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Reduction of protein kinase C ζ inhibits migration and invasion of human glioblastoma cells

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Abstract

Glioblastomas are the most aggressive forms of primary brain tumors with their tendency to invade surrounding healthy brain tissues, rendering them largely incurable. In this report, we used small-interference RNA technology to knock down the expression of protein kinase C (PKC) ζ, which resulted in specific and massive impairment of glioblastoma cell migration and invasion. We also explained the fundamental molecular processes of glioblastoma migration and invasion in which PKCζ is a participant. The silence of PKCζ expression likewise impaired the phosphorylation of LIN-11, Isl1 and MEC-3 protein domain kinase (LIMK) and cofilin, which is a critical step in cofilin recycling and actin polymerization. Consistent with the defects in cell adhesion, phosphorylation of integrin β1 was also dampened. Therefore, PKCζ regulated both cytoskeleton rearrangement and cell adhesion, which contributed to cell migration. Additionally, there was down-regulation of matrix metalloprotease-9 expression in siPKCζ/LN-229 cells, which coincided with decreased invasion both *in vitro* and *in vivo.* These results indicate that PKCζ is involved in the control of glioblastoma cell migration and invasion by regulating the cytoskeleton rearrangement, cell adhesion, and matrix metalloprotease-9 expression. Collectively, these findings suggest that PKCζ is a potential therapeutic target for glioblastoma infiltration.

Keywords: adhesion, F-actin, integrin β1, invasion.

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Among the malignant tumors of the adult CNS, glioblastomas are the most common; they have a median survival of less than one year. The highly lethal nature of this tumor is the consequence of an invasive phenotype, allowing tumor cells to infiltrate surrounding brain tissues. The initial treatment for many glioblastomas is maximal surgical resection; however, the widespread migration and invasion of tumor cells into the surrounding brain tissues render complete removal virtually impossible. Additionally, the molecular mechanisms that regulate glioblastoma invasion from primary tumor sites to the surrounding normal brain tissues remain poorly understood. Therefore, a better understanding of the glioblastoma cells' migratory and invasive mechanisms may result in new therapeutic approaches.

An over-expressed or hyperactive protein kinase C (PKC) is reported as one of the malignant CNS tumors' most distinguishing characteristics (da Rocha *et al.* 2002). PKC consists of 12 different isoforms, which can be sorted into three subfamilies based on their activation mechanisms: classic, novel and atypical PKC. PKCζ, belonging to the atypical PKC family, which has higher expression in glioblastoma cell lines than normal astrocytes has been reported to have an important role in glioblastomas (Acevedo-Duncan *et al.* 2004). Schih *et al.* reported that when human glioblastoma cell line U373 cells were transfected

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Abbreviations used: ECM, extracellular matrix; EGF, epithelial growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; LIMK, LIN-11, Isl1 and MEC-3 protein domain kinase; MMP, matrix metalloprotease; PI3K, Phosphatidyl inositol 3-kinase; PKC, protein kinase C; siRNA, small interference RNA.

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with anti-sense oligonucleotide sequences to the translation start sites of PKCα, β, γ, δ, ε, or ζ isoforms, only PKCζ and PKCα anti-sense oligonucleotide showed inhibition of vascular endothelial growth factor (VEGF) expression (Shih *et al.* 1999). PKCζ also plays an important role in determining cell polarity (Joberty *et al.* 2000). The isozyme-specific inhibitors demonstrated that PKCζ is involved in chemokine-triggered cell adhesion and actin assembly in polymorphonuclear cells (Laudanna *et al.* 1998; Giagulli *et al.* 2004). The potential importance of PKCζ as a downstream mediator in the Phosphatidyl inositol 3-kinase (PI3K) pathway has been emphasized by our recent finding, that is, it can itself function as a migration regulator in human breast cancers (Sun *et al.* 2005). However, it is not known whether PKCζ is necessary in glioblastoma cells motility and invasion. Likewise, the downstream events involved in atypical PKC-mediated motility have not been determined.

In the present study, we tested the hypothesis that PKCζ directly participates in glioblastoma cell migration and invasion. We down-regulated the PKCζ expression in glioblastoma LN-229 and U87 cells by PKCζ small interference RNA (siRNA); this effectively inhibited migration and invasion *in vitro* and *in vivo*. Our data further demonstrated that several key proteins, including integrin β1, cofilin, LIN-11, Isl1 and MEC-3 protein domain kinase (LIMK), and matrix metalloprotease (MMP)-9, were involved in PKCζ signaling pathways, which are critical for glioblastoma cell migration and invasion. Thus, our study suggests that PKCζ is required in glioblastoma migration and invasion, and is a potential therapeutic target molecule for glioblastoma infiltration.

Materials and methods

Cell culture and reagents

Cells from the human glioblastoma cell lines LN-229 and U87 were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 with 10% Fetal Bovine Serum (FBS) (complete medium). The microboyden chambers and membranes were acquired from Neuroprobe (Gaithersburg, MD, USA). The recombinant human epithelial growth factor (EGF) was obtained from R&D systems (Minneapolis, MN, USA), Gö6850 and chelerythrine chloride from Alexis Biochemicals (San Diego, CA, USA), the myristoylated and non-myristoylated pseudopeptides for PKCζ from Calbiochem (La Jolla, CA, USA). The antibodies against cofilin, P-cofilin, integrin β1, P-LIMK, LIMK1, β-actin, EGFR and PKCζ were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against P-integrin β1 (T788, ZP0351) were bought from ABZOOM (Dallas, TX, USA).

RNA interference

Following the manufacturer's instruction, transfection was performed by using Lipofectamine2000 (Invitrogen) with PKCζ-specific siRNA (insert GAGGAAGTGAGAGACATGTGT) and scramble sequence siRNA from Genescript (Piscataway, NJ, USA). The transfected cells were selected on the basis of their resistance to Hygromycin B (BD Biosciences, San Jose, CA, USA), and their expression level of PKCζ protein was monitored by western blotting to establish the cell lines stably expressed by the mutant proteins. Clones, in which the expression of PKCζ was effectively suppressed, were selected and used for the *in vivo* invasion study.

RT-PCR

Total RNA from cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed and PCR-amplified using the One Step RNA PCR Kit (AMV) from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The PCR procedure both with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PKCζ was done in 95°C for 1 min, 57°C for 1 min and 72°C for 1 min with a total of 35 cycles. The primer sequences were as follows: GAPDH (forward: 5′-ACCACAGTCCATGCCATCAC-3′; reverse: 5′-TCCACCACCCTGTTGCTGTA-3′), PKCζ (forward: 5′-CTGAGGAGCACGCCAGGTT-3′; reverse: 5′-ACGGGCTCG-CTGGTGAACT-3′).

Quantitative RT-PCR

Total RNA was extracted using Trizol (Invitrogen). cDNA was prepared using the First Strand Synthesis kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). For the quantitative RT-PCR (qPCR), the iQ SYBR Green kit was used (Bio-Rad, Hercules, CA, USA). The GAPDH gene was the internal control for all the qPCR experiments. The data were from three independent experiments, and the mean values with SD are presented. For PKCζ qPCR, the primers used are as follows:

forward:TTTACGCCATGAAAGTGGTGAAGA;
reverse: CAGGAGTGTAATCCGACCAGGAA.

Immunoprecipitation assay

Immunoprecipitation analysis was done as described (Fernandis *et al.* 2003). Details were shown in Appendix S1.

EGF activation and western blotting assay

Western blotting assay was performed as described by Sun *et al.* (2005). PKCζ activation assay was done by serum starving the cells for at least 3 h and subsequently activating them with 10 ng/mL EGF at different times. More details were shown in Appendix S1.

Chemotaxis assay

Chemotaxis assay was performed in a 48-well Boyden chamber as described by Sun *et al.* (2005). Details were shown in Appendix S1.

Invasion assay

A Boyden chamber invasion assay was performed as previously described by Albini *et al.* (1987). Details were shown in Appendix S1.

Scratch assay

One day before assay, cells were plated in 35-mm dishes at a density of 1×10^6 cells/mL to form a monolayer. An even trace was then lined out in the middle using a 10 μL pipette tip (Pixley *et al.* 2001). The cells were then incubated at 37°C in 5% CO_2, and the wounds were photographed at intervals until they were occluded by migrating cells.

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Adhesion assay

This assay was performed as described by Sun *et al.* (2005). Details were shown in Appendix S1.

Cellular F-actin measurement

Cellular F-actin measurement was carried out as described previously (Sun *et al.* 2005). Details were shown in Appendix S1.

Fluorescence microscopy

The cells were cultured one day prior to staining and starved in serum-free medium for 3 h. After stimulation with 50 ng/mL EGF at 37°C for 1 min, cells were fixed and then were incubated with Alexa-fluro 568 phalloidin for 30 min. Fluorescence of cells were visualized with the Nikon inverted fluorescent microscope (Nikon, Inc., Tokyo, Japan).

Tumor invasion assay *in vivo*

The invasion of glioblastoma cells was analyzed *in vivo* by subcutaneous inoculation as previously described (Blazquez *et al.* 2008). Male athymic Nu/Nu mice, 4- to 6-weeks-old, were purchased from Wei Tong Li Hua Experimental Animal Co. Ltd. (Beijing, China). All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Health. A total of 2×10^6 cells were inoculated subcutaneously into the mice's left flank. After 28 days, the tumors were excised. These were then fixed with 4% paraformaldehyde and embedded in paraffin after which they were sectioned to a thickness of 5 µm with a microtome, mounted on glass slides, and processed for H&E staining. A group of ten mice were used for each data point.

Statistical analysis

Prism 3.0 software (San Diego, CA, USA) was used for data analysis. Chi-square Test was used for *in vivo* analysis and the two-way ANOVA were used for two groups of data *in vitro*. All the results were generated from three independent experiments.

Results

Reduction of PKCζ impaired LN-229 cells migration

We first examined the expression of PKCζ in two glioblastoma cell lines, LN-229 and U87 cells. Western blotting analysis revealed the expression of PKCζ in both LN-229 and U87 cells at a level comparable to that of MDA-MB-231 cells, a type of human breast cancer cells (Fig. 1a). Regarding the specific involvement of PKCζ in EGF-induced migration of glioblastoma cells, we applied the siRNA technology to inhibit PKCζ expression. Specifically, LN-229 cells transfected with PKCζ siRNA plasmid were designated as siPKCζ/LN-229 cells, while siRNA construct containing a scrambled sequence was transfected into LN-229 cells to generate control cells, which were designated as scr/LN-229 cells. Transfected cells were selected by Hygromycin B resistance to establish cell lines that stably down-regulated PKCζ expression. Following Hygromycin B selection, infected cells were screened for PKCζ expression using western blotting analysis. As shown in Fig. 1(b), almost 70–80% of PKCζ protein levels were reduced in siPKCζ/LN-229 cells compared with those in scrambled scr/LN-229 cells or non-transfected cells. In addition, subsequent experiments using at least three independent batches of siPKCζ/LN-229 cells showed at least 70% PKCζ protein reduction. Meanwhile, the RT-PCR analysis detected a marked loss of PKCζ mRNA in siPKCζ/LN-229 cells, but not in scr/LN-229 cells or non-transfected cells which was consistent with the result of quantitive RT-PCR (Fig. 1b and c). On the other hand, western blotting analysis was applied to identify whether the transfection down-regulates cell surface EGFR expression. The analysis revealed considerable expression of EGFR on the cell surface of both glioblastoma and PKCζ down-regulated cell

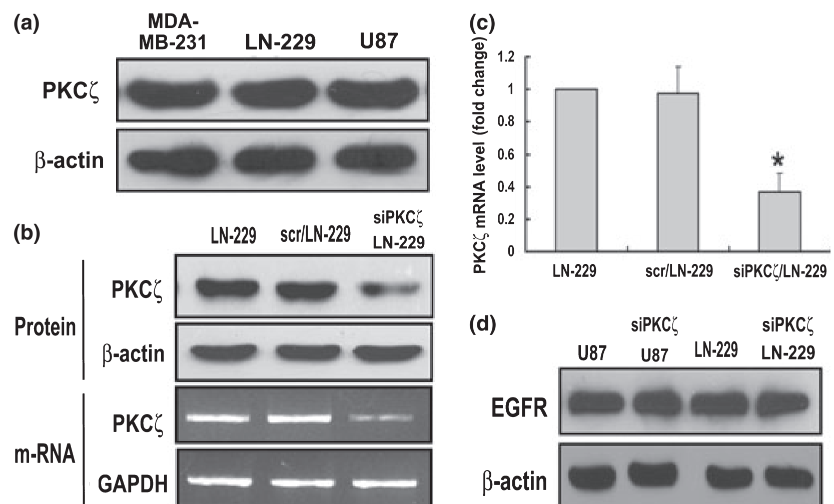

Fig. 1 (a) Comparison of the PKCζ expression in MDA-MB-231, LN-229, and U87 cells by western blotting; 20 µg of protein was loaded on each lane. (b) Western blotting and RT-PCR analysis of PKCζ expression in LN-229 cells. β-Actin, and GAPDH were used as internal control. (c) Quantitative PCR analyses showed the relative levels of PKCζ mRNA expression in LN-229, scr/LN-229 and siPKCζ/LN-229 cells. Data are given as mean ± SEM, $n = 3$. $*p < 0.05$, two-way ANOVA. (d) Protein expression of EGFR in U87, LN-229 cells, and their transfected groups by western blotting analysis; 5×10^5 cells were lysed and separated by 8% SDS-PAGE.

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lines, while siRNA plasmid transfection did not induce any detectable loss of surface EGFR (Fig. 1d).

Migration contributes to several important pathological processes, including immunity, wound repair, tumor formation, and metastasis. In order to examine how PKCζ affects cell migration, we performed cell chemotaxis assay and wound healing assay. Chemotaxis refers to directional cell movement dependent on a concentration gradient. A 48-well chemotaxis model was applied to perform EGF-induced 3-h cell chemotaxis. To test the hypothesis that atypical PKCζ plays an important role in glioblastoma cells chemotaxis we first examined the role of PKC family members in EGF-mediated chemotaxis using the specific inhibitors of different subtypes. As shown in Fig. 2(a), LN-229 cells were pretreated for 1 h with a spectrum of PKC inhibitors before being tested in 10 ng/mL EGF-induced chemotaxis assays. Chelerythrine chloride (Chel. C.), a specific inhibitor of all the PKC isotypes, severely impaired the EGF-induced chemotaxis in a dose-dependent manner, indicating that PKC isozymes were required. However, Gö6850, a specific inhibitor for classic and novel types of PKC, failed to inhibit chemotaxis. These results suggested that it was the atypical PKC and not the classic or novel PKC isotypes that was involved in the LN-229 cell chemotaxis. In contrast, Fig. 2(b) illustrates robust chemotaxis of the parental LN-229 cells and scr/LN-229 cells induced by EGF in a bell-shaped manner, although the chemotaxis of siPKCζ/LN-229 cells was impaired in response to PKCζ reduction. Also, EGF-induced chemotaxis in U87 cells was detected, consistent with our chemotaxis data in LN-229 cells. These results revealed that PKCζ was required for glioblastoma cell chemotaxis, while a concentration gradient was required for efficient migration of glioblastoma cells in response to EGF.

Scratch assay, an *in vitro* wound-healing assay, is another method of evaluating cell migration capacity. It was observed that scr/LN-229 cells migrated into the wound and resulted in wound closure within 26 h, whereas siPKCζ/LN-229 cells were significantly less motile (Fig. 2c) as supported by the delay in the mean distance of closure (Fig. 2d). Both the scr/LN-229 and siPKCζ/LN-229 cells were maintained in a medium supplemented with 0.5% FBS to block cell proliferation, which could otherwise account for gap closure. Taken together, our results obviously showed that PKCζ reduction by siRNA impaired the EGF-induced migration and that PKCζ was required for migration of glioblastoma cells.

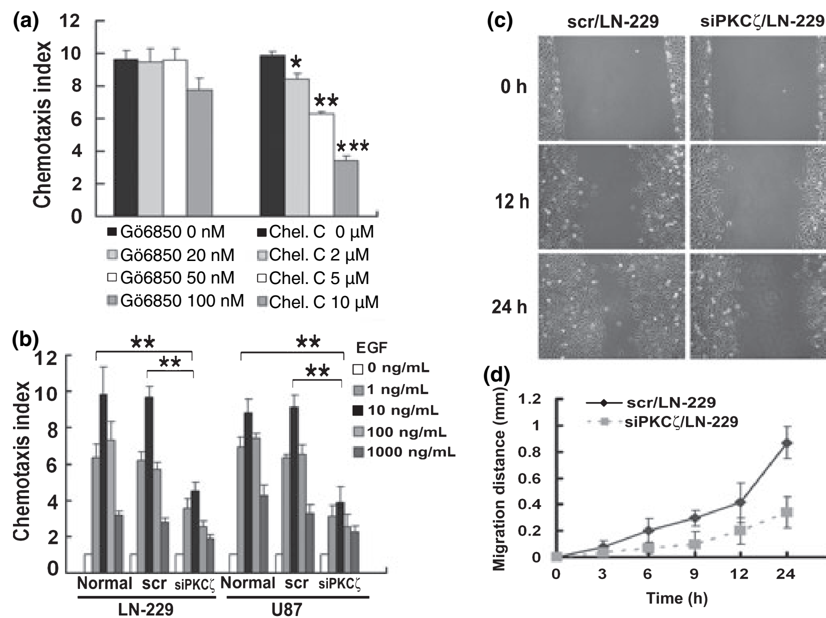

Fig. 2 Involvement of atypical PKC in EGF-induced chemotaxis in glioblastoma cell lines and PKCζ-deficient LN-229 cells display decreased motility. (a) EGF-elicited chemotaxis assay at the presence of Gö6850 (0 nM, 20 nM, 50 nM, 100 nM) and Chel. C. (0 μM, 2 μM, 5 μM, 10 μM). EGF was used at 10 ng/mL. (Data from three independent experiments summarized as mean ± SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two-way ANOVA.) (b) EGF-elicited chemotaxis of LN-229, U87 cells, and their siPKCζ transfected groups. (Data from three independent experiments summarized as mean ± SEM, $n = 3$. **$p < 0.01$, two-way ANOVA.) (c) Images of scratch assay of scr/LN-229 and siPKCζ/LN-229 cells at 0 h, 12 h, and 24 h (100×). LN-229 cells were grown to confluence on a 10-cm cell culture dish and the monolayer was wounded with a 10-μL pipette tip. (Data from three independent experiments.) (d) The migrating distance of the scr/LN-229 and siPKCζ/LN-229 cells at different time points in the scratch assay. (Data from three independent experiments, two-way ANOVA analysis, $p < 0.05$.)

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Reduction of PKCζ impaired F-actin polymerization of LN-229 cells

The actin cytoskeleton is a key mediator of cell polarization and the directed migration of cells toward a chemoattractant. The ability of cells to move implies dynamic remodeling of the actin cytoskeleton that governs the extension of cell protrusions and the retraction of the cell body (Ridley *et al.* 2003). Chemotaxis requires cytoskeletal reorganization and formation of filamentous actin (F-actin). In this regard, the F-actin polymerization assay was carried out to prove the hypothesis that the reduction of PKCζ could impair LN-229 cell migration by reducing the F-actin polymerization. In particular, LN-229 cells were stimulated at 0 s, 15 s, 30 s, 1 min, 2 min, 5 min with 50 ng/mL EGF. The cells were stained with Alexa-fluro 568 phalloidin and the levels of F-actin polymerization were detected by microplate fluorescence reader. An increase of the F-actin contents induced by EGF could be observed in scr/LN-229 cells but not in siPKCζ/LN-229 cells (Fig. 3a). Actin polymerization is most pronounced within 2 min after EGF stimulation, and two phases of typical actin polymerization were observed in the scr/LN-229 cells (Fig. 3b). The first phase peaked at 15 s and the second phase peaked at 60 s, which is consistent with previous reports (Chen *et al.* 2003; Wang *et al.* 2008). These two phases of actin polymerization display different dependencies and have unique roles during chemotaxis. Mouneimne *et al.* demonstrated that the first peak depended on phospholipase C (PLC)-γ and cofilin (Mouneimne *et al.* 2004) and the second peak depended on PI3K activity (Hill *et al.* 2000; Chen *et al.* 2003). In siPKCζ/LN-229 cells, the two phases were delayed and the two peaks were both lower than the control. As actin polymerization induced by EGF

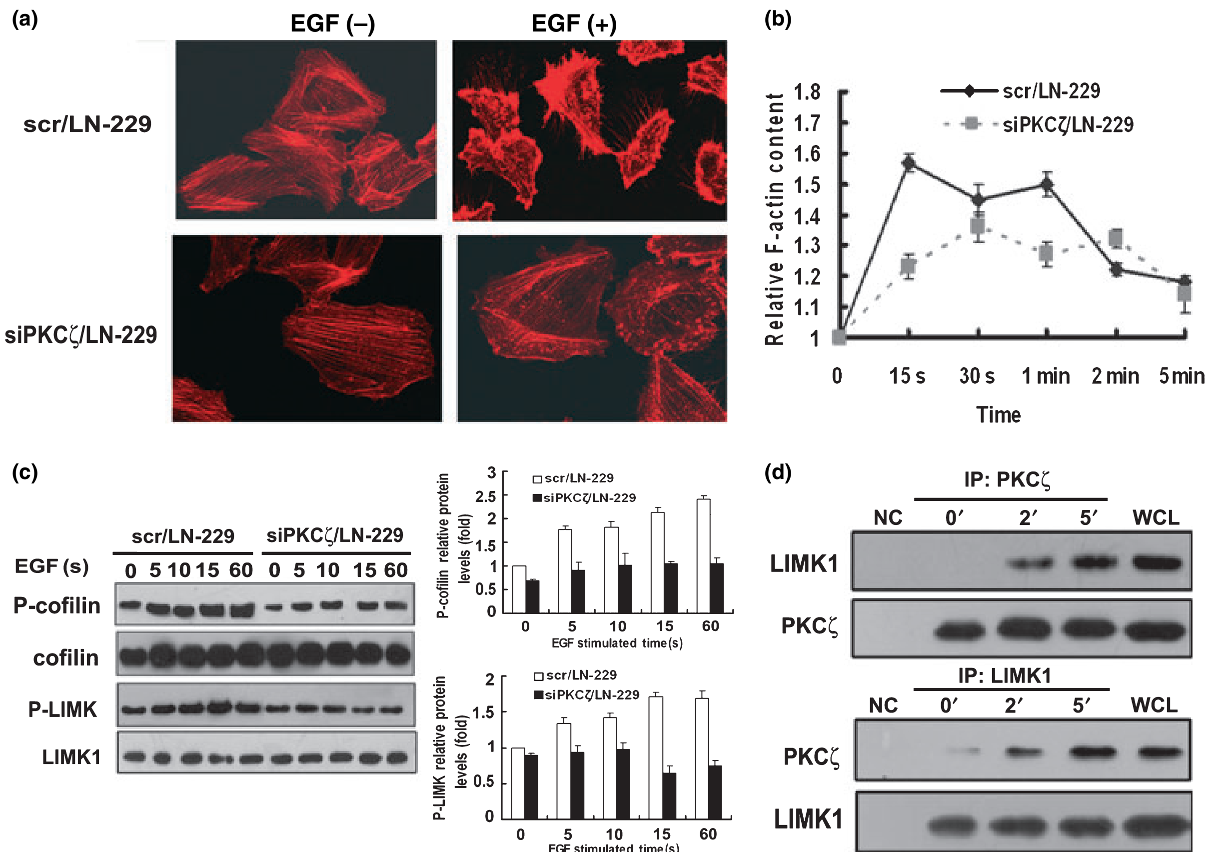

Fig. 3 Disruption of PKCζ by siRNA impaired actin polymerization in LN-229 cells. (a) Cytoskeleton rearrangement in scr/LN-229 cells and siPKCζ/LN-229 cells were imaged by fluorescence staining. The data were collected by representative images from three repeated experiments (400×). (b) Time course of relative F-actin content in scr/LN-229 cells and siPKCζ/LN-229 cells. Cells were treated with 10 ng/mL EGF for various times before fixation and staining of F-actin by using phalloidin conjugated to Alexa Flour 568. Data from three independent experiments ($n = 3$). (c) Western blotting analysis of phosphorylated LIMK and cofilin in total cell lysates from scr/LN-229 and siPKCζ/LN-229 cells after stimulating with 50 ng/mL EGF for 0 s, 5 s, 10 s, 15 s, and 60 s. LIMK1 and cofilin were used as loading control respectively. Data from three independent experiments ($n = 3$). (d) Immuno-precipitation analysis of LIMK1 and PKCζ. LN-229 cells were stimulated with 10 ng/mL EGF for 0 min, 2 min, and 5 min. Lane NC, immuno-precipitation without PKCζ or LIMK1 antibodies as negative control. Lane WCL, whole cell lysates. Each immuno-precipitation is a representative of three independent experiments.

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was significantly reduced in siPKCζ/LN-229 cells, this signified that PKCζ was required for EGF-induced rearrangement of the cytoskeleton.

LIM kinase and cofilin are the two critical factors involved in the regulation of F-actin dynamics; their phosphorylation mediates the regulation of chemoattractants inducing actin polymerization. To test the hypothesis that PKCζ mediates EGF-induced activation of LIMK and cofilin, which in turn regulates actin polymerization, we detected evidence for LIMK and cofilin activation. As seen in Fig. 3(c), reduction of PKCζ-inhibited EGF induced phosphorylation of LIMK and cofilin consistent with their defects in actin polymerization. Together, these findings indicated that the down-regulation of PKCζ expression in LN-229 cells resulted in a major cytoskeletal reorganization defect in response to EGF, possibly through regulation of LIMK/cofilin phosphorylation.

Our previous report has shown that the inhibition of PKCζ could impair the activation of LIMK in lung cancer cells (Liu *et al.* 2008). In this study, we examined the possibility of PKCζ interacting with LIMK1 directly. Immuno-precipitation analysis showed that treatment with EGF at 10 ng/mL for 2 min and 5 min induced co-precipitation of LIMK1 with PKCζ.. LIMK2 was not pulled down by PKCζ (data not shown). LIMK1 was co-precipitated with PKCζ by the anti-PKCζ antibody (Fig. 3d top panel), and PKCζ was co-precipitated with LIMK1 by the anti-LIMK1 antibody (Fig. 3d low panel). Lane whole cell lysates indicated that sufficient LIMK1 and PKCζ were present in cell lysates during immuno-precipitation assays. These results suggested that EGF induced direct interaction of PKCζ with LIMK1.

Reduction of PKCζ impaired adhesion of LN-229 cells

The rate of cell migration can be limited by the rate of rear retraction, and thus the dynamic formation and disassembly of cell–matrix adhesions are critical to cell migration (Ridley *et al.* 2003). We have shown F-actin polymerization changes induced by reduction of PKCζ in LN-229 cells; we also observed the effect of PKCζ on cell adhesion in LN-229 cells. Specifically, EGF at 10 ng/mL induced a more than two fold increase in the numbers of adhesive scr/LN-229 cells compared with siPKCζ/LN-229 cells at 5 min ($p < 0.05$). Disruption of PKCζ resulted in a marked reduction in numbers of adhesive cells in 5 min and 15 min. Although the numbers of adherent cells in scr/LN-229 cells and siPKCζ/LN-229 cells increased after incubated with EGF for 30 min, there was no statistically significant difference between these two groups (Fig. 4a). Integrin β1 is the main adhesion mediating molecule in tumor cells and its phosphorylation can promote cell attachment. Thus, we examined the EGF-induced activation of integrin β1 by western blotting analysis. As shown in Fig. 4(b), EGF-induced phosphorylation of integrin β1 was severely impaired in siPKCζ/LN-229 cells, consistent with the reduction in adhesion. Our results suggested that the PKCζ directly mediated migration signal pathways through regulation of EGF-induced cell adhesion in LN-229 cells.

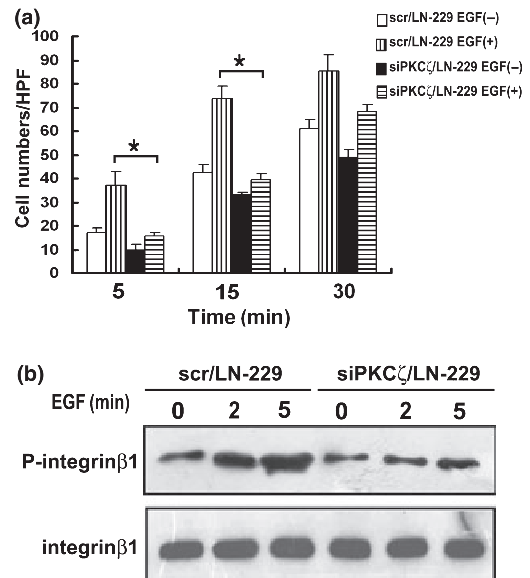

Fig. 4 Disruption of PKCζ by siRNA impaired adhesion of LN-229 cells. (a) Comparison of 10 ng/mL EGF-induced adhesion at 5 min, 15 min, and 30 min of scr/LN-229 cells to that of siPKCζ/LN-229 cells on fibronectin-coated coverslips. Cell numbers in each high power field (HPF) were counted in five fields on every coverslips under microscopy (200×) (Data from three independent experiments summarized as mean ± SEM, $n = 3$. *$p < 0.05$, two-way ANOVA.) (b) Western blotting analysis of phosphorylated integrin β1 in total cell lysates from scr/LN-229 and siPKCζ/LN-229 cells after stimulating with 10 ng/mL EGF for 0 min, 2 min, and 5 min. Integrin β1 was used as a loading control.

PKCζ pseudosubstrate blocked EGF-induced chemotaxis

To further confirm our observation by PKCζ siRNA (Fig. 2a), we investigated the role of PKCζ in glioblastoma cells by using a myristoylated pseudosubstrate, which is a proven specific inhibitor of PKCζ. Myristoylation of this pseudosubstrate was required to deliver this polypeptide through the plasma membrane (Laudanna *et al.* 1998). As shown in Fig. 5(a), the non-myristoylated peptide at 50 μM, which served as a negative control, had no detectible effect on EGF-induced chemotaxis in LN-229 cells. However, the addition of specific myristoylated peptide could apparently inhibit EGF-induced chemotaxis in a dose-dependent manner. The inhibitory effects were further confirmed by EGF-induced chemotaxis in U87 cells (Fig. 5b). Following this, we evaluated the inhibitory effect of a specific pseudosubstrate on cell adhesion. Within 5 min, treatment with 10 ng/mL EGF significantly impaired cell adhesion after the specific myristoylated PKCζ pseudosubstrate treated these two glioblastoma cell lines (Fig. 5c). Altogether, the results suggested that PKCζ was specifically required for EGF-induced cell migration and adhesion.

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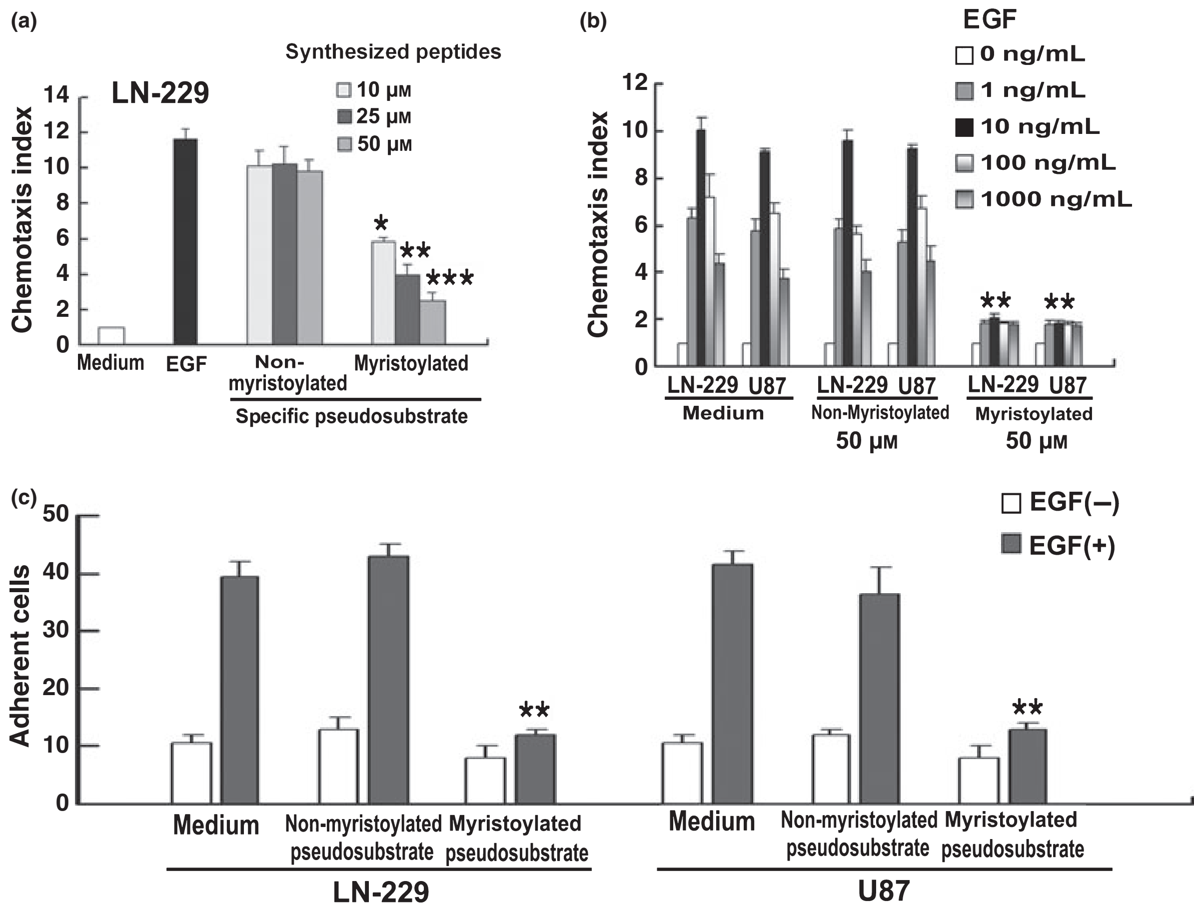

Fig. 5 Specific PKCζ pseudosubstrates blocked EGF-induced chemotaxis and adhesion in glioblastoma cells. (a) Blockage of chemotaxis in LN-229 cells by myristoylated PKCζ pseudosubstrate peptide under EGF stimulation (Data from three independent experiments summarized as mean ± SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two-way ANOVA.) (b) Inhibitory effect of 50 μM PKCζ pseudosubstrates on EGF-induced LN-229 and U87 glioblastoma cells chemotaxis. (Data from three independent experiments summarized as mean ± SEM, $n = 3$. **$p < 0.01$, two-way ANOVA.) Cells were collected and incubated with myristoylated pseudosubstrate peptide, non-myristoylated peptide, or PBS at 37°C for 45 min before chemotaxis assay for (a) or (b). (c) The impairment on cell adhesion with pseudosubstrate treatment. LN-229 and U87 cells were pretreated with myristoylated pseudosubstrate peptide, non-myristoylated peptide, or PBS at 37°C for 45 min before 10 ng/mL EGF stimulation. (Data from three independent experiments summarized as mean ± SEM, $n = 3$. **$p < 0.01$, two-way ANOVA.)

Reduction of PKCζ expression impaired glioblastoma cells invasion

Cell migration is essential in tumor invasion and metastasis. We have demonstrated that PKCζ plays a key role in LN-229 cells migration, hence we hypothesize that PKCζ has a pivotal effect on glioblastoma cells invasion. To measure the effects of PKCζ on glioblastoma cell invasiveness, we employed a Transwell invasion system. The system consisted of two fluid-filled stacked compartments separated by a porous membrane filter coated with Matrigel. It was then observed that the siPKCζ/LN-229 cells that invaded through the Matrigel were much less compared to the scr/LN-229 cells (Fig. 6a). The quantitative analysis of cell numbers revealed that the siPKCζ/LN-229 cells had a 4.3-fold lower invasion rate than that of the scr/LN-229 cells in response to the 10 ng/mL EGF (Fig. 6b).

Processes of tumor cell invasion into the stromal tissues are closely related to the interactions between tumor cells and the extracellular matrix (ECM). MMPs degrade the ECM at tumor-invasive fronts to overcome the ECM barrier, and subsequently enabling tumor cells to penetrate into adjacent brain structures. In the MMP family, the MMP-2 and MMP-9 are two of its important members. It has been reported that MMP-9 protein expression was inhibited by PI3K/Akt down-regulation (Lungu *et al.* 2008). In our current study, in order to detect the fundamental response of MMP-2 and MMP-9 to PKCζ deficiency, we applied western blot to show the protein levels of them in LN-229 cells. Of the two, we found that

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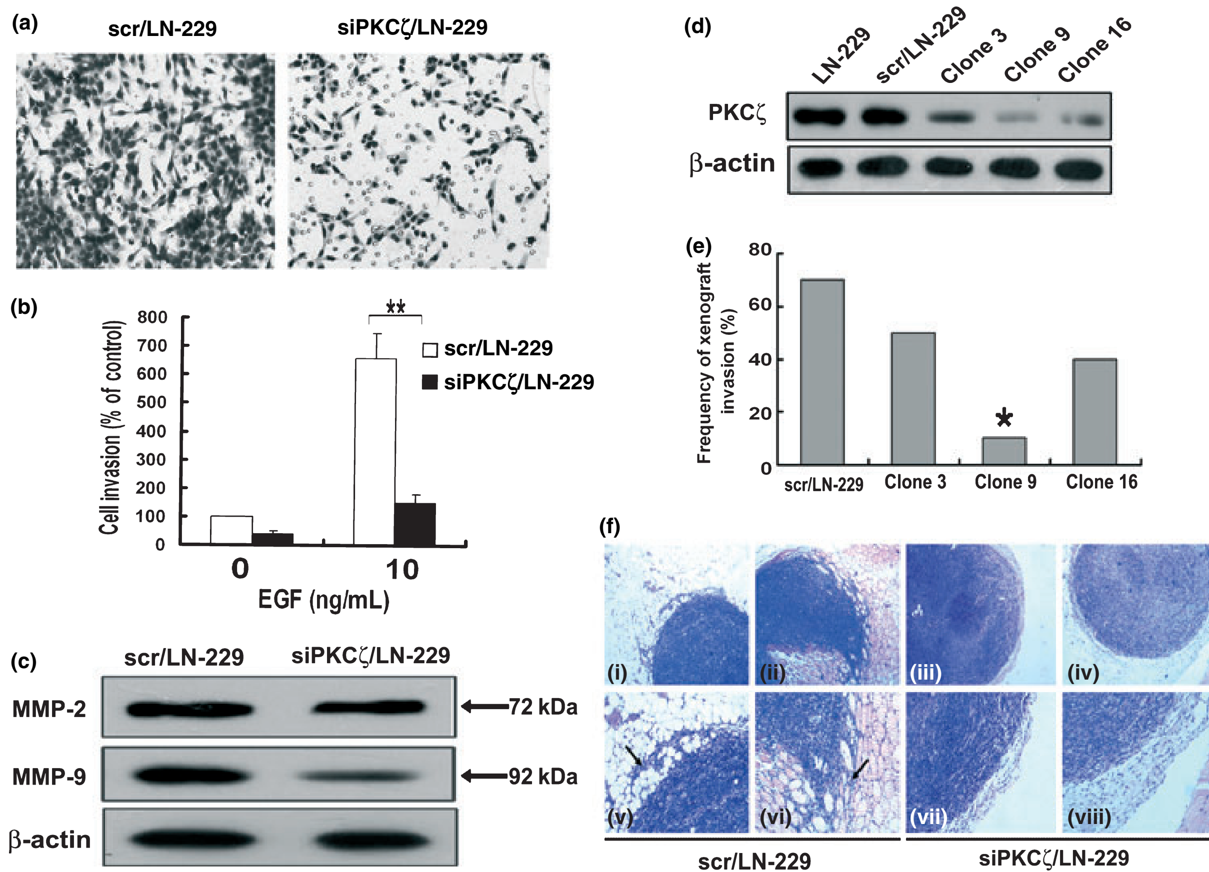

Fig. 6 Disruption of PKCζ by siRNA impaired invasion into matrigel in LN-229 cells and also impaired the invasion of glioblastoma cells *in vivo*. (a) Invasion was measured as described in 'Materials and Methods.' The cells that invaded through matrigel-coated transwell inserts toward chemoattractant were stained. Photographs were taken at a magnification of 200×. (b) The cells invading through the matrigel were counted under microscope in five pre-determined fields at 400×. Data are shown as mean ± SEM, $n = 3$. $^{**}p < 0.01$, two-way ANOVA. (c) Western blotting analysis of MMP-2 and MMP-9 expression in scr/LN-229 cells and siPKCζ/LN-229 cells. β-Actin was used as a loading control. (d) LN-229 cells were transfected with either control or PKCζ siRNA plasmid followed by the isolation of stable clones. Cell lysates of each representative clone were analyzed by western blotting using anti-PKCζ antibody and anti-β-actin antibody. (e) Frequency of xenograft invasion in control group and three clones of the PKCζ knockdown groups (10 mice for each group, $^{*}p < 0.05$, Chi-square Test). (f) Glioblastoma-xenografted tumors were sectioned for H&E staining to identify tumor regions. (i) and (ii): tumors of scr/LN-229-cells-injected mice group. (iii) and (iv): tumors of siPKCζ/LN-229-cells-injected mice group. Panel (v) and (vi) are enlarged figures of (i) and (ii), respectively. (Arrows indicate the invasive scr/LN-229 cells). Top row, 100×; bottom row, 200×.

MMP-9 expression in siPKCζ/LN-229 cells was significantly inhibited, while the MMP-2 expression remained almost unchanged (Fig. 6c). This is consistent with the previous report (Esteve *et al.* 2002).

Disruption of PKCζ by siRNA inhibited the invasion of glioblastoma cells *in vivo*

After we demonstrated, through the above results, that disruption of PKCζ decreased migration and invasion of LN-229 and U87 cells *in vitro,* we next used the subcutaneous mouse xenograft model to validate the role of PKCζ in glioblastoma invasion *in vivo*. In particular, the LN-229 cells were transfected with plasmid expressing either PKCζ siRNA or control plasmid, and several independent stable clones were isolated. Clone 3 showed about 40% reduction of PKCζ, while clone 16 and 9 presented about 70% and 85% reduction of PKCζ, respectively, compared with the LN-229 cells (Fig. 6d). Clones 3, 9, 16 and the scr/LN-229 clone cells were chosen for further *in vivo* tumor invasion assays. The frequency of xenograft invasion of 10 Nu/Nu mice in each group was compared. Group of clone 3 and clone 16 showed small differences compared with the control group, respectively ($p = 0.65$, $p = 0.37$); the frequency of xenograft invasion in clone 9 group was greatly decreased ($p = 0.02$) (Fig. 6e). Representative tissue sections of Haematoxylin and Eosin (HE) staining showed that multiple

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invasion sites of tumors were observed surrounding the tumor boundary in scr/LN-229 clone group, but not in mice injected with clone 9 cells (Fig. 6f).

Discussion

Protein kinase C is a family of enzymes that mediates the normal cellular signaling pathways downstream of growth factors receptors. It controls many cellular key functions, such as cell proliferation, survival, and migration (Donson *et al.* 2000). PKCζ, an atypical subtype of the PKC family, has been found to be involved in migration of breast cancer cells (Sun *et al.* 2005). The expression of PKCζ in glioblastomas was reported to be two times higher than the level in normal glial cells (Acevedo-Duncan *et al.* 2004). Although protein levels do not represent enzyme activity levels, the over-expression of PKCζ suggests it is involved in the malignant phenotype of glioblastomas. Therefore, elucidation of PKCζ signals associated with glioblastoma migration and invasion is crucial in revealing the mechanisms behind glioblastoma infiltration. In this study, we have shown that PKCζ is a key effector of migration and invasion in human glioblastoma cells. In addition, we also illustrated the fundamental molecular processes of glioblastoma migration and invasion in which PKCζ is a participant.

Directed cell migration and cell polarity are crucial in many biological processes, including wound healing and immune responses, as well as in pathological phenomena such as tissue invasion and metastasis formation. In our study, the down-regulation of PKCζ expression by siRNA in the chemotaxis and scratch assays severely impaired the migration ability of the LN-229 cells. This result suggested the general involvement of PKCζ in glioblastoma cell migration. In addition, actin polymerization and the subsequent formation of membrane protrusions are required for cell migration. This remodeling of the actin cytoskeleton is important for the motility and chemotaxis of cancer cells as it consequently influences the metastatic capability of these cells. Our results showed that PKCζ participates in actin polymerization to mediate cytoskeletal rearrangement, which plays an important role in glioblastoma cells migration and invasion.

Indeed, the cytoskeleton inside the cell assumes a significant task in cell migration and invasion. Regulation of actin polymerization of the cytoskeleton allows cells to control their shape and to move. Many studies have shown that LIMK phosphorylates cofilin, in turn regulates actin polymerization (Arber *et al.* 1998; Yang *et al.* 1998). Our results showed that the down-regulation of PKCζ expression in LN-229 cells resulted in attenuated phosphorylation of both LIMK and cofilin. Thus, PKCζ regulates phosphorylation of LIMK and cofilin in actin polymerization, which in turn influences migration of gliblastoma cells. Moreover, integrin β1 is a key adhesion molecule in regulating cell–cell and cell–matrix adhesions, thereby playing a critical role in different aspects of tumor formation and invasion. Results in our current study demonstrated that the PKCζ reduction led to the decrease in phosphorylation of integrin β1 in LN-229 cells. As a result, we speculate that the inhibition of PKCζ activation will further enhance a drastic decrease in integrin β1 recycling and finally lead to the impairment of cells adhesion. Taken together with our previous reports (Sun *et al.* 2005; Wang *et al.* 2008), we propose a simple signaling model: EGF-induced activation of PI3K/Akt/PKCζ pathways leading to rearrangement of cell cytoskeleton through LIMK/cofilin, and cell adhesion through integrin β1.

In the present study, not only did the cellular experiments *in vitro* indicate the reduction of PKCζ inhibited invasion of glioblastoma cells but the animal experiments as well. By invasion detection *in vivo*, the frequency of xenograft invasion in mice significantly decreased after PKCζ was significantly knocked down compared with the control. The efficiency of the PKCζ knockdown correlated with the extent of suppression of invasion. The invasive characteristics of glioblastoma cells depend mainly on the degradation of ECM by proteases and the induction of cell motility and invasion (Nakada *et al.* 2003). MMPs secreted by tumor cells can degrade the ECM at tumor-invasive fronts to overcome the ECM barrier. In fact, it is proposed that the high levels of MMP expression observed in high-grade gliomas serve to facilitate the tumor cells' invasion (Fillmore *et al.* 2001). MMP-2 and MMP-9 are considered to be particularly efficient with their ability to cleave type IV collagen, a major component of basement membrane (Timpl 1989). Moreover, increased production of MMP-2 and MMP-9 appeared to correlate with the malignant progression of human glioma *in vivo* (Forsyth *et al.* 1999; Wild-Bode *et al.* 2001). We demonstrated the significant decrease of MMP-9 expression in siPKCζ/LN-229 cells, while MMP-2 expression was constant. Of note, the involvement of MMP-9 in the PKCζ signaling pathway has been reported in various cell types, such as human breast cancer, pituitary adenoma, and non-small cell lung cancer (Wang *et al.* 2006; Hussaini *et al.* 2007; Lungu *et al.* 2008). While MMP-2 regulated by PKCα, PKCβ and PKCε were established in human melanoma, cardiac microvascular endothelial cells, and ciliary muscle cells, respectively. Accordingly, the functions of MMP-2 and MMP-9 most likely depend on different cell types or PKC isoforms. It has been reported that MMP-9 can form complex with β integrin, which is a part of the mechanism guiding migration in human THP-1 cells and microvascular endothelial cells and such migration is blocked by inhibitors of MMP-9 or β integrin, (Stefanidakis *et al.* 2004). In the present study, we speculated that the inhibition of MMP-9 expression by reduction of PKCζ maybe explains the inhibitory effect of PKCζ on glioblastoma cells invasion.

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PI3K/t-strain from AKR/J mouse (AKT)/PKCζ and the Ras/Raf/mitogen activated protein kinase kinase (MEK)/ Extracellular signal-regulated protein kinase (ERK) (MAPK) are the two most important signaling cascades frequently dysregulated in cancer. There is cross-talk between two such pathways (Grant 2008). Two research groups, Waugh Kinkade *et al.* and Carracedo *et al.*, observed that the simultaneous inhibition of MEK/ERK and the downstream factor of PI3K/AKT/PKCζ resulted in substantially enhanced anti-tumor effects both *in vivo* and *in vitro* (Carracedo *et al.* 2008; Kinkade *et al.* 2008). Furthermore, Alejandro J. demonstrated that over-expression of PKCζ modulates clonogenicity, cell adhesiveness, motility, and proteases secretion of normal murine mammary cells through the activation of the ERK/MAPK pathway (Urtreger *et al.* 2005). In our groups, the precise relationship between PI3K and MAPK signaling pathways remains under investigation.

In summary, we demonstrated that PKCζ is required for glioblastoma cell migration and invasion. These findings are highly important when contemplating novel treatments for glioblastomas, which have a tendency to diffuse, conveyed by single cells leaving the core lesion and invading healthy brain tissues, eventually creating recurrent tumors. Although the mechanism of PKCζ function in glioblastomas requires further clarification, our results potentially suggest that inhibition of PKCζ function would bring about progress in glioblastoma therapy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary material.

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