A Specific Role for AKT3 in the Genesis of Ovarian Cancer through Modulation of G_2-M Phase Transition

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Abstract

Ovarian cancer is the major cause of death from gynecological malignancy, and there is an urgent need for new therapeutic targets. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been strongly implicated in the genesis of ovarian cancer. However, to identify and evaluate potential targets for therapeutic intervention, it is critical to understand the mechanism by which the PI3K/AKT pathway facilitates ovarian carcinogenesis. Here, we show that AKT3 is highly expressed in 19 of 92 primary ovarian tumors. Strikingly, purified AKT3 exhibited up to 10-fold higher specific activity than AKT1, potentially amplifying the effects of AKT3 overexpression. Consistent with this finding, AKT3 levels in a range of ovarian cancer cell lines correlated with total AKT activity and proliferation rates, implicating AKT3 as a key mediator of ovarian oncogenesis. Specific silencing of AKT3 using short hairpin RNA markedly inhibited proliferation of the two cell lines with highest AKT3 expression and total AKT activity, OVCA429 and DOV13, by slowing G_2-M phase transition. These findings are consistent with AKT3 playing a key role in the genesis of at least one subset of ovarian cancers. (Cancer Res 2006; 66(24): 11718-25)

Introduction

Ovarian cancer is the major cause of death from gynecological malignancy. Although early-stage disease is often curable by surgery, nearly 70% of patients present with advanced disease and the majority of these patients die of their cancer (1, 2). Advanced ovarian cancer often responds to initial chemotherapy; however, the natural history of the disease is to progress toward resistance to these therapies (1, 2). Thus, understanding the mechanism of disease progression and the identification of new therapeutic targets is essential to improve patient outcome. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been implicated as a major determinant of oncogenic transformation in ovarian cancer. PI3K is up-regulated in 30% to 45% of ovarian cancers (3, 4), whereas ~36% of primary tumors show elevated AKT2 activity (5). The majority of these tumors were of high grade and late stage, implicating the kinase in tumor progression. AKT has also been shown to play a role in protecting ovarian cancer cells from cytotoxic drug-induced apoptosis (6, 7), raising the possibility that activation of the kinase may be associated with drug resistance and thus disease relapse. Indeed, AKT may contribute to tumorigenesis at multiple levels, with the kinase shown to play prominent roles in several processes considered hallmarks of cancer when deregulated, including proliferation control, cell survival, invasiveness, and angiogenesis (8, 9).

A recent study using a phosphospecific pan-AKT antibody (S473-P) found AKT activity to be elevated in 68% of primary ovarian tumors (10), significantly higher than the 36% revealed using AKT2-specific antibody (5). We hypothesized that up-regulation of AKT3 accounted for this discrepancy and that AKT3 may play a role in development of the disease. Consistent with this hypothesis, AKT3 protein is expressed in normal ovaries (11). Moreover, there is growing evidence that the differential expression of specific AKT isoforms is associated with individual tumor types. AKT1 activity is frequently elevated in breast and prostate cancers (12), whereas AKT2 has been shown to be amplified in ~35% of pancreatic carcinomas (13) and 36% of ovarian tumors exhibit elevated AKT2 activity (5). AKT3 has been shown to be up-regulated in estrogen receptor–deficient breast cancer cells and in androgen-independent prostate cancer cell lines (14). Analysis of primary breast tumors indicated that AKT3 levels were highest in estrogen receptor–negative tumors, implying that this isoform may contribute to a more aggressive phenotype (14). Strikingly, selective activation of AKT3 has recently been observed in 43% to 60% of sporadic melanomas (15).

In the present study, we have established a role for AKT3 in ovarian cancer. We show that AKT3 expression is elevated in 20% of primary ovarian cancers with 80% of these exhibiting high AKT activity by immunohistochemistry using the phosphospecific pan-AKT antibody, suggesting that this isoform characterizes a subset of ovarian tumors. Furthermore, we show that purified glutathione *S*-transferase (GST)–tagged AKT3 exhibited V_{max} values >10-fold higher than AKT1, indicating that overexpression of AKT3 would potentially have greater effects on downstream signaling than AKT1. Consistent with this finding, AKT3 expression in a range of ovarian cancer cell lines correlated with total AKT activity and proliferation rates in the presence and absence of growth factors, further implicating AKT3 as a key mediator of ovarian oncogenesis. Specific silencing of AKT3 expression using short hairpin RNA

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

B.E. Cristiano and J.C. Chan contributed equally to this work. Nicole Lundie was an outstanding Ph.D. student in Richard Pearson's laboratory who passed away tragically on August 1, 2003.

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doi:10.1158/0008-5472.CAN-06-1968

(shRNA) in the two cell lines (OVCA429 and DOV13) exhibiting highest total AKT activity markedly inhibited proliferation by slowing G_2-M phase transition without affecting cell growth or survival. Furthermore, restoration of the levels of activated AKT by overexpression of AKT1 failed to restore proliferation. Together, these findings are consistent with AKT3 activity being required for the genesis of at least a subset of ovarian cancers, providing a potential therapeutic target for the development of small-molecule inhibitors for second-line treatment of this devastating disease.

Materials and Methods

Cell culture and transfection. Cos-1 and HEK293 cells were maintained in DMEM plus 10% fetal bovine serum (FBS). OVCA429, DOV13, PEO-1, SKOV3, JAM, OVCA432, and OVHS-1 cell lines were cultured in RPMI plus 10% FBS. OVCAR3 cells were cultured in RPMI, 10% FCS, and 0.2 IU/mL insulin (Actrapid 100 IU/mL; Novo Nordisk, Denmark). HOSE cells were cultured in a 1:1 (v/v) mixture of Medium 199 and MCDB 105 (Sigma) plus 10% FBS. All cells were maintained in 5% CO_2 at 37°C. Cos-1 cells were transiently transfected with COOH-terminal hemagglutinin (HA)–tagged pCMV5 AKT1, pECE AKT2, or pCMV5 AKT3 using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Exponentially growing cells were cultured to a confluency of ~80%. Cells were serum starved by culturing in serum-free medium for 24 hours. HEK293 cells were transiently transfected with GST-tagged AKT1 and AKT3 using calcium phosphate as described previously (16).

Protein extracts. Cells were rinsed twice in ice-cold PBS and harvested in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 1% (v/v) NP40, 1 mmol/L EDTA, 50 mmol/L NaF, 40 mmol/L β-glycerophosphate, 1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride]. Cells were scraped and collected into microfuge tubes, cleared by centrifugation (4°C, 10 minutes, 13,000 × *g*), and the supernatant was retained. The protein concentration of the cell extracts was determined using DC Assay (Bio-Rad).

Western blot analysis. The affinity-purified sheep polyclonal antibodies described in Supplementary Fig. S1 were used at the following concentrations: 5 μg/mL AKT2 and 2 μg/mL AKT3. Polyclonal antibodies to Cdc2; phosphorylated (Tyr^{15}) Cdc2; phosphorylated rpS6; S6 kinase; phospho-AKT Ser^{473} or Thr^{308} (Cell Signalling, Danvers, MA); mouse anti-actin (clone C4; ICN, Costa Mesa, CA); and AKT1 (2H10; Cell Signalling) plus the anti-sheep (DAKO Corporation, Via Real Carpinteria, CA), anti-mouse, and anti-rabbit (Bio-Rad, Hercules, CA) horseradish peroxidase secondary antibodies were all used at a dilution of 1:2,000. The proteins were visualized by enhanced chemiluminescence.

Immunohistochemistry. Formalin-fixed, paraffin-embedded human primary ovarian tumor arrays were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was done by microwave irradiation for 15 minutes (medium/low setting) in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 10 minutes then rinsed with H_2O. Sections were treated with a biotin blocking system (DAKO), then incubated overnight at 4°C in primary antibody diluted in blocking solution [50 mmol/L Tris-HCl, 0.05% (v/v) Tween 20, 5% skim milk], anti-AKT3 antibody at 2 μg per section, and phospho-AKT (Ser^{473}) antibody (immunohistochemistry specific; Cell Signalling) at 1:50. The sections were then incubated in biotinylated link antibody for 15 minutes, peroxidase-labeled streptavidin for 15 minutes (LSAB+ kit, DAKO), 3,3′-diaminobenzidine substrate-chromogen solution for 10 minutes (DAKO), and counterstained with hematoxylin and mounted. Images were captured on a Zeiss Axioskop 2 microscope and SPOT RT slider diagnostic camera and manipulated identically using Adobe Photoshop 7.0. The intensity of staining was ranked from low (+) to high (+++). For a tumor sample to be given a particular score for AKT3 or active AKT expression, the majority of the cells in the section had to have this level of staining (>80%). Tumor samples were scored blind to their pathology.

AKT activity assay. AKT activity assays were done as described previously (17) with three different peptide substrates: RPRAATF (17), Crosstide (GRPRTSSFAEG; ref. 18), and RIRTQFSFSLQERQLRG (16). The units of activity are expressed as picomoles of ^{32}Pi transferred per minute per milligram of protein added (pmol/min/mg). K_m and V_{max} values were derived using the nonlinear data analysis program Enzfitter.

Purification of GST-AKT. The active GST-AKT fusion protein was purified by glutathione-Sepharose chromatography of extracts from transiently transfected HEK293 cell as described in refs. (19, 20).

Proliferation analysis. Cells were seeded in a six-well cell culture plate at 0.1×10^6 per well (OVCA429, PEO-1, SKOV3, DOV13, OVCA432, JAM, and OVHS-1) or 0.2×10^6 per well (HOSE and OVCAR3). Cells were maintained in either complete medium (RPMI 1640 + 10% FBS) or serum starved for 48 hours after plating by culturing in serum-free medium (RPMI 1640 only). Proliferation was followed for 10 days by counting cell number using a Sysmex CDA500 automated cell counter (Sysmex, Japan). The medium on the cells was changed daily.

shRNA. AKT3-specific and control hairpins were cloned into the pSuper.retro (pSR) vector, which directs the synthesis of short-interfering RNAs as previously described (21). The sequences of hairpins are as follows: AKT3Y 5′-AACCAGGACCATGAGAAACTT-3′, AKT3Z 5′-ACAGATGGCTCATTCATAGGA-3′, and control 5′-TAGCAAGCGATCACAAGTACA-3′.

AKT suppression with RNA interference. OVCA429 and DOV13 cells were plated at a density of 1.0×10^6 in 94-mm plates. The pSR hairpins were cotransfected 48 hours after plating with pEGFP-C1 (Clontech, Mountainview, CA) and AKT1 (pCDNA3-HA AKT1) or constitutively active S6 kinase (pCMV5 CA S6K; ref. 22) where indicated by calcium phosphate (20). The brightest 50% of green fluorescent protein–expressing cells were collected by fluorescence-activated cell sorting (FACS) 48 hours after transfection and replated into six-well plates (0.1×10^6 per well). Cells were harvested every 24 hours in triplicate for Western blot analysis, AKT activity assay, proliferation, apoptosis analysis, cell volume (Sysmex), and cell cycle analysis.

Cell cycle and phospho–histone H3 analysis. Cells were harvested, fixed in 70% ethanol, incubated with 20 μg/mL propidium iodide, and analyzed using FACSCalibur interfaced with Modfit software to determine percentage of cells in the different cell cycle phases. Alternatively, fixed cells were permeabilized with 0.25% Triton X-100, incubated with anti-phospho-histone H3 antibody (Upstate, Charlottesville, VA) for 3 hours then with anti-rabbit Alexus 488 for 30 minutes, and counterstained with propidium iodine for 30 minutes. The sample was analyzed by FACSCalibur interfaced with the FCS Express software.

Statistical analysis. Values are expressed as mean ± SD of at least two separate experiments. Data were compared by the unpaired Student's *t* test with P values <0.05 considered significant.

Results

AKT3 is frequently expressed in primary ovarian epithelial tumors. To examine our hypothesis that up-regulation of AKT3 plays a role in the development of ovarian cancer, we developed an AKT3-specific antipeptide antibody (Supplementary Fig. S1). The AKT3 antibody was used for immunohistochemical analysis of tumor arrays containing 92 primary ovarian tumors. Figure 1*A* shows an example of strong AKT3 staining (*left*) that is abolished by preincubation of the antibody with antigenic AKT3 peptide (*right*). In parallel, the levels of active total AKT were determined by immunohistochemistry of serial sections with the S473-P antibody. Figure 1*B* (*left*) shows representative examples of strong AKT3 staining in two separate grade 2 serous tumors that also exhibited strong phospho-AKT staining (*right*). No stromal staining was observed with the AKT3 antibody under the conditions used. Overall, AKT3 is highly expressed in 19 of 92 (20%) primary ovarian tumors tested compared with low expression levels in 69 of the remaining 74 tumors and negligible expression in the remaining five tumors. Fifteen of 19 tumors exhibiting high AKT3 expression also exhibited the highest AKT activity by immunohistochemistry (Table 1).

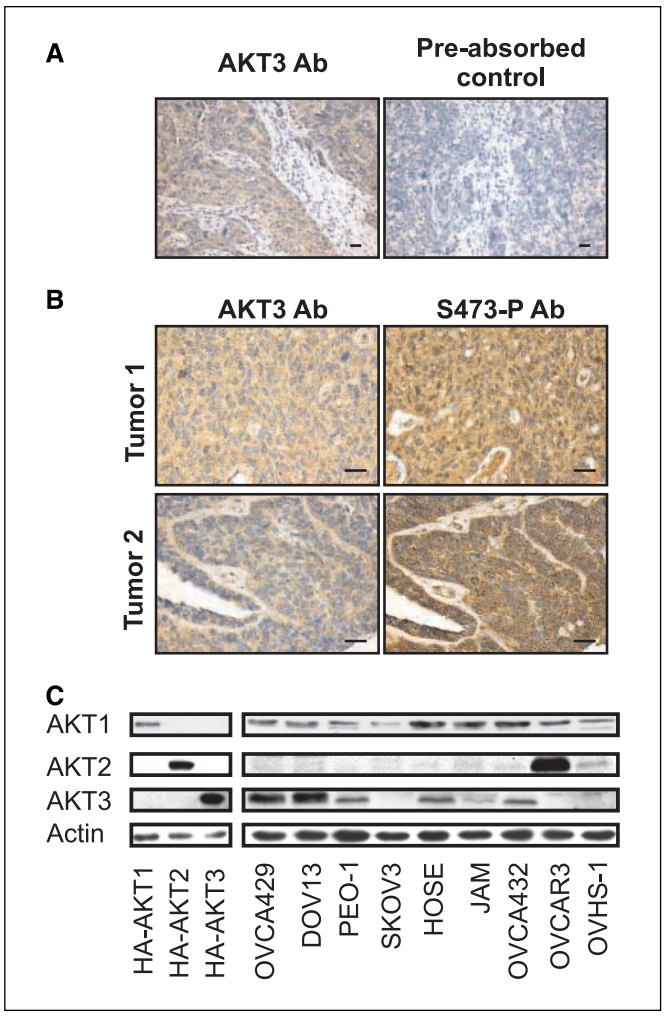

Figure 1. Immunohistochemistry and Western analysis of endogenous expression of AKT family members and active AKT in primary ovarian tumor samples and ovarian cancer cell lines. *A,* confirmation of the specificity of the AKT3-specific antibody (*AKT3 Ab*) by immunohistochemical peptide competition analysis. Primary antibody was incubated overnight with the AKT3 antigenic peptide (5 μmol/L) before immunohistochemistry. *B,* representative examples of the differences in staining intensity observed with the AKT3 and S473-P antibodies (*S473-P Ab*) in grade 2 serous ovarian tumors. All images were manipulated identically by using Adobe Photoshop 7.0. *Bar*, 25 μm. *C, left,* to confirm the specificity of the AKT antibodies, Cos-1 cells were transiently transfected with HA-tagged AKT1, AKT2, or AKT3. Protein extracts (10 μg) were resolved by SDS-PAGE; transferred to polyvinylidene difluoride membrane; and immunoblotted with AKT1, AKT2, and AKT3 isoform-specific antibodies. *Right,* representative Western blot of AKT isoform expression in eight ovarian cancer cell lines (40 μg of extract) and a nontumorigenic control cell line (*HOSE*). Actin was used as a loading control.

Comparison of expression levels by tumor type reveals that 9 of 45 serous tumors (20%), 6 of 24 (25%) endometrioid tumors, and 4 of 15 (26%) of a group of uncommon tumor types, including clear cell, germ cell, Brenner, and mesodermal mixed tumors, as well as adenocarcinoma, exhibit high AKT3 expression. None of the nine mucinous tumors exhibited high AKT3 expression. Positive S473-P staining also correlated with the high AKT3 expression in these tumor types; 89% serous, 67% endometrioid, and 75% other tumors that have high AKT-3 also have high S473-P staining. Taken together, these data show that AKT3 expression is frequently up-regulated in ovarian cancer tissue and that AKT3 expression correlates with total AKT activity in ovarian tumor samples, suggesting that AKT3 might specifically contribute to the pathology of ovarian cancer.

AKT3 is expressed in a range of ovarian cancer cell lines. To establish defined systems for analysis of possible mechanisms by which deregulation of AKT3 could contribute to ovarian cancer, we next characterized the expression patterns of the individual isoforms in whole-cell lysates from eight ovarian cancer cell lines and the nontumorigenic control cell line, HOSE (Fig. 1*C*). The specificity of the AKT1, AKT2, and AKT3 antibodies was shown by Western blot analysis of extracts prepared from Cos-1 cells transiently expressing HA-tagged versions of each AKT isoform (Fig. 1*C, left*). AKT3 expression was observed to vary across the cell lines tested (Fig. 1*C, right*), with obvious expression in HOSE cells and in five of nine ovarian cancer cell lines tested. The highest levels of AKT3 were detected in OVCA429 and DOV13 cells, whereas intermediate levels of expression were observed in PEO-1, HOSE, OVCA432, and JAM cells. No AKT3 protein expression was detected in SKOV3, OVCAR3, and OVHS-1 cell lines. Increased expression of AKT3 in OVCA429 and DOV13 correlated with apparent gene duplication in these cell lines (Supplementary Fig. S2). Conversely, AKT2 expression was only detected in two of the cell lines tested; OVHS-1 had a low level of expression, whereas OVCAR3 highly expressed this isoform. AKT1 was expressed in all cell lines tested, although at variable levels, with highest expression in HOSE, JAM, and OVCA432, and medium/low expression in the remaining cell lines.

AKT3 exhibits elevated intrinsic catalytic activity. Some isolated reports indicate that AKT3 may also exhibit elevated intrinsic kinase activity compared with other isoforms (23, 24), potentially amplifying the effects of up-regulation of this isoform. To definitively address this possibility, we purified GST-tagged AKT3 and AKT1 exogenously expressed in HEK293 cells and determined their kinetics of phosphorylation of three distinct peptide substrates. In all cases, AKT3 exhibited V_{max} values >10-fold higher than AKT1, indicating that overexpression of AKT3 may have greater effects on downstream signaling than AKT1. It should be noted that AKT3 also exhibited 4- to 5-fold higher K_m values (Supplementary Fig. S3), indicating that this amplified signaling may be confined to a subset of substrates that colocalize with the kinase, in particular subcellular compartments, thus giving a high local concentration (16, 25).

AKT3 expression correlates with AKT activity in ovarian cancer cell lines. To explore the correlation between AKT3 expression, activity, and cellular phenotype, we next determined the total AKT activity in exponentially growing cultures of each cell type (Fig. 2*A*). Consistent with the kinetic analysis, the two cell lines expressing highest levels of AKT3, OVCA429 and DOV13, contained >5-fold higher AKT activity than the other cell lines. No AKT activity was detected in OVCAR3 cells that largely express AKT2. The activity levels measured by direct kinase assay are mirrored by Western blotting with the activity state-dependent phosphospecific antibodies recognizing the kinase phosphorylated at Ser^{473} or Thr^{308}. Immunoprecipitation kinase assays using AKT3 immune sera essentially mirrored total AKT activity data consistent with AKT3 being the major contributor to AKT activity in ovarian cancer cells (data not shown).

AKT3 activity correlates with cellular proliferation capacity. AKT has been implicated in the control of cell growth, cell cycle progression, and cell survival (9, 26, 27)—all processes that combine to dictate how cell populations increase in number (proliferate) under defined conditions. To determine the correlation between

AKT activity and cell proliferation, changes in cell number with time were monitored for each cell line in normal growth medium containing 10% FCS (Fig. 2*B*). Consistent with a direct role for AKT3 in mediating tumor phenotype, the cells with highest AKT3 expression and hence highest AKT3 and total kinase activity (OVCA429 and DOV13) proliferated fastest in the presence of growth factors and grew to higher density compared with control HOSE cells.

AKT3 expression correlates with constitutive AKT activity and the ability of ovarian cancer cell lines to proliferate in the absence of growth factors. One hallmark of cancer cells is the ability to survive and proliferate despite withdrawal of growth factors and nutrients (9, 28, 29). Hence, we determined total AKT activity levels in extracts from ovarian cancer cells starved of growth factors for 24 hours and correlated these with cellular proliferation (Supplementary Fig. S4). Both OVCA429 and DOV13 cell lines exhibited constitutive activity in the absence of growth factors, as did SKOV3 cells. Proliferation rates again reflected total and AKT3 activity levels. OVCA429 and DOV13 exhibited a marked growth advantage, largely due to their ability to grow to high density, consistent with a role for AKT signaling in allowing anchorage-independent growth (30).

Inhibition of AKT3 expression reduces ovarian cancer cell proliferation. To determine whether high levels of AKT3 activity are required to confer the proliferative advantage of these cells and to determine the mechanism by which this is achieved, we used shRNA to specifically knock down AKT3 expression in OVCA429 and DOV13 cells. shRNA constructs targeting two distinct regions of AKT3 (3Y and 3Z) were designed and shown to reduce AKT3 expression by >95% in both OVCA429 and DOV13 cells. Importantly, the 3Z hairpin showed negligible effects on AKT1 expression in either cell line, whereas the 3Y hairpin reduced AKT1 expression in DOV13 cells (Fig. 3*A*), emphasizing the importance of choosing multiple target sequences. These cell lines do not express AKT2. The reduction in AKT3 expression resulted in an equivalent reduction in levels of activated, phosphorylated AKT by Western blot analysis and direct kinase activity (Fig. 3*A* and *B*, respectively), further confirming that AKT3 expression is the major contributor to total AKT activity in these cells. Specific down-regulation of AKT3 expression resulted in marked inhibition of cell proliferation in both cell lines (Fig. 3*C*). When these experiments are repeated in the absence of growth factors, both hairpins not only abolish proliferation but also prevent replating by inducing anoikis, making further analysis impossible (data not shown). Overexpression of exogenous AKT1 in both OVCA429 and DOV13 cells restored the levels of active AKT phosphorylated on Ser^{473} and Thr^{308} to above those in control cells (Fig. 4*A*), but failed to rescue cell number (Fig. 4*B*).

Mechanism of AKT3-dependent ovarian cancer cell proliferation. Given the role of AKT3 in regulating apoptosis in melanoma cell lines (15) and the sensitivity of the OVCA429 and DOV13 cells with depleted AKT3 levels to anoikis, we examined whether enhanced apoptosis might explain the reduced cell proliferation in serum-stimulated cells following shRNA-mediated silencing of AKT3 (Supplementary Fig. S5*A*). Apoptosis rates of OVCA429 cells were unaffected by shRNA-induced knockdown of AKT3, whereas DOV13 rates were marginally increased.

AKT3 has been implicated in the regulation of neuronal cell size via specific signaling to the mammalian target of rapamycin (mTOR)/S6K/rpS6 growth control pathway (11). Consistent with this, down-regulation of AKT3 activity correlated with reduced rpS6 phosphorylation in OVCA429 and DOV13 cells (Supplementary Fig. S5*B*), suggesting that decreased rpS6-dependent cell growth may account for the proliferation defect. However, we observed no difference in cell size following AKT3 depletion (Supplementary Fig. S5*C*). Furthermore, although expression of recombinant constitutively active S6K1 restored phosphorylation of

Table 1. Comparison of AKT3 expression and AKT activity in primary ovarian tumors

Tumor type	Tumor grade	Number	Low AKT3	Low AKT3 S473-P		High AKT3	High AKT3 S473-P		No AKT3
				Low	High		Low	High	
Serous	Grade 1	4	4	4	—	—	—	—	—
	Grade 2	17	12	7	5	5	1	4	—
	Grade 3	24	19	9	10	4	—	4	1
Endometroid	Grade 1	4	4	3	1	—	—	—	—
	Grade 2	11	8	4	4	2	1	1	1
	Grade 3	9	3	1	2	4	1	3	2
Mucinous	Grade 1	5	5	2	3	—	—	—	—
	Grade 2	3	3	2	1	—	—	—	—
	Grade 3	—	—	—	—	—	—	—	1
Other	Grade 1	6	3	2	1	3	1	2	—
	Grade 2	2	2	—	2	—	—	—	—
	Grade 3	7	6	4	2	1	—	1	—
Total		92	69	38	31	19	4	15	5

NOTE: Immunohistochemistry was done using antibodies to AKT3 and active AKT (S473-P) on four primary ovarian tumor arrays containing 1 to 2 μm of a tumor that were serially sectioned (a total of 92 tumors with known pathology). The intensity of staining was ranked from low (+) to high (+++), and samples were scored blind to their pathology to eliminate any bias. "Other" includes clear cell, Brenner, mesodermal mixed, and germ cell tumors as well as adenocarcinoma.

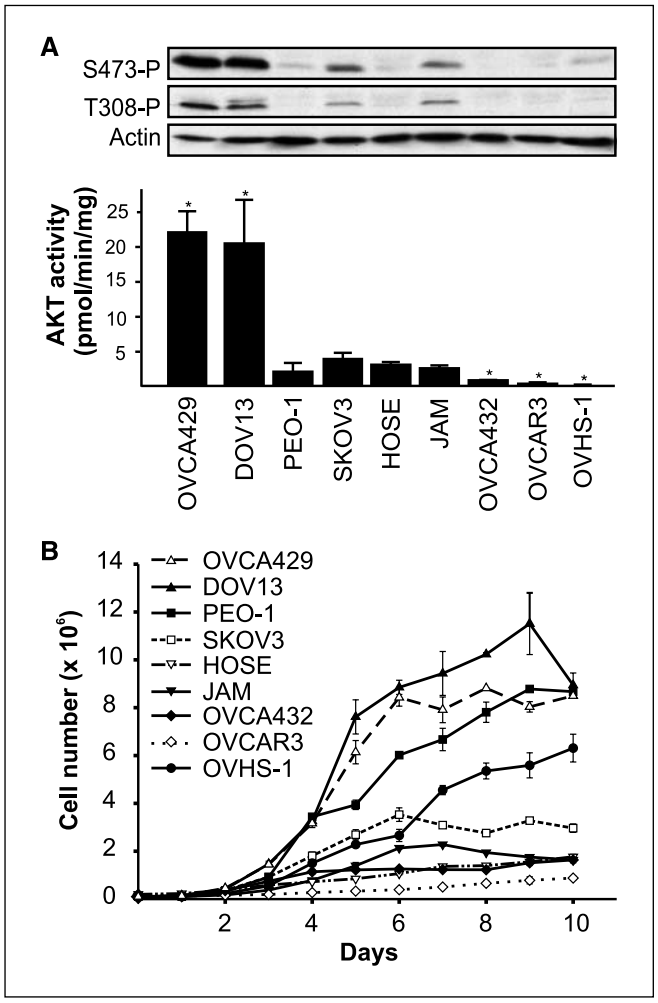

Figure 2. Analysis of AKT activity and proliferation rate in exponentially growing ovarian cell lines. *A,* total AKT activity was determined by Western analysis (40 μg of extract) using S473-P and T308-P antibodies (*top*; actin was used as a loading control), and a direct kinase assay using RPRAATF as substrate (*bottom*) in exponentially growing ovarian cancer cell lines and HOSE. *Columns,* mean (n = 6); *bars,* SD. Statistical analysis of difference from HOSE cells was done by Student's t test: *, $P < 0.005$. *B,* the proliferation rates of the ovarian cell lines were determined by counting cell number in the presence of growth factors. *Points,* mean (n = 3); *bars,* SD.

rpS6 during AKT3 knockdown, it was not sufficient to rescue the proliferation defect (Supplementary Fig. S5*D*).

AKT has also been implicated in the control of cell cycle progression (and hence proliferation) by phosphorylation of key regulators of both G_1-S and G_2-M transition (for review, see ref. 31). To assess whether AKT3 down-regulation induced specific effects on the cell cycle, we monitored cell cycle profiles by FACS analysis after 48 and 72 hours of shRNA treatment. Reduced AKT3 activity in OVCA429 (Fig. 5*A*) and DOV13 (Supplementary Fig. S6) cells results in a marked accumulation of cells in G_2-M. This accumulation correlated with increased levels of phosphorylated histone H3 (Fig. 5*B*), indicating a requirement for AKT3 early in mitosis (32). AKT has been shown to mediate G_2-M transition via inhibition of phosphorylation of Cdc2 on the negative regulatory site, Tyr^{15} (33, 34). Consistent with this model, Tyr^{15} phosphorylation is elevated whereas total Cdc2 expression is unchanged in AKT3-depleted cells (Fig. 5*C*). These data indicate that AKT3 is critical for the proliferation of OVCA429 and DOV13 cells via activation of G_2-M transition, and suggests that inappropriate activation of AKT3 may promote ovarian cancer through G_2-M–targeted increases in cell cycle progression.

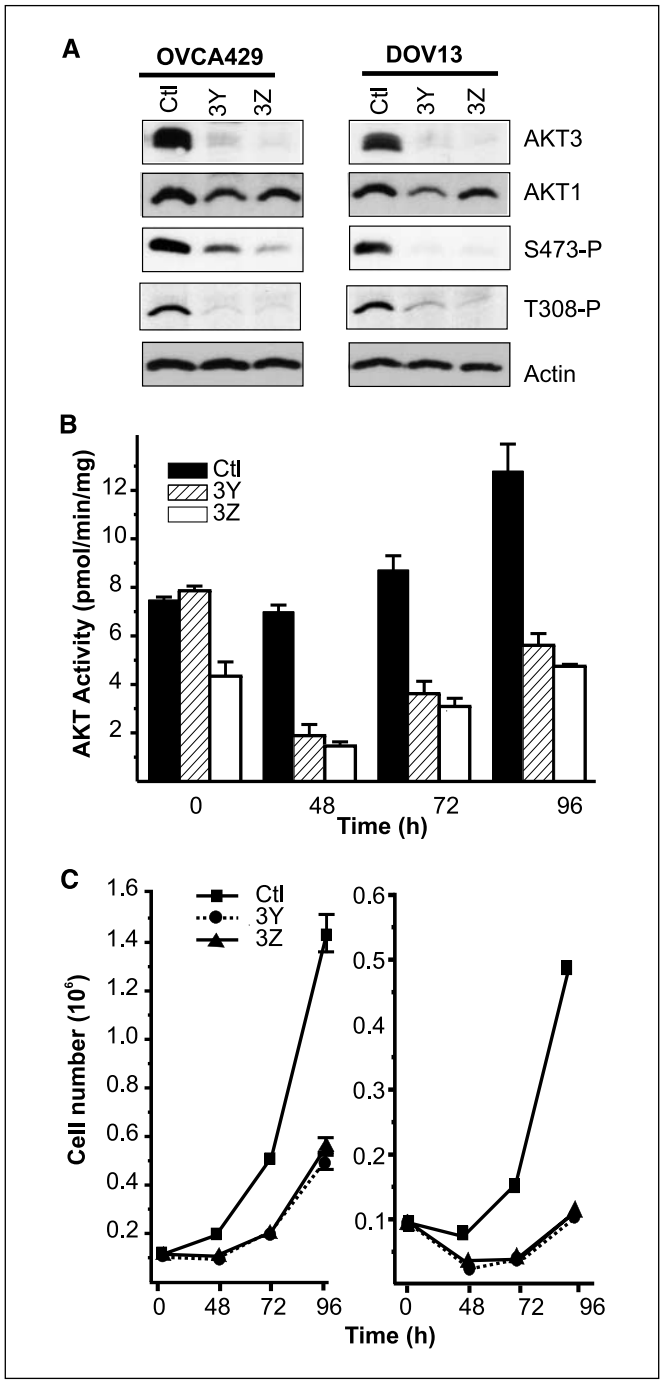

Figure 3. Inhibition of AKT3 expression results in decreased total AKT activity and proliferation rate. Cells were transiently transfected with the shRNA including GFP, FACS sorted, replated, and harvested at the indicated times. *A,* expression of AKT3, AKT1, S473-P, T308-P, and actin in OVCA429 and DOV13 cell lines was determined by Western analysis (10 μg of extract). Actin was used as a loading control (*Ctl*). *B,* total AKT activity in OVCA429 was determined by direct kinase assay with RPRAATF as substrate. Samples were assayed in duplicate. *Columns,* mean (n = 2); *bars,* SD. *C,* representative data for cellular proliferation of OVCA429 (*left*) and DOV13 (*right*) cell lines, determined by cell number at the time points indicated (n = 5).

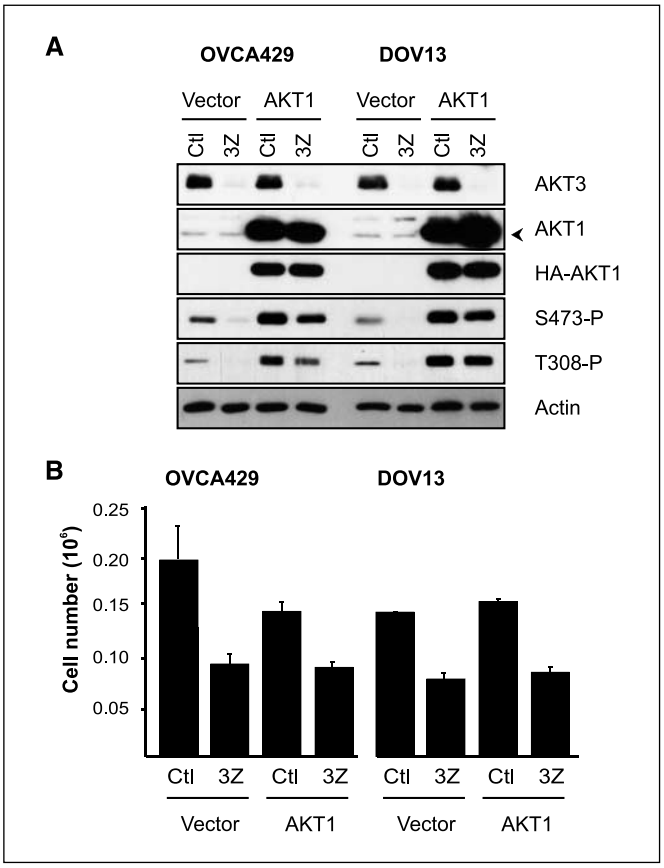

Figure 4. AKT1 does not rescue reduced cell number with inhibition of AKT3 expression. OVCA429 and DOV13 cells were transiently transfected with the shRNA to AKT3 (3Z), including GFP ± pCDNA3-HA AKT1; FACS sorted; replated; and harvested at 48 hours. *A,* representative Western blot (10 μg of extract) of expression of AKT3, AKT1, HA-tagged AKT1, S473-P, T308-P, and actin (n = 2–3). *B,* cell number was determined by Sysmex analysis in triplicate (n = 2–3).

Discussion

In the present study, we showed that AKT3 expression is detectable in 93% of primary ovarian tumors and is specifically elevated in 20%. Increased AKT3 expression was associated with high levels of phosphorylated AKT in 80% of cases; thus, elevated, active AKT3 characterizes a distinct subset of ovarian tumors. The basal level of AKT3 expression in the majority of tumors is consistent with AKT3 playing a role in normal ovarian epithelial cell physiology. This concept is supported by observations that normal mouse ovaries contain significant levels of AKT3 protein (11) and HOSE cells also express AKT3 (Fig. 1*C*). AKT3 expression correlates with AKT activity and ovarian cancer cell proliferation in both the presence and absence of growth factors, emphasizing its potential role in driving at least a subset of ovarian cancer. Our preliminary studies indicate that somatic mutation of AKT3 is rare in ovarian cancer;[10] hence, it is likely that the combination of deregulated PI3K signaling and increased AKT3 expression is critical in the genesis of ovarian tumors. Indeed, a high percentage of primary ovarian tumors exhibits mutated or amplified PI3K (3, 4).

[10] I.G. Campbell et al., in preparation.

A number of studies have used the general PI3K inhibitor LY294002 to show the importance of PI3K/AKT in the development of a range of tumors, including ovarian cancer (10). Other studies have relied on dominant-negative forms of the kinase that do not address the possibility of isoform-specific signaling (11). Here, we show that specific down-regulation of AKT3 expression using shRNA constructs targeting separate regions of the AKT3 mRNA markedly reduced total AKT activity and cell proliferation in two different ovarian cancer cell lines. This observation confirms that AKT3 is the predominant isoform in these cells and conclusively shows a critical role for AKT3 signaling in ovarian cancer cell proliferation.

AKT is a pleiotropic enzyme that provides a hub to signaling cascades controlling cell survival, growth, and proliferation as well as nutrient responses and glucose metabolism (35). In the present study, we show that decreased expression of AKT3 reduced total AKT activity by up to 90% without affecting the rate of apoptosis. This is in contrast to results reported for malignant

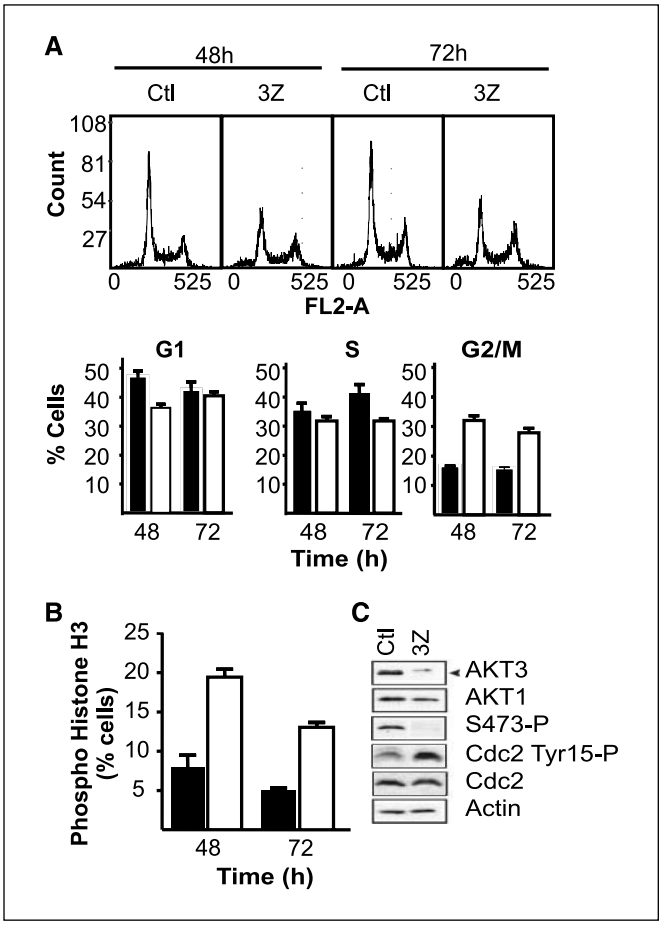

Figure 5. Inhibition of AKT3 expression causes a G_2-M delay. Cells were transiently transfected with the shRNA including GFP, FACS sorted, replated, and harvested at 48 and 72 hours. *A,* typical FACS data (*top*) and cumulative data (*bottom*) illustrating cell cycle phases as determined by propidium iodide staining and analysis using the FACSCalibur interfaced with Modfit (samples were assayed in triplicate, n = 2). *Solid columns,* control; *open columns,* 3Z shRNA. *B,* phospho-histone-H3 levels analyzed in triplicate by FACS. *Columns,* mean (n = 2); *bars,* SD. *Solid columns,* control; *open columns,* 3Z shRNA. *C,* representative Western analysis of AKT3, AKT1, S473-P, Tyr[15] phosphorylation of Cdc2 (*Cdc2 Tyr[15]-P*), Cdc2, and actin expression at 72 hours (10 μg of extract, n = 2).

melanoma where activation of AKT3 was implicated in disease development and targeted reduction of AKT3 activity stimulated apoptosis (15). Thus, AKT3 can signal via distinct pathways, depending on cellular context. Indeed, it is becoming clear that whereas AKT signals to a plethora of crucial signaling pathways, distinct isoforms can signal via defined subsets of these pathways and that these subsets can vary depending on cellular and tissue context (11). For example, AKT3, but not AKT1, is crucial for activation of the mTOR/S6K signaling pathway in brain (11). Furthermore, cellular context is important for AKT1 signaling where $AKT1^{-/-}$ livers were smaller due to reduced cell number whereas $AKT1^{-/-}$ hearts were smaller entirely because of reduced cell size (11).

Whereas studies in knockout mice revealed that AKT3 was required for determination of both cell number and size (11, 36), we found no effect of reduced AKT3 activity on cell size in ovarian cancer cell lines. The basis of this difference between the phenotype of the mouse knockout and that of the shRNA-treated cells does not seem to be via differential effects on mTOR/S6K–dependent growth signaling, as both studies show reduced rpS6 phosphorylation in response to reduced AKT3 signaling. Indeed, activation of the mTOR/S6K pathway is not sufficient to confer the growth advantage observed in these ovarian cancer cells as rescue of rpS6 phosphorylation by exogenous expression of constitutively active S6K1 failed to rescue cell proliferation after down-regulation of AKT3.

In fact, here, we show that the predominant role for AKT3 in ovarian cancer cell lines is in driving proliferation by promoting the G_2-M transition. In eukaryotic cells, the onset of mitosis is controlled by activation of the cyclin B/Cdc2 protein kinase. The activity of this complex is inhibited by phosphorylation of Cdc2 on Tyr^{15} by WEE1Hu, and on Thr^{14} and Tyr^{15} by Myt1 (37). Conversely, the complex is activated by dephosphorylation of these sites by the Cdc25 family of phosphatases (38). Here, we show that reduced AKT3 expression results in increased phosphorylation of Cdc2 on Tyr^{15}, explaining the observed delay in G_2-M transition. Consistent with this observation, AKT has been shown to promote G_2-M transition through phosphorylation and inactivation of the Wee1 kinase family members Myt1 (33) and WEE1Hu (34), via their subsequent binding to 14-3-3 proteins and the resultant cytoplasmic sequestration. Studies are ongoing to confirm that AKT3 targets the Wee1 kinases in the ovarian cell system and to define the relevant Wee1 family member.

Constitutive activation of AKT has been shown to overcome the G_2-M arrest induced by γ-irradiation and DNA damage (39), raising the possibility that inhibition of AKT3 in conjunction with DNA-damaging agents may improve antitumor response in ovarian cancer. Two DNA damage–activated kinases, chk1 and chk2, are inactivated by AKT phosphorylation, leading to activation of Cdc25 and Cdc2 and resistance to DNA damage (38, 40). It will now be important to investigate whether constitutive activation of AKT3 in ovarian cancer can confer resistance to DNA-damaging agents and whether targeted suppression of AKT3 can affect chk1 and chk2 activities in the absence of these reagents.

One key question is whether the effects of specific knockdown of AKT3 expression reflects the existence of crucial AKT3-dedicated pathways or is due to the effect of reduced total AKT activity. This was difficult to resolve due to the lack of AKT2 and low levels of AKT1 activity in OVCA429 and DOV13 cells. However, restoration of active AKT levels by exogenous expression of AKT1 failed to rescue cell proliferation, consistent with AKT3 playing a specific role. Mouse knockout studies indicate distinct but overlapping functions for the individual isoforms. Although knockout of individual AKT isoforms give distinct phenotypes without compensatory increases in the expression of the other isoforms (36, 41, 42), the AKT1 with AKT2 (43) and AKT1 with AKT3 (44) double knockouts give more severe phenotypes indicative of functional redundancy. Evidence for distinct signaling pathways to and from individual AKT isoforms is beginning to accumulate. Reduction of PTEN expression in melanocytes activates AKT3 apparently without affecting the activity of AKT1 or AKT2 (15). Although the mechanism for this selective activation remains to be elucidated, it provides an intriguing precedent.

As with isoform-specific activation, the basis of this apparent selective signaling remains to be elucidated. Recent studies using synthetic peptides indicate that AKT3 may also exhibit elevated intrinsic kinase activity (23, 24). Strikingly, in this study, we used purified preparations of AKT1 and AKT3 to show that AKT3 exhibits far greater specific activity than AKT1, potentially amplifying the effects of overexpression of this isoform, especially in the context of cells with constitutive activation of PI3K signaling. If this is correct, it will be important to interpret the results of screens for tumor levels of phosphorylated AKT with measurement of isoform expression. Indeed, this may aid in patient stratification and selection of patients to be treated more aggressively, or with isoform-specific inhibitors when they become available.

The PI3K/AKT pathway is obviously an attractive candidate for the development of anticancer drugs, and much effort is being placed in generating AKT inhibitors in many laboratories (45, 46). The possibility of distinct modes of activation of individual isoforms and the very different intrinsic kinetic variables of AKT1 and AKT3 raise the possibility of generating isoform-specific, small-molecule inhibitors. Indeed, the generation of allosteric inhibitors selective for AKT1 and AKT2 have recently been reported (46, 47). Given the present findings, development of an AKT3-specific, small-molecule inhibitor would provide the potential for second-line treatment of a significant subset of ovarian cancers without potential side effects of pan-AKT inhibition, such as deregulation of glucose metabolism.

In conclusion, this study has provided novel insight into the mechanism by which the PI3K/AKT pathway signals in primary ovarian tumors and ovarian cancer cells. We have identified a potentially crucial role for AKT3 in driving the genesis of a subset of tumors via stimulation of G_2-M phase transition and cell proliferation. In addition to identifying AKT3 as a new potential therapeutic target for second-line therapy, these studies allow an additional method of subclassification of primary tumors to inform treatment decision and have provided the tools for further analysis of the role and mechanism of isoform-specific signaling in ovarian cancer.

Acknowledgments

Received 5/30/2006; revised 8/28/2006; accepted 10/19/2006.

Grant support: National Health and Medical Research Council of Australia grants 166908 and 251688 (R.D. Hannan), and 350299 and 251688 (R.B. Pearson).

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