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Knockdown of Ras-Related Protein 25 (Rab25) Inhibits the In Vitro Cytotoxicity and In Vivo Antitumor Activity of Human Glioblastoma Multiforme Cells

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Ras-related protein 25 (Rab25) is a member of the Rab family, and it has been reported to play an important role in tumorigenesis. However, its direct involvement in human glioblastoma multiforme (GBM) is still unclear. The aim of the current study was to investigate the potential role of Rab25 in the growth, proliferation, invasion, and migration of human GBM. Our results showed that Rab25 expression was significantly higher in human GBM cell lines compared with a normal astrocyte cell line. In vitro functional studies revealed that knockdown of Rab25 reduced cell proliferation and inhibited invasion and migration of GBM cells. In vivo experiments showed that knockdown of Rab25 attenuated the tumor growth in nude mice. Finally, knockdown of Rab25 significantly inhibited the phosphorylation levels of PI3K and AKT in GBM cells. Taken together, these findings indicate that Rab25 may act as a tumor promoter in human GBM and that approaches to target Rab25 may provide a novel strategy to treat this disease.

Key words: Ras-related protein 25 (Rab25); Glioblastoma multiforme (GBM); Migration; Invasion

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary central nervous system tumor, which accounts for nearly half of all intracranial primary tumors¹. GBM is characterized by its invasiveness and also by its resistance to radiation and chemotherapy. In general, this disease is associated with an overall poor prognosis², and the 5-year survival rate for patients with GBM is less than 10%³. The main obstacle to the successful treatment of GBM is the development of cellular drug resistance and the fact that GBM cells rapidly intrude into the brain, which results in alteration of the normal brain parenchyma architecture⁴. Despite advances in our understanding of the molecular genetics and biology of GBM, therapeutic progress has remained limited⁵. As such, there remains an urgent need to further investigate the molecular mechanisms of glioma, which can provide the rational basis for new strategies to treat this life-threatening disease.

The Rab protein family consists of small GTP-binding proteins⁶. There is a growing body of evidence indicating that Rab proteins play an important role in a variety of human diseases, including cancer⁷⁻⁹. Ras-related protein 25 (Rab25) is one of the members of the Rab family, and it is involved in the pathogenesis of human cancer.

Increased gene expression of Rab25 has been correlated with poor prognosis in prostate and ovarian cancers. In contrast, other studies have shown that reduced Rab25 gene expression was associated with breast cancer¹⁰⁻¹². Recently, the loss of Rab25 gene expression has been shown to promote intestinal tumors in mice and colon adenocarcinoma in humans¹³. These studies, taken together, suggest that Rab25 plays an important role in tumor progression. Several mechanisms have been proposed to define the potential effect of Rab25 expression on cancer. Cheng et al. reported that Rab25 gene overexpression by increasing cell biomass inhibited apoptosis and autophagy¹⁴. Other studies have suggested that Rab25 expression is associated with chloride ion channel 3-regulated tumor invasion¹⁵. However, the direct role of Rab25 on human GBM has yet to be well defined. With this in mind, the goal of the current study was to investigate the expression and function of Rab25 in human GBM.

MATERIALS AND METHODS

Cell Culture

The human GBM cell lines (U87MG, U373, U343, and LN229) and the normal human astrocyte cell line SVG were purchased from the American Type Culture Collection (Manassas, VA, USA) and were grown in

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Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). All cells were maintained in a humidified atmosphere containing 95% O₂ and 5% CO₂ at 37°C.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from all cell lines using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed using a SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR (qRT-PCR) was conducted in triplicate using iQ Supermix (Bio-Rad, Hercules, CA, USA) on the C1000 Touch TM Thermal Cycler CFX96 Real-Time System (Bio-Rad). The following primers were used: Rab25, 5'-GCTGCTGTCAAGGCTCAGAT-3' (sense) and 5'-CCCCTGCAACCACGATAGTA-3' (anti-sense); β -actin, 5'-CCGTGAAAAGATGACCCAGATC-3' (sense) and 5'-CACAGCCTGGATGGCTACGT-3' (anti-sense). For relative quantification, the levels of individual gene mRNA transcripts were first normalized to the control β -actin. The differential expression of these genes was then analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

GBM cells were rinsed with PBS and rapidly lysed with protein lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM DTT], and then transferred immediately to microcentrifuge tubes and sonicated for 20 s. Protein yield was quantified using the Quick Start Bradford Protein Assay (Bio-Rad). Equivalent amounts of cellular protein (80–100 μ g) were separated by SDS-PAGE and transferred to PVDF membranes, and immunodetection was performed with tubulin as the loading control. The antibodies against Rab25, E-cadherin, N-cadherin, vimentin, p-PI3K, PI3K, p-AKT, AKT, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After washing with TBST, blots were then incubated with horseradish peroxidase-linked secondary antibodies (Invitrogen) at room temperature for 1 h. The blots were visualized by super ECL and quantified by the Quantity ONE (Bio-Rad) software.

RNA Interference and Transfection

Short hairpin RNA targeting Rab5a (sh-Rab25) and nontargeting control siRNA (sh-NC) were synthesized by RiBo Biotech (Guangzhou RiBo Biotech, Guangzhou, P.R. China). For transfection, 5×10^4 cells were seeded in each cell of a 24-well microplate, grown for 24 h to reach 30%–50% confluence, and then incubated with sh-Rab25 or sh-NC and Lipofectamine 2000 reagent (Invitrogen) in 100 μ l of serum-free DMEM, according to the manufacturer's instructions.

Cell Proliferation Assay

Cell proliferation was analyzed using the MTT assay. In brief, cells (1×10^4 cells/well) were seeded onto a 96-well plate and incubated for 1, 2, 3, and 4 days. At the indicated time points, 20 μ l of MTT (5 mg/ml) was added to each well, and the plates were incubated for another 4 h. The supernatants were then removed, and 150 μ l of DMSO was added to terminate the reaction. The absorbance value (OD) was measured at 490 nm on a microplate reader.

Cell Migration and Invasion Assays

The Matrigel invasion assay was performed using a 24-well Transwell chamber. The inserts were coated with 20 μ l of Matrigel (1:5 dilution). Forty-eight hours after transfection, the cells were trypsinized, suspended in 100 μ l of serum-free medium, and transferred to the upper chamber. Medium supplemented with 10% FBS was added to the lower chamber. After 16 h of incubation, the noninvading cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were stained using hematoxylin. The number of invading cells was counted under the microscope.

For the migration assay, the inserts were not precoated with Matrigel, and the other steps were the same as outlined above.

In Vivo Tumor Xenograft Assay

U87MG cells transfected with sh-Rab25 or sh-NC resuspended in PBS (0.1 ml) were subcutaneously injected into the right flank of nude mice ($n=6$ for each group) at 1×10^6 cells/0.1 ml (coordinates: 2 mm anterior, 2 mm lateral, 2.5 mm depth from the dura). Tumor size was measured every 7 days using a caliper and calculated using the formula: volume = length \times width² \times $\pi/6$. Approximately 4 weeks after inoculation, mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg), and the tumors were weighed. All surgeries were performed under sodium pentobarbital anesthesia, and every effort was made to minimize animal suffering.

Statistical Analysis

Statistical analysis was performed using Prism 5 and the one-way ANOVA. Statistical significance was determined at the level of $p < 0.05$.

RESULTS

Rab25 Is Highly Expressed in Human GBM Cell Lines

We examined the expression of Rab25 in four human GBM cell lines (U87MG, U373, U343, and LN229) by qRT-PCR assay. The expression of Rab25 at the mRNA level was significantly increased in the four GBM cell lines when compared to the mRNA expression in normal human astrocytes SVG ($p < 0.05$) (Fig. 1A). Western immunoblot

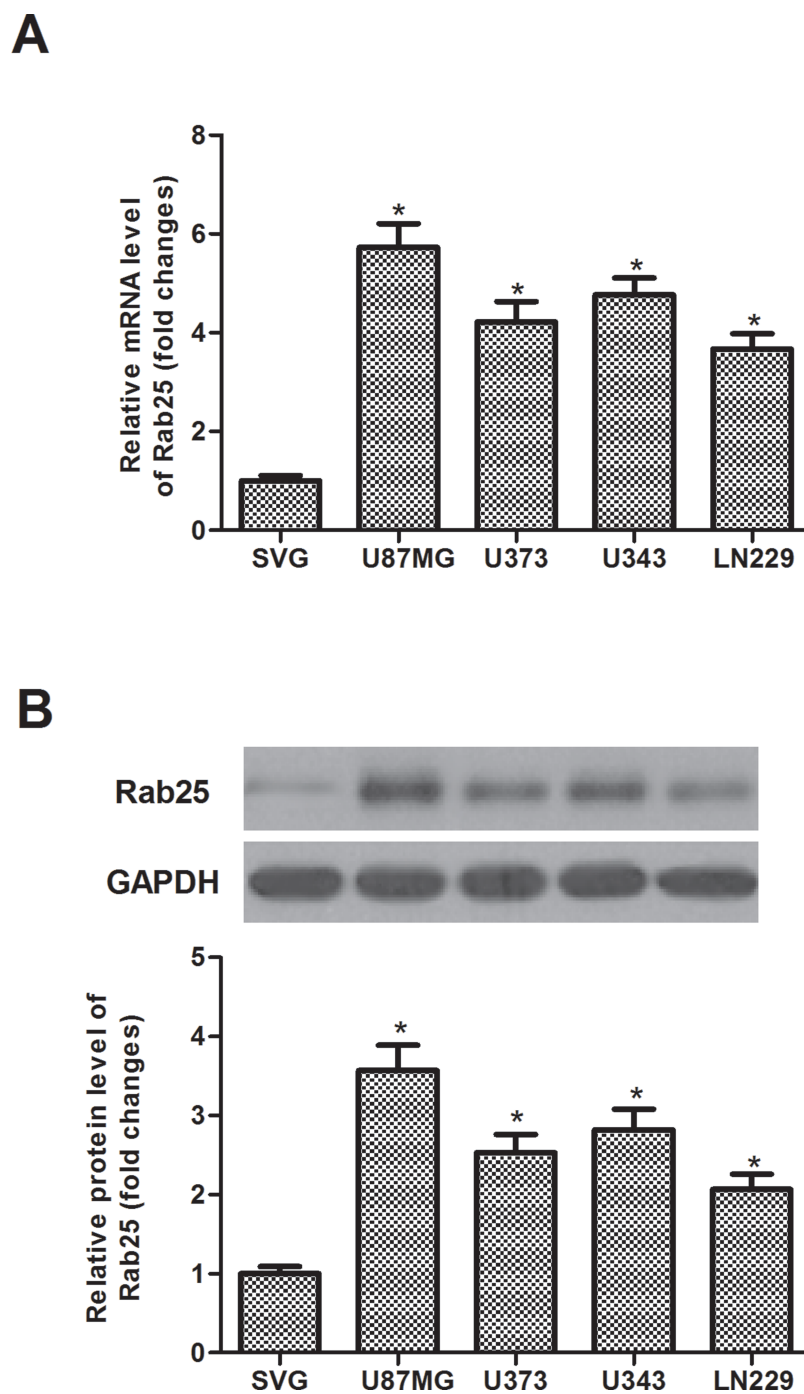


Figure 1. Rab25 expression in human GBM cell lines (U87MG, U373, U343, and LN229). (A) mRNA levels of Rab25 in four human GBM cell lines (U87MG, U373, U343, and LN229) were significantly higher than that in normal human astrocyte SVG cells. $*p < 0.05$. (B) Protein levels of Rab25 in four human GBM cell lines (U87MG, U373, U343, and LN229) were significantly higher than that in normal human astrocyte SVG cells. $*p < 0.05$.

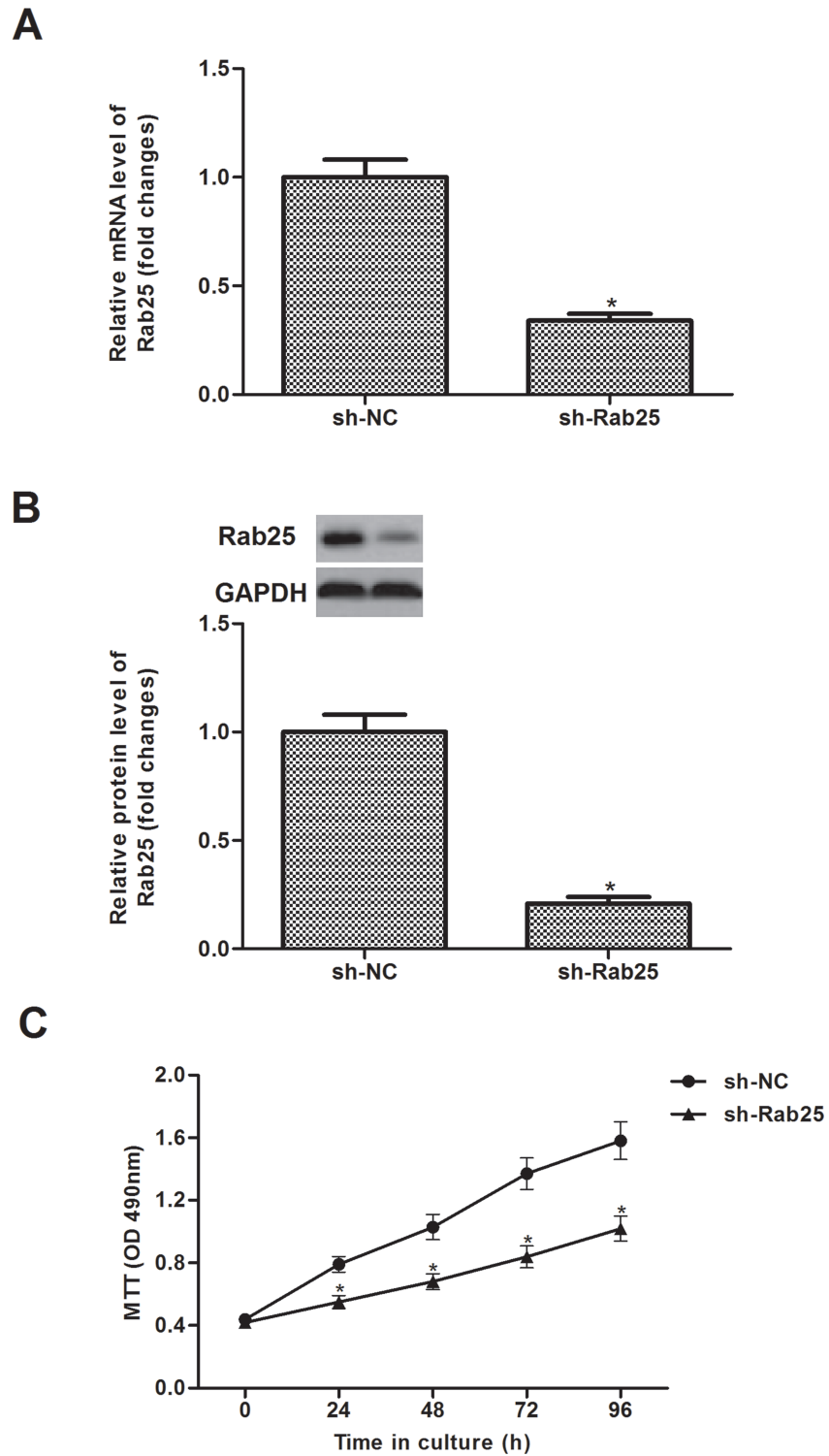


Figure 2. Knockdown of Rab25 inhibits GBM cell proliferation in vitro. U87MG cells were transfected with sh-Rab25 or sh-NC for 48 h. (A) Rab25 mRNA expression was significantly lower in cells transfected with sh-Rab25. (B) Rab25 protein expression was significantly lower in cells transfected with sh-Rab25. (C) Cell proliferation was measured by the MTT assay. * $p < 0.05$.

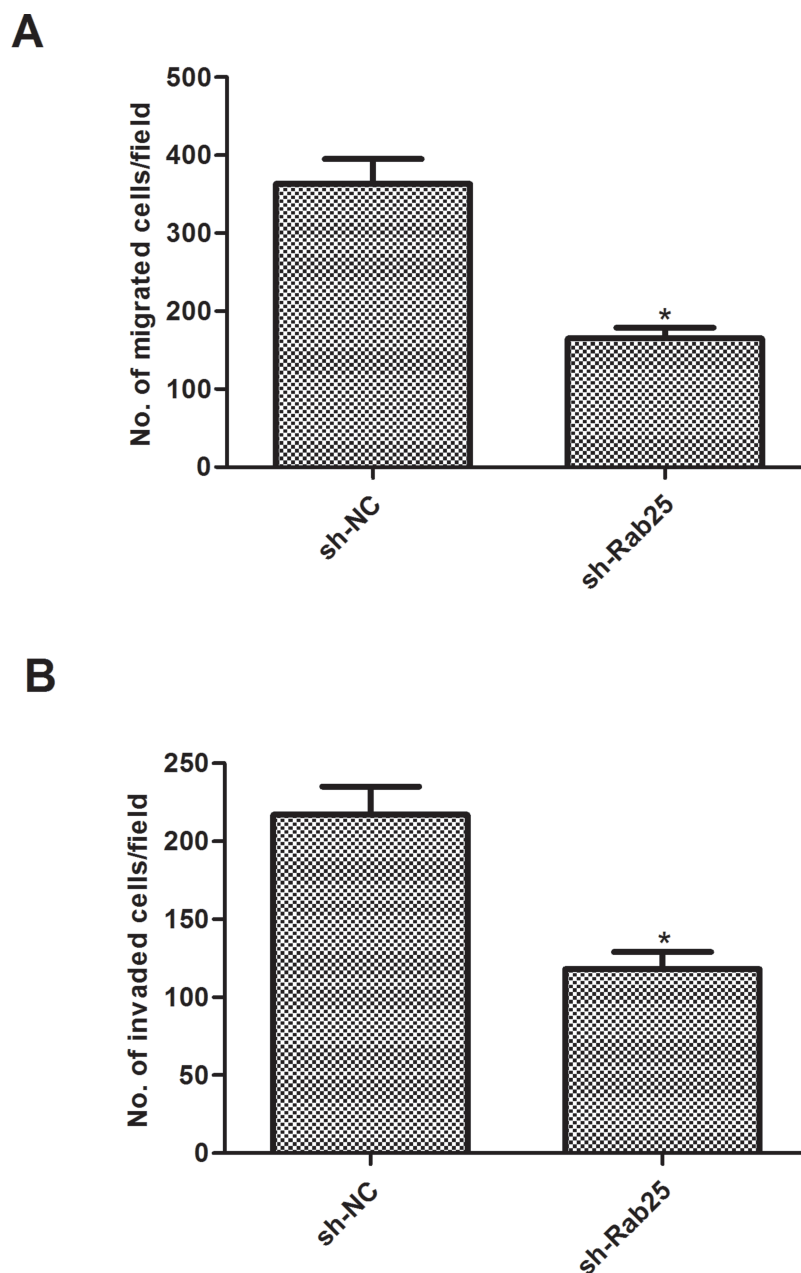


Figure 3. Knockdown of Rab25 inhibits GBM cell migration and invasion in vitro. U87MG cells were transfected with sh-Rab25 or sh-NC for 48 h. (A) The Transwell migration assay showed that the number of migrated cells was significantly lower in the knockdown of Rab25 group than in the sh-NC group. (B) The Matrigel invasion showed that the number of invaded cells was significantly lower in the sh-Rab25 group than in the sh-NC group. * $p < 0.05$.

analysis showed that Rab25 protein expression was also significantly increased when compared to expression in the SVG astrocyte cell line ($p < 0.05$) (Fig. 1B).

Knockdown of Rab25 Inhibits GBM Cell Proliferation In Vitro

For Rab25 knockdown experiments, sh-Rab25 or sh-NC was transfected into U87MG cells. As seen

in Figure 2A, transfection of sh-Rab25 significantly downregulated the Rab25 mRNA levels, and Western blot analysis confirmed that the reduced expression of Rab25 mRNA resulted in reduced Rab25 protein expression of Rab25 (Fig. 2B). The effect of sh-Rab25 on the proliferation of U87MG cells was then determined using the MTT assay. Following Rab25 knockdown, the proliferation of U87MG cells was clearly inhibited in a

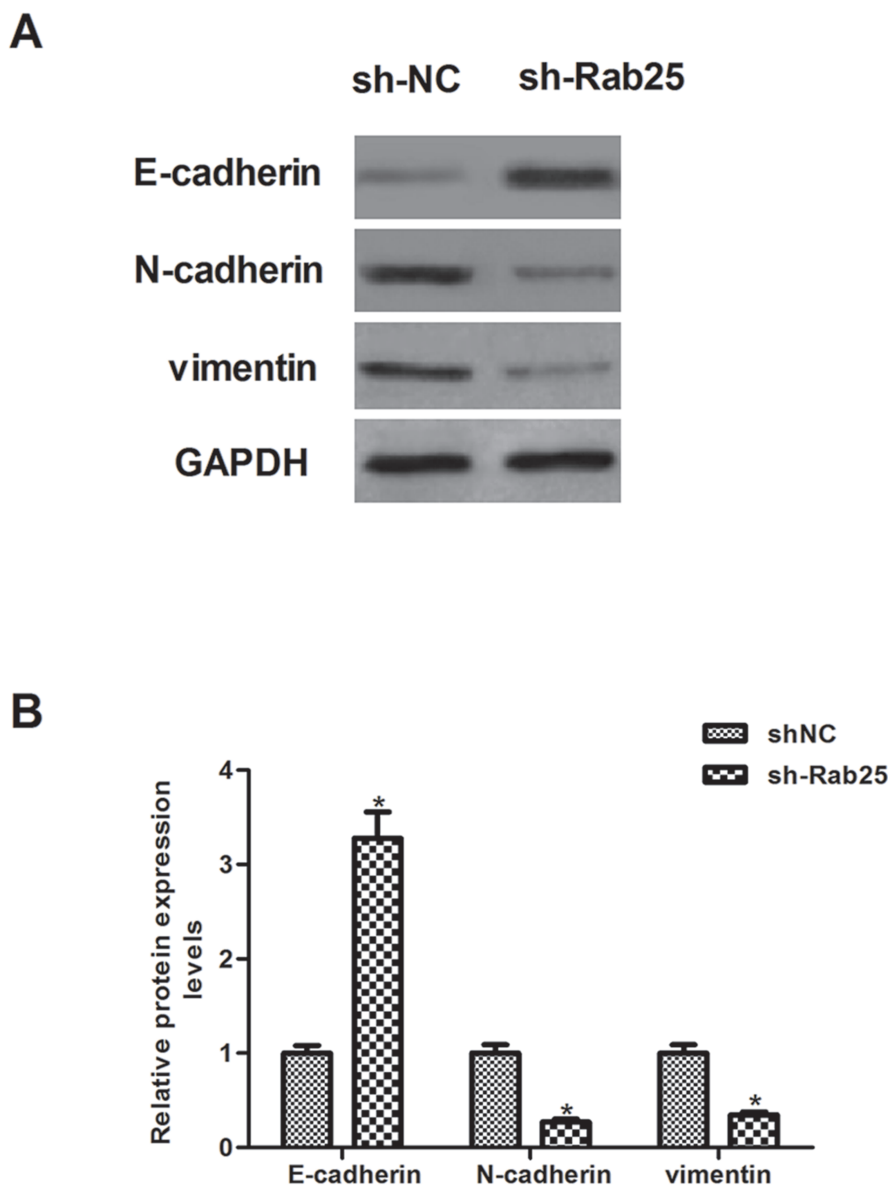


Figure 4. Knockdown of Rab25 inhibits EMT phenotype in GBM cells. U87MG cells were transfected with sh-Rab25 or sh-NC for 48 h. (A) Western blot analysis was used to evaluate the protein levels of E-cadherin, N-cadherin, and vimentin. (B) Statistical analysis of E-cadherin, N-cadherin, and vimentin levels in the sh-NC and sh-Rab25 groups. * $p < 0.05$.

time-dependent manner when compared to cells transfected with sh-NC (Fig. 2C).

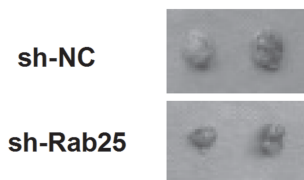
Knockdown of Rab25 Inhibits GBM Cell Migration and Invasion In Vitro

Invasion and migration are two well-characterized features of tumor cells. The effect of sh-Rab25 on cell migration was investigated using the Transwell migration and invasion system. As seen in Figure 3, Rab25 knockdown was associated with significant inhibition of U87MG cell migration (Fig. 3A) and invasion (Fig. 3B).

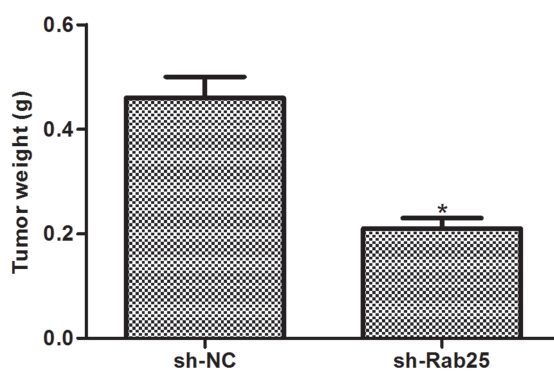
Knockdown of Rab25 Inhibits EMT Phenotype in GBM Cells

The increased metastatic potential of hypoxic cancers has been shown to result from morphological changes associated with epithelial-to-mesenchymal transition, a process referred to as EMT. As shown in Figure 4, E-cadherin protein levels in sh-Rab25-transfected U87MG cells were significantly higher than that in sh-NC U87MG cells. In contrast, N-cadherin and vimentin protein levels in sh-Rab25 U87MG cells were significantly lower in the sh-Rab25-transfected cells when compared to U87MG cells transfected with sh-NC.

A



B



C

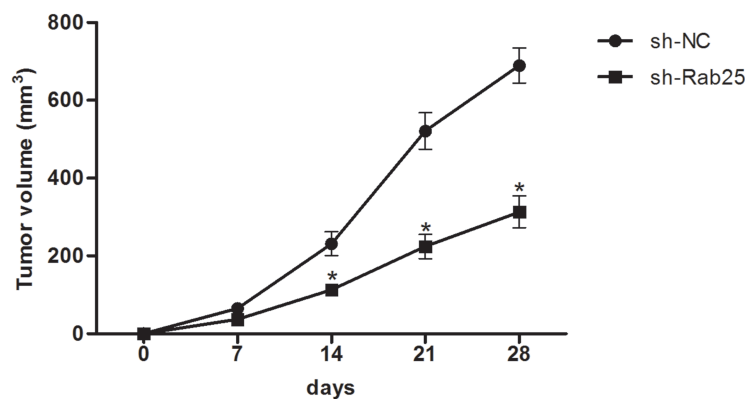


Figure 5. Knockdown of Rab25 inhibits the growth of GBM in vivo. U87MG cells transfected with sh-Rab25 or sh-NC were subcutaneously injected into the right flank of nude mice. (A) Photograph showing excised tumors in representative mice in each group on day 25 after treatment. (B) At 25 days after injection, the animals were euthanized, and the tumors were weighed. (C) The tumor volumes were monitored every 5 days. * $p < 0.05$.

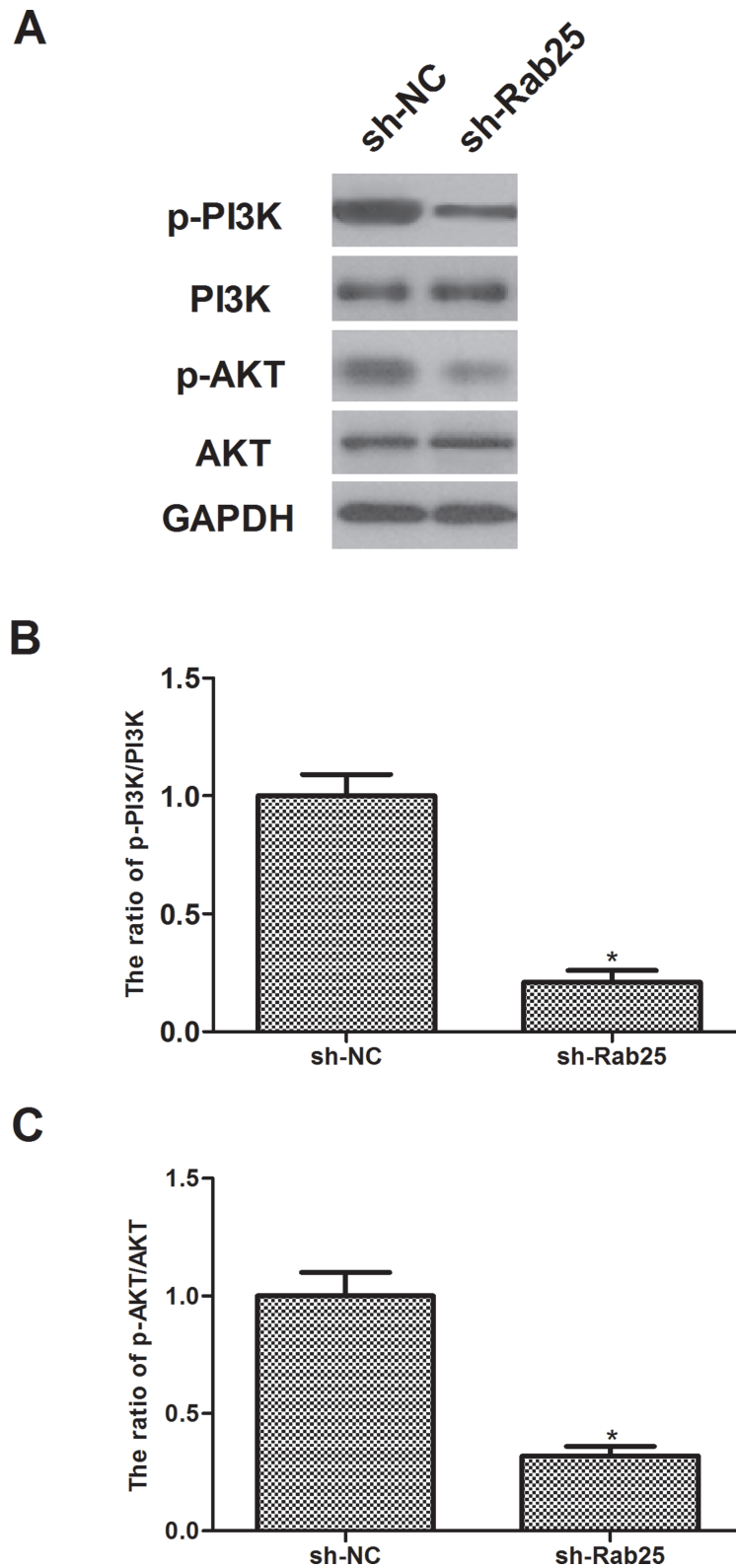


Figure 6. Knockdown of Rab25 inhibits activation of the PI3K/AKT pathway in GBM cells. U87MG cells were transfected with sh-Rab25 or sh-NC for 48 h. (A) Western blot analysis was used to evaluate the phosphorylation levels of PI3K and AKT in the knock-down and sh-NC groups. (B) Statistical analysis of PI3K phosphorylation levels in the control group and the knockdown Rab25 group. (C) Statistical analysis of AKT phosphorylation levels in the sh-NC and sh-Rab25 groups. * $p < 0.05$.

Knockdown of Rab25 Inhibits the Growth of GBM In Vivo

In order to investigate the effects of Rab25 on GBM growth in vivo, U87MG cells transfected with sh-Rab25 or sh-NC were injected into the flanks of nude mice, and tumor growth was determined after 4 weeks. As shown in Figure 5A, Rab25 knockdown significantly suppressed tumor growth of Balb/C nude mice compared with the control group. In addition, sh-Rab25 significantly reduced tumor weight when compared to control mice (Fig. 5B). Tumor size was also significantly inhibited in U87MG cells transfected with sh-Rab25 when compared with control tumors (Fig. 5C).

Knockdown of Rab25 Inhibits the Activation of the PI3K/AKT Pathway in GBM Cells

The PI3K/AKT signaling pathway is another important signaling pathway that mediates cancer growth and development⁷. We therefore examined the effect of Rab25 on the activation of PI3K/AKT signaling in U87MG cells. As shown in Figure 6, knockdown of Rab25 markedly inhibited the levels of p-PI3K and p-AKT in U87MG cells when compared with the sh-NC group, but had no effect on total PI3K and AKT protein expression.

DISCUSSION

Previous studies have demonstrated the potential role of Rab25 in tumorigenesis. With respect to cervical cancer, increased expression of Rab25 is associated with reduced survival¹⁶. Rab25 and Rab11 had 63% homology, Rab25 genes across 9 kb in its 5'-upstream area rich in the GC zone. Goldenring et al. showed that Rab25 expression was absent in heart, brain, skeletal, and muscle tissues, but present in the gastrointestinal mucosa, lung, and kidney tissues¹⁷. Schaner et al. observed an increased Rab25 protein expression level in ovarian cancer and was associated with increased invasive potential. As has been reported in cervical cancer, increased Rab25 expression was associated with reduced survival¹⁸. Rab25 expression is increased under various conditions where there is increased environmental pressures, including serum deprivation and exposure to genotoxic stresses such as chemotherapy and radiation therapy^{19,20}.

In the present study, we investigated the potential role of Rab25 in human GBM. Our studies show that Rab25 serves as a positive regulator of glioma cell growth, proliferation, invasion, and migration. Inhibition of Rab25 expression through shRNA knockdown resulted in inhibition of tumor cell invasion and migration, an effect similar to that observed with Rab25 knockdown in prostate cancer and liver cancer cells^{21,22}. There are two potential mechanisms for the inhibitory effect of Rab25 on cell migration. It is conceivable that alterations in Rab25 expression lead to

downstream consequences in the expression of other key cellular proteins that are directly involved, such as N-cadherin and vimentin, which mediate EMT and an increased capacity for invasion and migration. The second mechanism may be related to the fact that inhibition of Rab25 expression results in significant inhibition of growth and proliferation of GBM tumor cells, and the reduced number of tumor cells leads to a reduced ability for invasion and migration.

Aberrant activation of AKT has been observed in several different human cancers, and such abnormal activation of AKT signaling has also been observed in gliomas and other brain tumors. Growth factors and cytokines activate lipid kinase PI3K, which then generates phosphatidylinositol 3,4,5-triphosphate (PIP 3). PIP 3 AKT protein kinases can then be transported to the plasma membrane^{23,24}. mTORC2 phosphorylates AKT at Ser-473 residues, and this leads to enhanced cell survival, proliferation, cytoskeletal organization, and activation of cell metabolic pathways^{25,26}. Constitutive activation of the PI3K/AKT/mTOR signaling pathway can thereby mediate the process of tumorigenesis. In addition, activation of this pathway has also been associated with the development of cellular drug resistance to chemotherapy and targeted therapy²⁷. In this study, we provide evidence that knockdown of Rab25 inhibits phosphorylation levels of PI3K and AKT in GBM cells, which suggests that there is a linkage between Rab25 and PI3K/AKT signaling. Moreover, these findings suggest that the effect of Rab25 on the proliferation and tumorigenesis in GBM cells is mediated, at least in part, through the PI3K/AKT signaling pathway.

In summary, our findings demonstrate that Rab25 has a positive effect on promoting human GBM cell growth and proliferation and that inhibition of Rab25 expression results in inhibition of growth and progress of GBM cells along with inhibition of invasion and metastasis. This study provides new insights into the key signaling pathways involved in GBM growth and proliferation, and our findings provide the rational basis for developing new strategies to treat human GBM.

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