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Original Article

Knock-down Akt3 inhibits ovarian cancer cell growth and migration

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Abstract: The serine/threonine protein kinase (Akt) consists of three isoforms: Akt-1, -2 and -3. Their over-expressions have been detected in human cancer and recent studies have shown the contribution of Akt to cancer pathogenesis, but their roles in ovarian cancer growth and metastasis remind clarified. In the present study, we investigated the impact of specific silencing of Akt1 and Akt3 on human ovarian cancer cell proliferation, colony growth and migration in vitro, as well as tumor growth in vivo by employing ovarian cancer cells SK-OV-3. Transient as well as stable silencing of Akt3 resulted in a strong inhibition of mammalian target of rapamycin (mTOR) signaling associated with a decrease in cellular growth and colony formation, leading to the inhibition of tumor growth in the xenograft model. Furthermore, functional assays revealed that silencing Akt3 suppressed ovarian cancer cell migration, and invasion in vitro. Altogether, these results indicate that Akt3 plays an important role in ovarian cancer progression and can be a promising target for ovarian cancer therapy.

Keywords: Akt3, ovarian cancer, proliferation, mTOR

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy and is associated with limited overall survival, due to problems in early detection and therapy. Despite considerable efforts to improve early detection, and advances in chemotherapy, metastasis remains a major challenge in the clinical management. The high mortality related to ovarian cancer is thought to be because of the advanced stage of disease at presentation. Tumor progression toward increasing metastatic potential is a complex, multistep process and requires the coordinated expression of metastasis-promoting genes and the down-regulation of metastasis-suppressing genes. Metastatic colonization requires disseminated cells to initiate context-dependent signaling cascades that allow them to survive, enter the cell cycle, and proliferate to become metastases.

Akt, also known as protein kinase B (PKB), is a family of three serine/threonine kinases Akt1, Akt2, and Akt3 [1]. The Akt pathway is among the most frequently deregulated pathway in cancer and appear to play crucial roles in cancer cell growth and survival, which suggesting a key role in carcinogenesis. Aberrant activation of the Akt pathway occurs in virtually every type of human malignancy through either activating mutations in PIK3CA, which encodes the catalytic p110α kinase subunit, or loss-of-function mutations, deletions, or promoter methylation silencing of PTEN, a negative regulator of phosphoinositide 3-kinase (PI3K). More rarely, an activating mutation of Akt1 or Akt3 leads to PI3K/PTEN-independent pathway activation.

Frequent coactivation of the PI3K/AKT and RAS/MEK/ERK pathways has also been seen in a number of different tumor types, including melanoma, prostate, and lung cancer. Akt is activated by several growth factors, including epidermal growth factor (EGF), insulin-like growth factors (IGFs), and leptin via the 3-phosphoinositide-dependent protein kinase-1 (PDK1)/PI3K pathway. In addition, the mammalian target of rapamycin (mTOR) directly phosphorylates Akt on Ser473 and facilitates Thr308 phosphorylation and Akt activation by PDK1 [2]. In turn, activated Akt phosphorylates and inhibits glycogen synthase kinase-3β (GSK-3β), leading to increased stability of cyclin D1 and c-Myc, two critical mediators required for tumor cell cycle progression. In addition, Akt protects cells against apoptosis via phosphorylation of the IκB kinase leading to the activation of the nuclear factor-kappa B (NF-κB) survival factor, and inactivation of several pro-apoptotic factors, including pro-apoptotic protein BAD and caspase-9 [3]. As a consequence, Akt promotes tumor resistance to both chemotherapy and radiotherapy. All these functions of Akt make this signaling element an attractive target for cancer therapy. It has been established that the Akt cascade is linked to the actions of c-src, c-kit, c-met and other transforming pathways initiated by the HER and IGF receptors [4]. Accordingly, the anti-cancer activity of several humanized function-blocking antibodies and tyrosine kinase inhibitors such as Herceptin and Gleevec, respectively targeting ErbB2/HER2 and abl/c-kit, rely at least in part on their impact on the PI3K-Akt pathways.

The three Akt isoforms Akt1, -2, -3 are ubiquitously expressed in normal and tumor tissues. Compared to Akt1, Akt3 is abundant in insulin-responsive tissues. Akt3 isoform is predominantly expressed in brain, heart, kidney, lung, breast, prostate, and colon [5]. Akt3 and Akt2 share respectively 81 and 83% primary sequence homology with Akt1, suggesting overlapping signaling functions for the three Akt isoforms [6]. Overexpression of Akt has been reported in metastatic human colorectal, breast, thyroid, and prostate cancer cells. These results suggest that COX-2 has a significant role in the generation and progression of solid tumors and the inhibition of COX-2 may inhibit the growth of a variety of solid malignancies. Therefore, downregulation of COX-2 in cancer cells may prove useful in improving clinical outcomes in cancer patients. There are limited data regarding the role of Akt in ovarian cancer, but Akt was reported to be overexpressed in most human ovarian cancers. RNA interference (RNAi) is a powerful method for gene inactivation and cancer gene therapy. The basic mechanism of RNAi starts with a long double-stranded RNA that is processed into small interfering RNAs (siRNAs) of ~21 nt. The advantage of RNAi technology is that it can be used to target a large number of different genes, which are involved in a number of distinct cellular pathways. The technology of RNA silencing is poised to have a major impact on the treatment of human disease, particularly cancer. Thus, we undertook the present study with the following aim to evaluate the functional role of Akt in ovarian cancer metastasis. The present study demonstrated that RNAi can effectively silence Akt3 gene expression and inhibit the growth and metastasis of ovarian cancer cells, which indicates that there is a potential of targeting Akt as a novel gene therapy approach for the treatment of ovarian cancer.

Materials and methods

Cell culture

Human ovarian carcinoma SK-OV-3 cells were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS, Shanghai, China). Cells were maintained in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with antibiotics (penicillin 50 U/ml; streptomycin 50 μg/ml) (Invitrogen, Cergy Pontoise, France) and with 10% fetal bovine serum (FBS, Biowest, Nouaille, France) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNA interference

Cells were seeded in 35 mm petri dishes (5×10^4 cells) and allowed to attach for 24 h. Transfections were carried out in 1 ml Opti-MEM using DharmaFECT siRNA transfection reagent (Thermo Fisher Scientific, Dharmacon Products, Lafayette, CO, USA), according to the manufacturers' protocols. After 24 h incubation with the Akt1 and Akt3 siRNA or control oligonucleotides, 1 ml of 10% FBS containing medium was added [7].

Stable silencing of Akt in SK-OV-3 cells

Cells were seeded at a density of 2×10^4 into 96-well plates and allowed to attach for 24 h. Cells were transduced with SMARTvector 2.0 Lentiviral shRNA particles targeting Akt1, Akt3 or SMARTvector 2.0 Non-Targeting control particles (Dharmacon Thermo Scientific, US). Selection of cells stably expressing Akt1-shRNA, Akt3-shRNA and the control-shRNA started 72 h post-transfection. Growth medium was replaced with fresh selection medium containing 10 μg/mL of puromycin. Puromycin-containing medium was refreshed every 3 days, and selection of stable cells expressing Akt1-shRNA, Akt3-shRNA or control-shRNA was completed after approximately 4 weeks [8].

One solution cell proliferation assay

SK-OV-3 cells viability was determined by CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). Cells were seeded in 96-well cell culture plates and after 24 h transfected with the Akt1-siRNA, Akt3-siRNA or the control sequence (control-siRNA). SK-OV-3 cells were stably transduced with Akt1-shRNA, Akt2-shRNA or control sequences (control-shRNA). At the times indicated, 20 μl one solution reagent were added to each well and incubation was continued for additional 4 h. The absorbance was measured at 490 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). The effect of siRNA or shRNA on cell viability was assessed as the percent of cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability.

Western blot analysis

Total cellular proteins were isolated using RIPA buffer (25 Mm Tris. HCl pH 7.6, 1% NONIDET P-40, 1% sodium deoxycholate, 0.1% SDS, 0.5% protease inhibitors cocktail (Sigma, Steinheim, Germany), 1% PMSF, 1% phosphatase inhibitors cocktail (Thermo Scientific, Rockford, USA). The whole cell lysate was recovered by centrifugation at 14,000 rpm for 20 minutes at 4°C to remove insoluble material and 25-50 μg of proteins were separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electro-transferred onto a nitrocellulose membrane, blocked with 5% non-fat milk and probed with Akt1, Akt3, mTOR, phospho-mTOR, FOXO1, phospho-FOXO1, NF-κB, phospho-NF-κB, GSK-3β, phospho-GSK-3β and GAPDH antibodies overnight at 4°C. The blots were washed, exposed to secondary antibodies and visualized using the ECL system (Perkin, Waltham, MA, US). The quantifications of intensities were normalized to the corresponding GAPDH bands' intensities. This densitometry analysis was done using ImageJ software.

Colony formation assays

1 ml per well of 1% low melting temperature agar (Bio-Rad) dissolved in distilled water was poured into wells of a 6-well cell culture dish and allowed to set at 4°C for 5 minutes then incubated at 37°C for 30 min. A second layer (2.9 ml/well) containing 0.7% of low melting agar dissolved in growth media containing stably transfected cells with Akt1-shRNA, Akt3-shRNA or with control-shRNA (5×10^3 cells/ml) was placed on top of the first layer and allowed to set at 4°C for 5 minutes. After 30 minutes to 1 hour in a humidified incubator at 37°C, growth medium (2 ml) was added on top of the second layer and then left at 37°C for 2 weeks. Medium was changed twice a week. At the end of the experiment, colonies were stained for 30 min with 1% crystal violet stain, and incubated with PBS overnight to remove excess stain. The colonies were photographed and scored.

Tumor growth assay in vivo

Six-week-old nude mice (nu/nu, Shanghai Slack laboratory animal co., LTD) were maintained under specified pathogen-free conditions. SK-OV-3 cells transiently transfected with Akt3-siRNA or control sequences (Control-siRNA) (1×10^6 cells) and SK-OV-3 cells stably transduced with Akt3-shRNA or control sequences (Control-shRNA) (1×10^6 cells) were injected subcutaneously into the lateral flank of the mice. Tumor dimensions were measured with calipers every week. Tumor volumes (V) were calculated during five weeks using the formula: $V=a \times b^2/2$, with "a" being the length and "b" the width of the tumor. The animals were sacrificed 25 days after inoculation. The animal experiments were performed in accordance with the protocol approved by the animal ethics committee and the Institutional Animal Care at the Guangzhou Medical University.

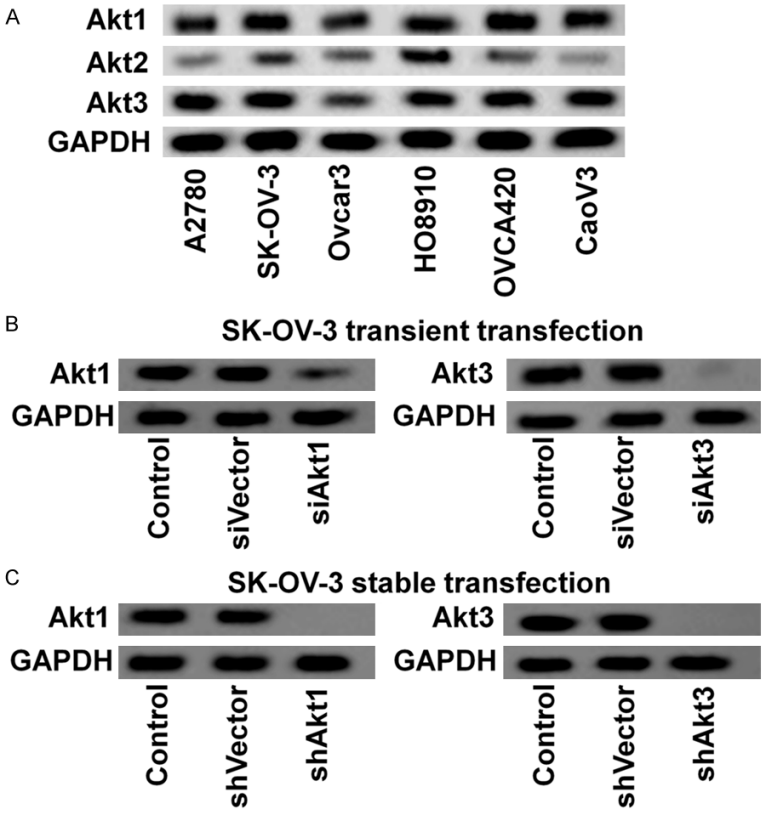

Figure 1. Analysis of selective Akt1 and AKt3 silencing in SK-OV-3 cells. A. Western blot analysis of the three Akt isoform in the ovarian cancer cells. B. Akt1 and Akt3 protein expression in SK-OV-3 cells transiently transfected with siRNA targeting Akt1 and Akt3 transcript respectively or with its siVector oligonucleotide. C. Akt1 and Akt3 protein expression in SK-OV-3 cells stably transduced with control-shRNA, Akt1 shRNA or Akt3 shRNA. Data are representative of three independent experiments.

Statistical analysis

Results were expressed as means ± SD of the indicated data. The difference between experimental and control values were assessed by ANOVA followed by Dunnett's post-hoc multiple comparison test. For the cell proliferation of Akt-1 and -3 transient silenced cells, tumor growth volume and weight, and ganglia weight data, the difference between experimental and control values were assessed by the unpaired Student's t-test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ indicate a significant difference.

Results

Validation of Akt1 and Akt3 silencing in ovarian cancer cells

Western blot analysis revealed that the three Akt paralogs are expressed in a panel of ovarian cancer cell lines. We observed a strong expression of Akt1 and Akt3 in these cells whereas the Akt2 signal was weak in all cells and barely detectable in A2780 cells. In comparison with other cells, Ovcar3 cells expressed a low level of Akt2 and a very low level of Akt3 (**Figure 1A**). We decided to use the highly tumorigenic and metastatic ovarian cancer cells SK-OV-3 to investigate the role of Akt1 and Akt3 isoforms on ovarian cancer progression. SK-OV-3 cells were transfected with the Akt1-siRNA or Akt3-siRNA (Eurogentec), at 100 nM. Two days after transfections, Akt1 and Akt3 protein levels were efficiently silenced in the SK-OV-3 cells (**Figure 1B**). Transfection with the control-siRNA had no effect on Akt1 and Akt3 protein levels. Next, SK-OV-3 cells were stably transduced with two designs of SMARTvector 2.0 Lentiviral shRNA particles targeting Akt1 and Akt3. Control cells were transduced with SMARTvector 2.0 Non-Targeting control particles. The puromycin-resistant clones were selected and analyzed by western-blot to verify Akt1 and Akt3 silencing. The shRNA targeting Akt1 and Akt3 induced an approximately 99% decrease in the Akt1 and Akt3 protein level respectively. There was no impact on Akt1 and Akt3 protein in the cells transduced with shRNA control particles (control-shRNA) in comparison with parental SK-OV-3 (**Figure 1C**).

Akt3 silencing decreased SK-OV-3 cell proliferation

To elucidate the effects of the shRNA on SK-OV-3 ovarian cancer cell proliferation, cell proliferation was determined by One solution cell proliferation assay. Transient silencing of Akt1 had only a limited impact on cell proliferation. However, transient silencing of Akt3 significantly reduced SK-OV-3 cell proliferation rates

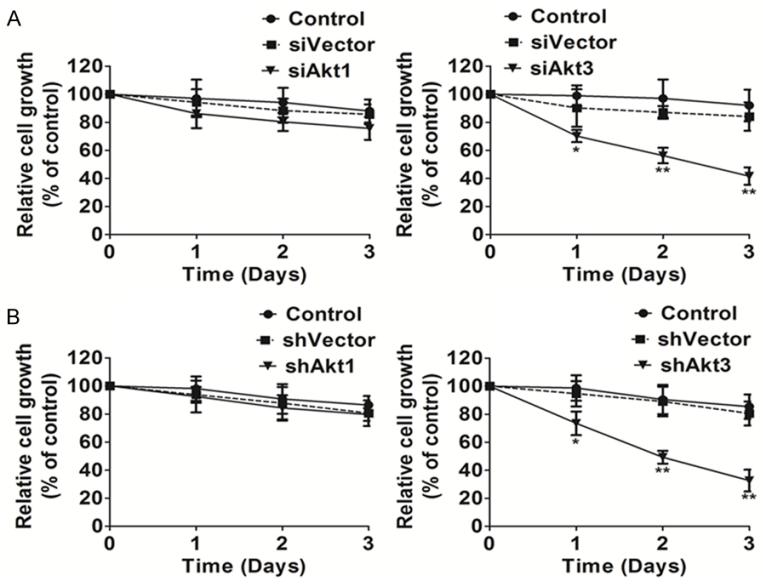

Figure 2. Impact of Akt1 and Akt3 silencing on the proliferation of SK-OV-3 cells. A. Cells were transiently transfected with siRNAs targeting the transcripts encoding Akt1, Akt3 or with its siControl sequences. On days 1, 2 and 3 following the transfection, cell proliferation was quantified in silenced Akt1 and Akt3 cells and compared to the growth curve of the control siRNA cells. B. SK-OV-3 cells stably transduced by control-shRNA, AKT1-shRNA or control-shRNA, Akt3-shRNA. On days 1, 2, and 5 following the transfection, cell proliferation was assayed by one solution cell proliferation assay. Data are means ± SD from 3 independent experiments. Statistical differences obtained at $*P < 0.05$, $**P < 0.01$ compared with control.

(**Figure 2A**), as compared to the control-shRNA. To confirm the ability of Akt3 to interfere with cancer cell proliferation, control-shRNA cells and their stably silenced counterpart's cells Akt1 and Akt3 were compared for their growth rates. As shown in **Figure 2B**, stable silencing of Akt1 has little consequence on the proliferation rate of the SK-OV-3 cells. However, Akt3 stably silenced cells exhibit very significantly slower proliferation rates (**Figure 2B**). These data demonstrate that the inhibition of Akt3 by shRNA can inhibit the proliferation of SK-OV-3 cells.

Akt3 silencing decreased SK-OV-3 cell colony growth in soft agar

In addition, colony formation assay was performed to determine cell proliferation in vitro. Similarly, Akt1 knock-down had no impact on colony formation in soft agar (**Figure 3A**) whereas Akt3 silencing strongly inhibited the ability of SK-OV-3 cells to form colonies in soft-agar (**Figure 3B**). As shown in **Figure 3B**, the size of single colony in cells infected with shAkt3 was much smaller than that in cell infected with control-shRNA, and the total number of colonies in 6-well plates was remarkably decreased in SK-OV-3 cells transduced with shAkt, in comparison with the control. The very low impact of Akt1 silencing on cell proliferation and colony growth in vitro suggests that it is unlikely that Akt1 has an important function in the growth of SK-OV-3 derived tumors. Our data prompted us to investigate the impact of Akt3 silencing on the growth of SK-OV-3 human lung tumor xenografts in immunodeficient mice.

Impact of stable Akt3 silencing on tumor growth in vivo

To evaluate the effects of *Akt3* on tumor growth in vivo, SK-OV-3 cancer cells stable transfection with the appropriate shRNA and then SK-OV-3 cancer cells were injected subcutaneously in nude mice. No major side effects were noted throughout the study (data not shown). Selective silencing of Akt3 reduced SK-OV-3 tumor volume significantly, compared with SK-OV-3 cells transfected with control-shRNA as measured 25 days after tumor cell injection (**Figure 4A**). A similar difference was found in tumor weight at the end of the experiment (**Figure 4B**). Moreover, there was no effect on body weight of mice (**Figure 4C**).

Effect of Akt3 silencing on the expression and phosphorylation of FOXO1-mTOR signaling

It has been reported that Akt plays a role in mTOR signaling in breast, colon, and gastric cancer cells. Accordingly, we analyzed the FOXO1-mTOR signaling pathway that was mediated by Akt3 and that was associated with cell growth. To further investigate the FOXO1-mTOR signaling pathway that mediated Akt3 expression, we assessed the expression of total and phosphorylated FOXO1-mTOR and its downstream transcription factors, including NF-κB and GSK-3β in SK-OV-3 cells transfected with

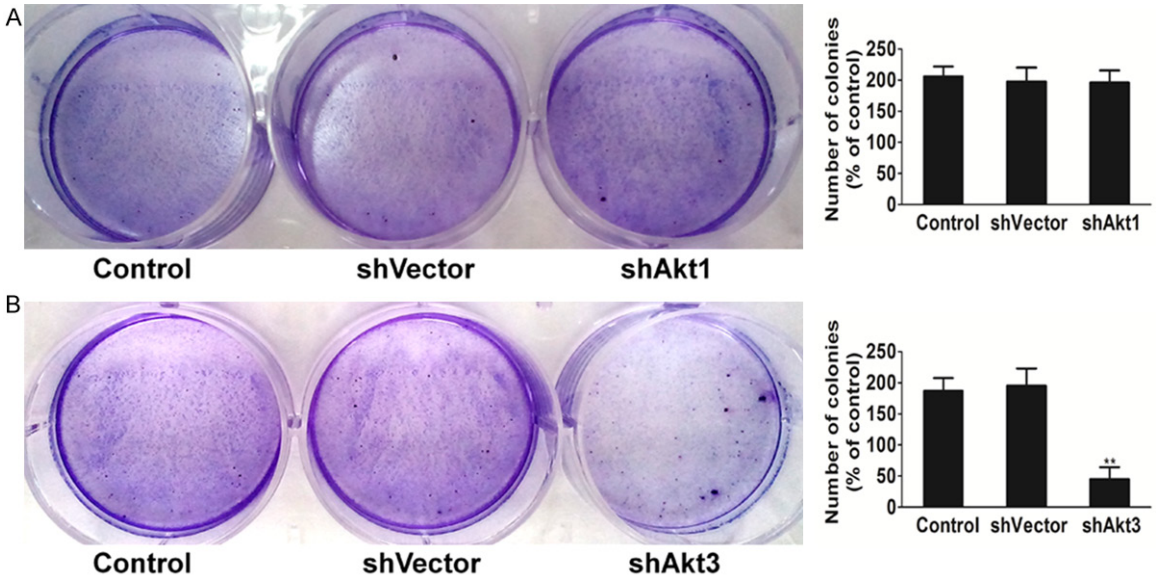

Figure 3. Impact of Akt1 and Akt3 silencing on SK-OV-3 colonies' growth. Anchorage-independent colonies growth of SK-OV-3 cells stably silenced for Akt1 (A) and Akt3 (B) is shown. Cells were plated in 0.7% soft agar. Two weeks later, colonies were stained with crystal violet and counted. Data are means ± SD from 3 independent experiments. Statistical differences obtained at $^{**}P < 0.01$ compared with control.

either siVector or siAkt3 by western blotting. In this context, we demonstrated that the inhibition of cell proliferation, colony formation, and tumor growth resulting from the silencing of Akt3 was correlated with a significant decrease of FOXO1, mTOR, NF-κB and GSK-3β phosphorylation whereas the total expression was not significantly affected by the knock-down of Akt3 (**Figure 5**).

Discussion

The serine/threonine kinase Akt isoforms are over-expressed and activated in many cancers including breast, lung, liver and colon [9]. The importance of Akt in tumor growth and progression is indisputable and is the most promising target for cancer therapy, but its specific roles in ovarian cancer progression still remain unclear. In this study, we demonstrate that the Akt3 isoform plays an important role in ovarian cancer cell proliferation, colony in vitro, as well as tumor growth in vivo. In our cellular model, we also demonstrated that Akt3 isoform is mainly involved in cellular signaling.

Our data demonstrate that Akt3 is necessary for ovarian cancer cell proliferation, colony and tumor growth in accordance with the decrease in mOTR signaling [10]. Numerous reports from other laboratories are in agreement with our conclusions. It has been reported that suppression of Akt3 expression in human lung adenocarcinoma cell line A549 resulted in notable inhibition of cell proliferation and colony growth [11]. Similarly, silencing of Akt3 in neuroblastoma and gliomas impaired cell proliferation and colony growth. It has also been reported that loss of Akt3 decreased the proliferation of Pten wild-type astrocytes [12]. In breast cancer, it has been demonstrated that Akt3 is the most relevant isoform to cell proliferation and colony growth and the stable ablation of Akt3 in mammary tumors of MTB-IGFIR transgenic mice delayed tumor onset and growth rate [13]. It has also been reported that silencing of Akt3 resulted in decreased IGF-IR mediated cell proliferation as well as ovarian and hepatocellular carcinoma cell proliferation and growth. In another study, transient silencing of Akt3 had a small but non-significant impact on H460 colony growth. It has also been reported that in fibroblasts Akt3 has no function in cell proliferation [14]. On the contrary, a very recent study using a viral oncogene-induced mouse model of lung cancer, showed a dramatic increase in tumorigenesis in $Akt3^{-/-}$ mice due to the enhancement of cell proliferation and inhibition of apoptosis [17, 18]. Our results are in agreement with the large majority of studies suggest-

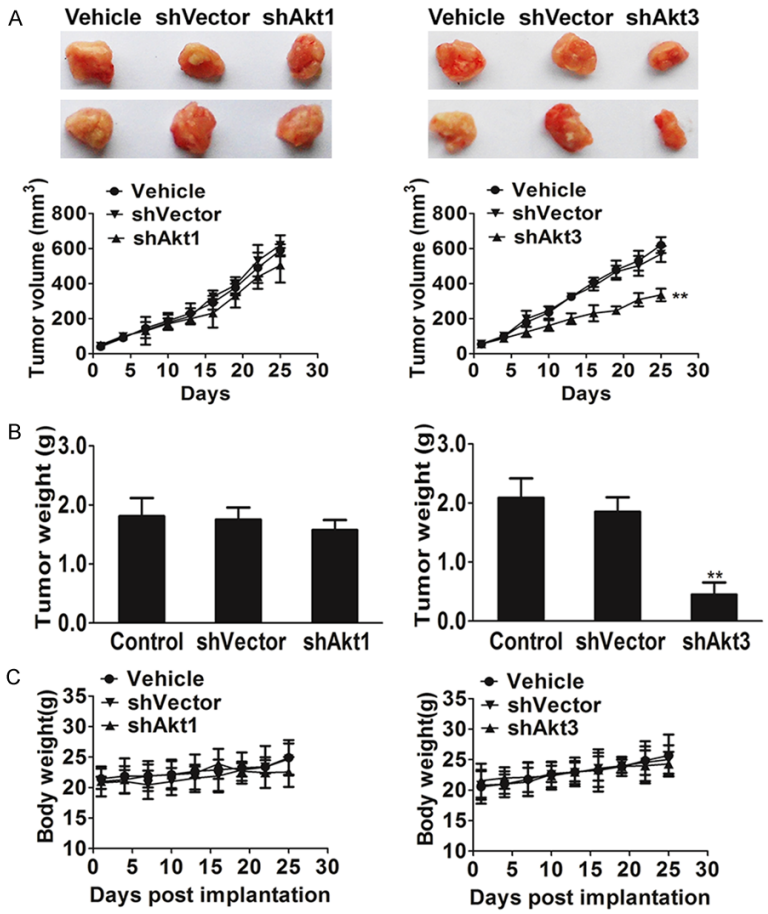

Figure 4. Impact of Akt3 silencing on xenografted tumor growth in nude mice. A. Nude mice were xenografted subcutaneously into the lateral flank with SK-OV-3 cells stably transduced with Akt3-shRNA or control sequences (Control-shRNA). Tumor volumes (mm^3) were measured for a total of 25 days. B. Mice were then sacrificed and tumors weighed. C. There was no significant difference in body weight in the three groups. Data points represent the mean ± SD of six mice per group. Statistical differences obtained at $^{**}P < 0.01$ compared with vehicle.

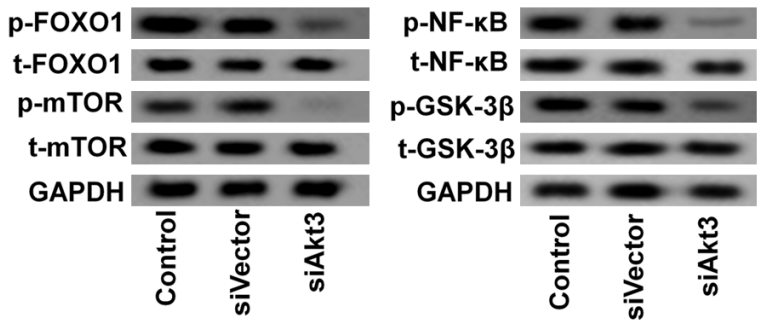

Figure 5. Impact of Akt3 silencing on Rb expression and phosphorylation. Representative western blots of the expression and phosphorylation of FOXO1, mTOR, NF-κB and GSK-3β in Akt3-siRNA SK-OV-3 cells in comparison with control-siRNA cells. Data are representative of three independent experiments.

ing that Akt3 plays a major role in cancer cell proliferation and consequently in tumor growth.

Our data are also in line with a very recent publication indicating that the inhibition of cell proliferation by the PI3K/AKT inhibitor LY294002 was, at least in part, mediated by the decrease in mOTR phosphorylation [15]. In this context, another study reported the hypo-phosphorylation of FOXO1, mTOR, NF-κB and GSK-3β in Akt3-depleted cells. We also show that Akt1 has only a minimal role in SK-OV-3 ovarian cancer cell proliferation and has no impact in colony growth. Similarly, it has been reported that silencing of Akt1 resulted in a moderate inhibition of A549 human lung adenocarcinoma cell proliferation and colony growth [16]. However, in a transient Akt1 silencing study, Akt-1 had a marked role in A549 and NCI-H460 NSCLC colony growth. It has also been reported that deletion of Akt1, but not Akt3 prevented lung tumor progression in a tobacco carcinogen-induced model and in a genetic mutant K-ras model. In conclusion, we believe in the potential pharmacological impact of selective Akt3 inhibitors to decrease ovarian tumor growth.

Disclosure of conflict of interest

None.

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