequencing

cDNA libraries were prepared by KAPA Stranded mRNA-Seq Kit. One microgram of total RNA was used as starting material. Manufacturer's protocol was followed. In brief, Poly-A containing mRNA was collected using poly-T oligo-attached magnetic beads. The purified mRNA was fragmented to 200–300 bases by incubating at 94 °C for 6 min in the presence of magnesium ions. The fragmented mRNA was then applied as template to synthesize the first-strand cDNA using random hexamer-primers and reverse transcriptase. In the second strand cDNA synthesis, the mRNA template was removed, and a replacement strand was generated to form blunt-end double-stranded cDNA. The cDNA underwent 3′ adenylation and indexed adaptor ligation. The adaptor-ligated libraries were enriched by 10 cycles of PCR. The libraries were denatured and diluted to optimal concentration. Illumina NovaSeq 6000 was used for Pair-End 151 bp sequencing. Raw reads collected were pre-processed and filtered. Reads with ≥40 bp were considered and mapped to the reference genome using STAR Version 2.5.2 database with default parameters. Expressions were quantified by RSEM Version 1.2.31 software. Finally, EBSeq Version 1.10.0 software was used to identify the differentially expressed genes.

Tumorigenicity assays

For in vitro colony formation assay, U87-MG cells and *PIK3CD* knockout clones were counted and seeded (500 per dish) onto 6-well plates and incubated at 37 °C. Fresh culture medium was replaced every 2 days. After 21 days, cells were washed with PBS, stained with Giemsa, and colonies with more than 50 cells were counted.

For in vivo tumorigenic assay, 100 µL cell suspension of control and knockout cell clones (2×10^6 cells) were subcutaneously injected into the lower flanks of female BALB/c nude mice (aged 6–7 weeks, $n = 6$/group). The experiment was performed in accordance with the protocols approved by Xiamen University Animal Ethics Committee and complied with the ARRIVE guidelines. Tumor growth was determined by calliper measurement and when they reached 1.2 mm in diameter, the animals were sacrificed under inhaled anesthesia (isofluorane). The tumor volume was determined via the modified ellipsoid formula: (tumor length × tumor width^2)/2. All efforts were made to minimize the suffering of the animals. The xenograft tumors were fixed in 10% buffered formalin, embedded in paraffin, then cut into serial sections for hematoxylin and eosin (H&E), and immunohistochemical staining. Primary antibodies used include anti-GFAP and anti-Ki67 (MaiXin-Bio). Dako REAL EnVision Detection System was used for detection. For orthotopic implantation, mice were injected into their right caudate nucleus with 2 µL of 2.0×10^5 of U87-MG, SD2, or SD13 cells. The bodyweights of mice were monitored every 3 days, and they were sacrificed on day 21. Brains derived from the three groups of mice were fixed in 4% paraformaldehyde and processed for H&E staining.

Statistical analyses

Each quantitative experiment was repeated ≥3 times. All statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). All results are presented as mean ± SD and were compared using a two-tailed Student's t-test. $p \le 0.05$ was considered statistically significant.

RESULTS

Glioma malignancy is positively correlated with PIK3CD expression

To investigate the relationship between *PIK3CD* expression and human glioma malignancy, we analyzed RNA sequencing data from TGGA based on *PIK3CD* expression in GBM (WHO IV) and LGG (WHO II and III). The results showed that the level of *PIK3CD* expression in GBM samples was significantly higher than in the LGG samples (Fig. 1A). Consistent with expression pattern, the survival probability was shown significantly lower in GBM cases compared with LGG regarding *PIK3CD* expression (Fig. 1B). Further analysis confirmed that the high *PIK3CD* expression level is correlated with poor overall survival both in GBM (non-significant) and LGG (significant) (Fig. 1C, D). Taken together, these data indicated that *PIK3CD* could take part in GBM pathogenesis. We further performed Western blot analysis on cell lines derived from glioma patients with different malignant degrees and the results showed similar expression differences at protein levels (p110δ) as the TCGA analysis of GBM (Fig. 2A). There is a correlation between p110δ and Thr-308 Akt phosphorylation in the high grade U87-MG cell line; therefore, we used it for our subsequent experiments.

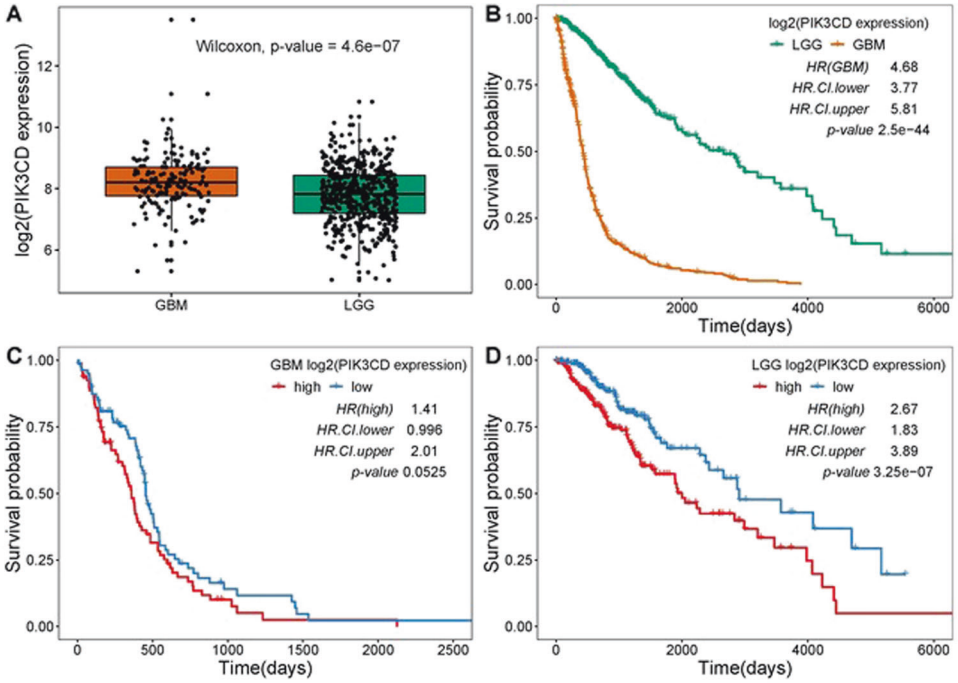

Fig. 1 ***PIK3CD*** **expression is associated with different grades and overall survival of glioma patients. A** *PIK3CD* expression is associated with GBM and LGG. The *p*-value is based on Wilcoxon rank-sum test. **B** Kaplan–Meier survival analysis comparing the *PIK3CD* expression of GBM and LGG. **C**, **D** Kaplan–Meier survival analysis comparing the high and low sets which were partitioned with the *PIK3CD* median expression of GBM and LGG, respectively. The *p*-values and hazard ratio (95% confidence interval) are estimated using Cox-regression with age at diagnosis and gender as covariates.

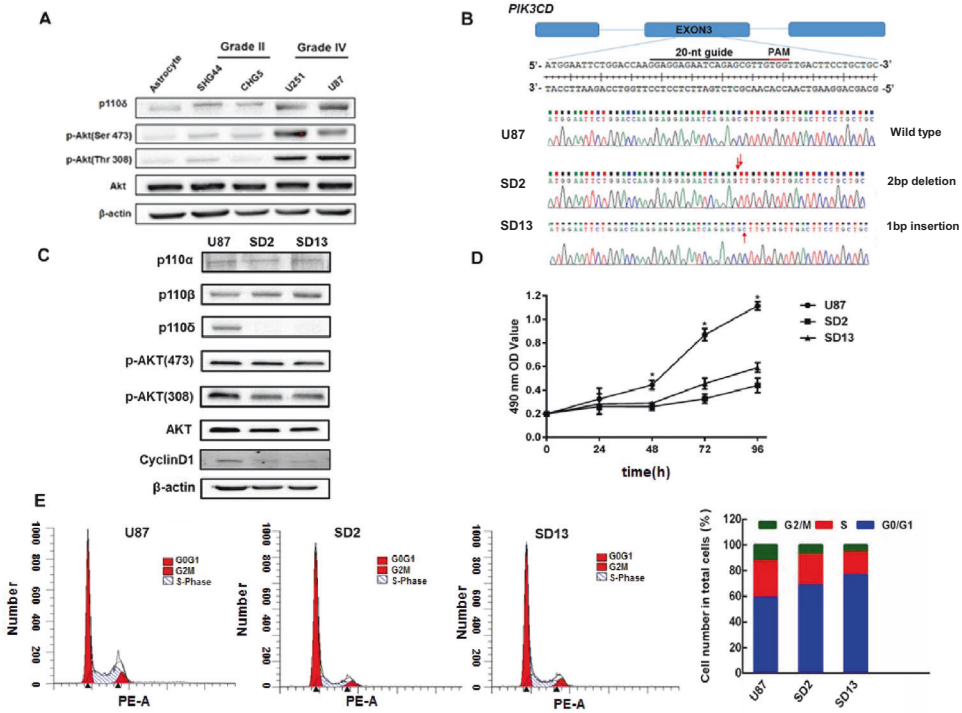

Fig. 2 ***PIK3CD*** **gene expression in different glioma cell lines and the generation of** ***PIK3CD*** **knockout U87-MG glioma cell clones. A** Western blot detection of p110δ, Akt and p-Akt in different glioma cell lines. **B** CRISPR/Cas9 introduced deletion and insertion mutation in the target exon of cell clones SD2 and SD13, respectively. **C** Western blot analysis of p110α, p110β, p110δ, Cyclin D1, and p-AKT protein levels. β-actin used as loading control. **D** Proliferation abilities of *PIK3CD* knockout clones compared with parent cell line (*$p < 0.05$). **E** Cell cycle analysis of *PIK3CD* knockout clones.

Successful knockout of PIK3CD and correlation studies suggest its role in gliomagenenesis

We employed CRISPR/Cas9 technology to completely knockout the *PIK3CD* gene in the U87-MG cell line. Sequencing data showed that 2 isolated clones have different genetic modifications at exon 3 of *PIK3CD*: (1) deletion: 2 bp (SD2), and (2) insertion: 1 bp (SD13) (Fig. 2B). Exome sequencing further confirmed these indel mutations leading to frameshift variants (see Supplementary Fig. 1S). Western blot analysis showed that the expression of p110δ was totally obliterated in the two cell clones but the expression of the other PI3K Class IA isoforms, p110α and p110β, was not affected (Fig. 2C). Interestingly, the level of p-Akt was differentially altered at Thr308 but not Ser473 in both knockout clones. The MTS proliferation assay showed that the two knockout cell clones have significantly lower proliferative capability compared with U87-MG control (Fig. 2D). Flow cytometric analysis of the two knockout cell clones showed a decrease in S-phase of the cell cycle (Fig. 2E). As cyclin D1 plays major roles at the G1 to S-phase transition, we performed Western blot analysis and confirmed its expression is reduced in the knockout clones (Fig. 2C). The migration ability of the knockout clones is lower than the parent U87-MG cells at both 16 and 24 h (Fig. 3A). Trans-well invasion assay also showed that U87-MG cells have higher invasion ability than the knockout clones (Fig. 3B). These results collectively indicated that *PIK3CD* is important in glioma cell proliferation, migration and invasion. The reduced proliferation, migration and invasion exhibited by the *PIK3CD* knockout clones were further examined by colony formation assay in vitro and tumor formation in vivo. The colonies formed by the knockout cells (SD2 and SD13) were significantly less than the parental U87-MG cells (Fig. 3C). Consistent with the in vitro results, SD2 and SD13 cells did not form any noticeable xenograft tumor even 26 days after implantation (see Supplementary Fig. 2SA). Whereas xenograft tumors could be clearly observed 7 days after implantation in the U87-MG control group. The body weight between groups was not significantly different during tumor development. Hematoxylin and eosin staining of cells in the xenograft tumors confirmed the presence of prominent nuclei surrounded by a thin rim of cytoplasm (see Supplementary Fig. 2SA). The cells were poorly differentiated and highly proliferative as indicated by low glial fibrillary acidic protein expression and Ki67 (a marker expressed by proliferative cells) staining, respectively. Orthotopic implantation experiments were also conducted with similar results (Fig. 3D). Thus, these results demonstrated that *PIK3CD* knockout could attenuate the proliferation, migration, and invasion of glioblastoma cells both in vitro and in vivo.

RT^2 profiler PCR arrays and Western-blot show PIK3CD takes part in cell movement by regulating the expression of PAK3 and PLEK2

Focal adhesion molecules and epithelial to mesenchymal transition play important roles in cytoskeleton assembly/disassembly that ultimately control tumor cell movement[19]. Using the RT^2 PCR arrays on genes involved, we identified several of them were significantly down-regulated in knockout cells (Fig. 4A). Among them, *PAK3* and *PLEK2* were found highly down-regulated in knockout cells. Western blot analysis confirmed that PAK3 and PLEK2 were highly down-regulated at protein levels (Fig. 4B), which is supported by qRT-PCR analysis (Fig. 4C). Using the GBM cohort's data set in the TCGA database, we conducted correlation analysis and found that *PIK3CD* expression is positively associated with *PAK3* and *PLEK2* expression

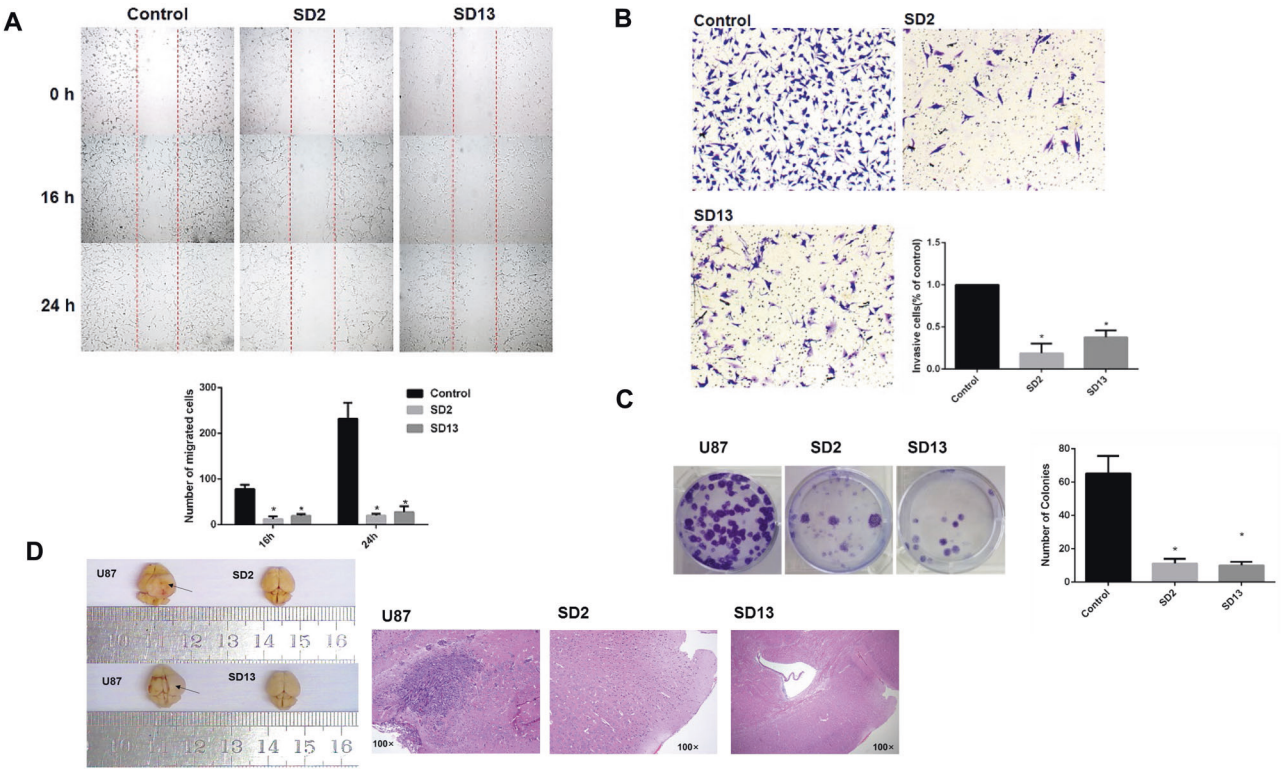

Fig. 3 ***PIK3CD*** **knockout inhibits glioma cell migration, invasion and xenograft tumor formation.** The migration (**A**) and invasion (**B**) abilities of *PIK3CD* knockout clones were much reduced compared with parental U87-MG cells. Representative results from at least three independent experiments are shown (Mean ± SD, $*p < 0.05$). Magnification of the photomicrographs (x100). **C** The in vitro colony formation ability of the *PIK3CD* knockout cells was significantly impaired (Mean ± SD, $*p < 0.05$). **D** Orthotopic implantation showing xenograft tumor formation was inhibited in mice injected with the *PIK3CD* knockout cells. Arrows indicate the tumor formed in mouse brain injected with U87-MG. H&E histology confirmed the inability of PIK3CD knockout cells to form any tumor in the mouse brain.

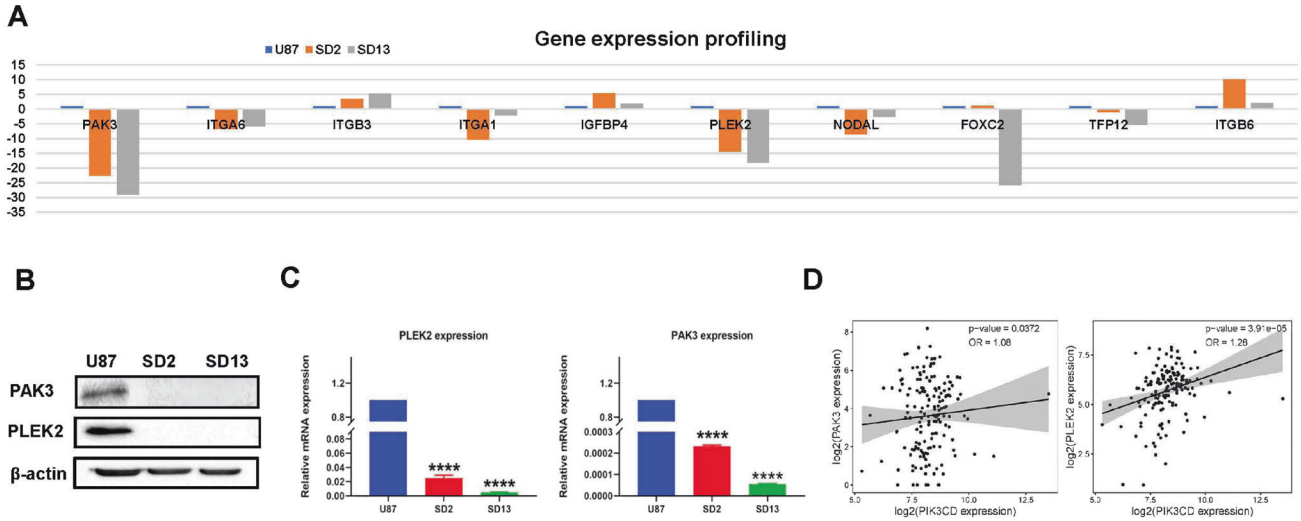

Fig. 4 ***PIK3CD*** **knockout impairs PAK3 and PLEK2 gene expression. A** Gene expression profiling of Human Focal Adhesions and Epithelial to Mesenchymal Transition genes by RT^2 Profiler PCR array. *PAK3* and *PLEK2* gene activity were highly suppressed in knockout cells. **B** Western blot analysis of PAK3 and PLEK2 protein levels. β-actin used as loading control. **C** Analysis of PAK3 and PLEK2 mRNA levels by qRT-PCR. **D** The correlation analysis of *PAK3* and *PLEK2* expression levels with *PIK3CD* based on the TCGA database.

(Fig. 4D). Thus, the oncogenic roles of *PIK3CD* in GBM could be exerted through PAK3 and PLEK2 activity.

Global Transcriptome and pathway analysis suggest novel roles of PIK3CD in glioma cell movement and growth

To investigate the global impact of *PIK3CD* expression on glioma cell biology, we performed the transcriptomic profiling on the control U87-MG and knockout cell lines SD2 and SD13. The total expressed genes are shown in Fig. 5A, and the hierarchical clustering analysis indicated that the *PIK3CD* knockout leads to substantial changes in the expression profile of U87-MG. The Venn plots (Fig. 5B) showed the overlapped up/down regulated genes between U87-vs-SD2 and U87-vs-SD13. List of differentially expressed genes are shown in Supplementary Table 1S. The overlapped differentially regulated genes (DEGs), 1125 up- and 1822 down-regulated, were ranked with the average $\log_2$FC of U87-vs-SD2 and U87-vs-SD13. *PAK3* and *PLEK2*, two genes of major interest, were significantly down-regulated both in SD2 and SD13 cells. The ingenuity pathway analysis (IPA) software predicted a number of cellular functions and pathways are deregulated after *PIK3CD* knockout (Summarized in Table 1). Within the molecular and cellular functions category, the sub-categories related to changes in cell movement topped the list in both knockout clones. We further queried the association of DEGs with GO enrichment analysis and interestingly identified the axonogenesis pathway as one of the most highly over-represented biological process related to the cell movement of glioma cell (Fig. 5C). To better understand the interplay among DEGs in axonogenesis pathway, we generated the PPI networks using the STRING tool (Fig. 5D). It showed that the *PIK3CD* and *PAK3* were the remarkable nodes with the connections in the axonogenesis pathway. We further examined the RNA-Seq data by IPA interaction analysis of DEGs with canonical pathways and found that several genes including, *FYN*, *Cdc42*, actin-related protein G2, *MMP9,* and *CCL5* have direct interactions with *PAK3*. Among them, *FYN* acts as upstream regulator of *PIK3CD*. We also identified integrins as upstream regulators of *PIK3CD* by IPA analysis. The molecular signaling cascade affected after *PIK3CD* knockout is illustrated in Fig. 6. *PIK3CD* knockout suppresses the activity of *PAK3* and *PLEK2*, which in turn affects GBM migration and invasion by down-regulating the expression of *MMP9*, *ACTG2,* and *CCL5*. Together, our RNAseq analysis indicates the profound effect of *PIK3CD* on GBM progression and consolidates our in vitro and in vivo findings.

DISCUSSION

A number of signaling pathways are deregulated in GBM pathogenesis and among them PI3K plays a very critical role[20]. The catalytic subunits of PI3K are not functionally dispensable, therefore, determining the specific roles of each subunit is considered an effective strategy to tackle GBM. Substantial research has been performed regarding isoform-specific roles of PI3Ks in cancers including GBM[21–23]. PI3K p110δ has a major function in immune cell regulation[24], but it is also expressed in a variety of malignancies such as breast cancer[25], colorectal cancer[13], and leukemia[26]. We previously reported that inhibition of PI3K p110δ activity in GBM by siRNA or pharmacological agent suppressed their migration and invasion ability[14]. In agreement with our findings, Schulte et al. showed that high expression of PI3K p110δ in GBM is related to erlotinib resistance[15]. In this study, we found that PIK3CD protein expression is positively related with the malignant grade of glioma and dependent on p-Akt status. In view of PIK3CD protein expression in GBM cell lines, we further evaluated the activity of *PIK3CD* gene by CRISPR/Cas9 gene knockout strategy. Successful knockout of *PIK3CD* gene demonstrated that PI3K p110δ acts through phosphorylation of Akt at Thr308 while not affecting other class IA PI3Ks, which is consistent with previous findings[14,15]. Further phenotypic study revealed *PIK3CD* knockout clones not only exhibited reduced migration, invasion and proliferation but their colony formation ability was also suppressed significantly. This agrees with a study showing PI3K p110δ induced tumor growth and apoptosis in breast cancer[25]. In another study, dual inhibition of PI3Kδ/γ affected apoptosis and increased survival rate in multiple myeloma[27]. In agreement with our results, another study indicated that pharmacological inhibition of PI3K p110δ effectively reduced viable GBM cell numbers[11]. To date, the role of PI3K p110δ in GBM growth in vivo has never been studied. This is the first study to show that knockout of *PIK3CD* not only inhibited migration, invasion and proliferation in vitro but also suppressed GBM growth in vivo. Our findings highlighted the diverse functions of the PI3K p110δ isoform in gliomagenesis.

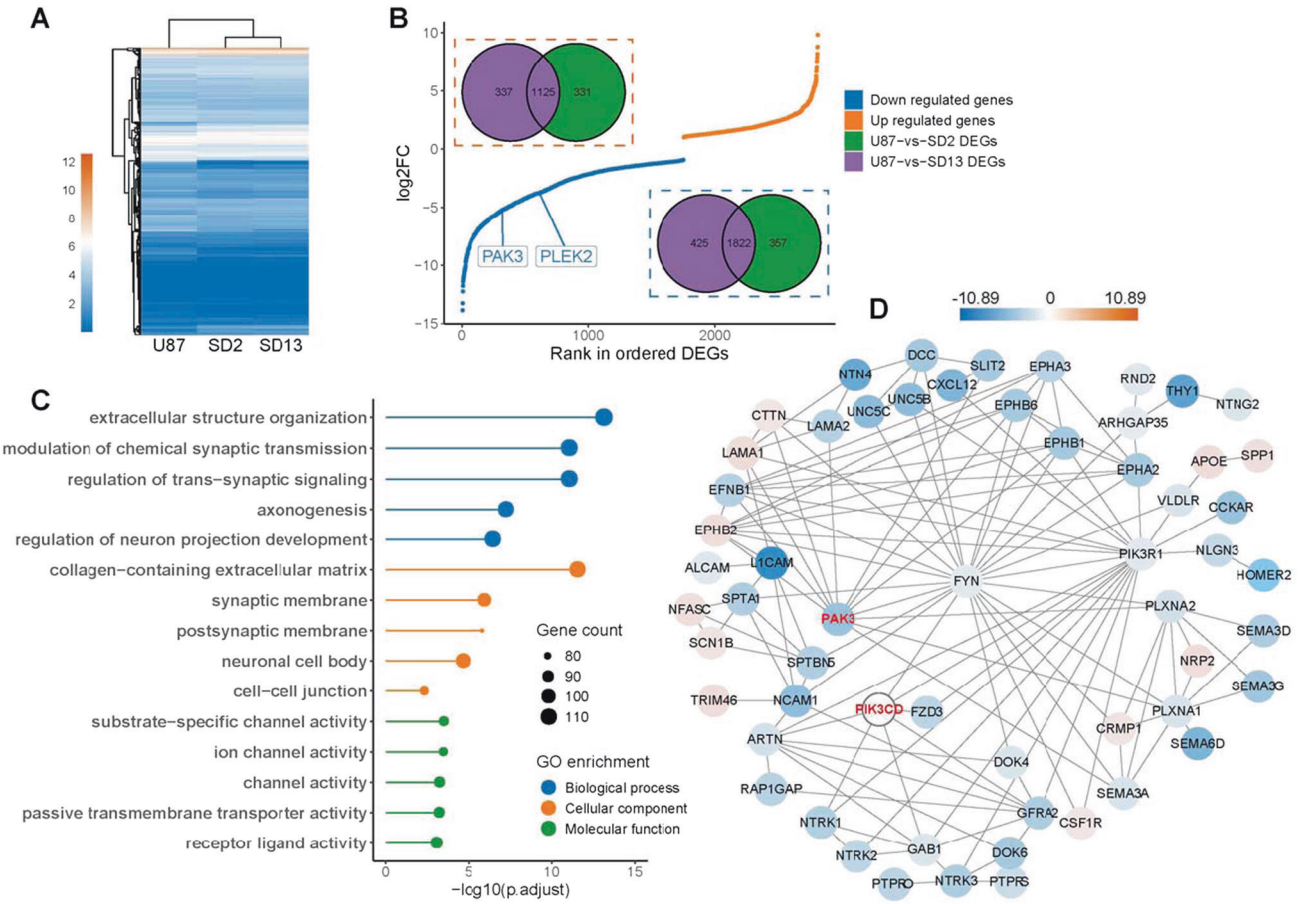

Fig. 5 ***Pathway and Gene Ontology analysis of RNAseq results.*** **A** Heatmap showing all the gene expressions ($log_2(x + 1)$) in U87, SD2 and SD13. The orange color represents up-regulated genes and the blue represents down-regulated genes. Clustering was performed using complete method based on the Euclidean distance. **B** The Venn plots show the overlapped up-/down-regulated DEGs between U87-vs-SD2 and U87-vs-SD13. The 1125 up- and 1822 down-overlapped regulated DEGs were ranked with the average log_2FC of U87-vs-SD2 and U87-vs-SD13. **C** The overlapped DEGs are respectively enriched in top 5 biological processes, cellular components and molecular functions based on the gene ontology enrichment analysis. The different dot size represents the different enriched gene count. **D** PPI network of DEGs in axonogenesis pathway. The *PIK3CD* and *PAK* nodes are highlighted in red.

Cytoskeletal rearrangement through the assembly of PI3K regulated proteins can modulate cell shape and movement[28]. The resulting cytoskeletal changes such as filopodia and lamellipodia formation will ultimately affect cell migration and invasion[29]. Pleckstrin-2 (*PLEK2*), a homolog of pleckstrin-1 (*PLEK1*), can promote cell spreading by regulating actin rearrangement[30]. Unlike *PLEK1*, *PLEK2* activity does not depend upon PKC-regulated phosphorylation but in a PI3K dependent manner[31]. Our results showed that *PLEK2* expression is down-regulated in *PIK3CD* knockout cells compared with control U87-MG cells at both mRNA and protein levels, suggesting *PLEK2* is one of the downstream targets of PI3K p110δ in regulating glioma cell migration and invasion. Another important component of the PI3K signaling cascade is the PAK (p-21 activated kinase) family of kinases (*PAK1* to *PAK6*)[32]. Among them, *PAK3* is preferentially expressed in neuronal cells[33] and involved in neuronal function. Ample evidence indicates that upon activation by Rho GTPases, Rac and Cdc42, PAKs can participate in cancer tumorigenesis through promotion of cell growth, survival, migration and invasion[34–36]. The cross talks between PI3Ks and PAKs are evident in various types of tumors. A recent study showed that *PAK4* overexpression in breast cancer is associated with PI3K overexpression and ultimately lead to tumor growth[37]. Another study demonstrated that following epidermal growth factor stimulation, the levels of PI3K and *PAK1* increased simultaneously[38]. Our data revealed that *PAK3* is down-regulated to a great extent in *PIK3CD* knockout cells at both mRNA and protein levels. Together, these results indicated that *PLEK2* and *PAK3* regulation by *PIK3CD* might be responsible for GBM invasiveness and aggressiveness.

Quantitative gene expression analysis by RNA-Seq provides vital information about gene function in both physiological and pathological conditions. In-depth analysis of DEGs between parental and knockout clones demonstrated reduced cell movement, migration and invasion in both knockout clones compared with control U87-MG cells, which further strengthen our in vitro findings. The role of axonogenesis/neurogenesis in cancer growth is well evident throughout the literature[39,40]. Single cell analysis of glioma indicates axonogenesis related ligands and receptors are expressed in glioma tumors[41]. In this sense, our RNA-Seq dataset identified the involvement of axonogenesis pathway in glioma cell movement. Matrix metalloproteinase 9 (*MMP9)* and chemokine C-C ligand 5 (*CCL5)* involve in glioma progression through enhancing cell proliferation and migration, respectively[42,43]. Actin gamma smooth muscle 2 (*ACTG2)* plays significant role in cancer metastasis and migration[44]. Our in-depth bioinformatics identified *MMP9*, *ACTG2,* and *CCL5* as final targets of *PIK3CD*, works via *PAK3* and *PLEK2*, and their involvement in GBM migration and invasion.

Table 1. Number of genes affected by *PIK3CD* knockout and their relationship with cellular and molecular functions.

Cellular function	*p*-value	Activation z-score	Molecules
U87vsSD2			
Cell movement	1.56E-20	−6.253	242
Migration of cells	7.09E-18	−5.973	215
Invasion of cells	1.34E-09	−3.571	102
Cell movement of tumor cell lines	0.00000021	−3.923	98
Chemotaxis	0.000000834	−3.115	62
Invasion of tumor cell lines	0.00000117	−3.605	76
Migration of tumor cell lines	0.00000127	−3.351	82
Cell spreading	0.00000798	−2.755	36
U87vsSD13			
Cell movement	4.26E-24	−4.567	270
Migration of cells	2.14E-22	−4.725	244
Invasion of cells	6.39E-14	−1.943	122
Migration of tumor cell lines	6.24E-11	−2.336	102
Invasion of tumor cell lines	6.16E-10	−2.144	92
Cell movement of tumor cells	9.35E-09	−0.944	33

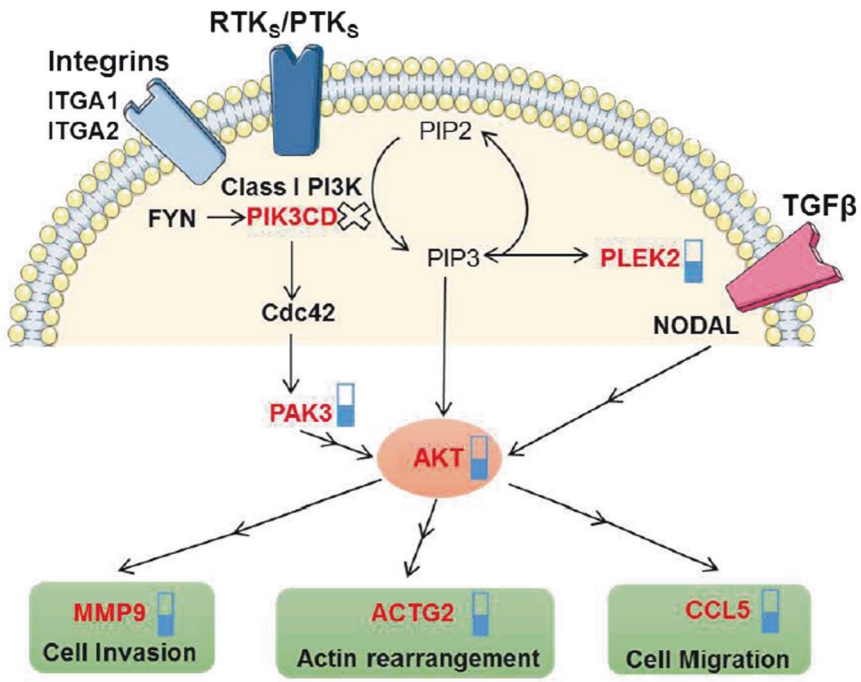

Fig. 6 Schematic representation of the down-regulated signaling cascades resulting from *PIK3CD* knockout. *PIK3CD* activation by external stimuli can directly activate *PLEK2* and indirectly stimulate *PAK3* via cdc42. The resulting *PAK3* activation subsequently affect *MMP9*, *ACTG2* and *CCL5* expression through activating AKT. Elevated *MMP9*, *ACTG2* and *CCL5* expression can modulate GBM cell invasion, actin rearrangement and cell migration simultaneously.

These RNAseq data further suggested that PI3K p110δ is important in the regulation of glioma cell movement, migration and invasion through its action on at least two targets, *PAK3* and *PLEK2*.

There is a recent report showing that the DNA profile of the current U87-MG cell line obtained from ATCC differs from that of the original cells[45]. Nevertheless, it is still a bona fide human glioblastoma cell line that is widely used for GBM studies. We have sequenced the U87-MG cell line used in our study and confirmed it is IDH wild-type as is the case for most glioma cell lines. Although more than one GBM cell lines should be used, we have chosen two *PIK3CD* knockout clones that have different mutations instead to confirm our findings are reproducible.

In conclusion, PI3K p110δ may promote GBM tumorigenesis through axonogenesis/Akt signaling pathway leading to *PAK3* and *PLEK2* up-regulation. As *PIK3CD* also influences other signaling pathways in GBM development, thus, simultaneous inhibition of *PIK3CD*, *PAK3*, and *PLEK2* may be a valid strategy to counteract GBM progression.

DATA AVAILABILITY

The corresponding author is committed to provide the raw RNAseq data and other materials upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, resources and visualization, writing - review and editing: SSTT; investigation: WS, ZA, and JTG; writing - original draft preparation: WS, ZA, and JTG; writing - review and editing: SSTT. All authors have read and agreed to the published version of the manuscript.

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COMPETING INTERESTS

The authors declare no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal ethics approval was obtained from Xiamen University Animal Ethics Committee and the experiments performed were complied with the ARRIVE guidelines.

ADDITIONAL INFORMATION

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