Inhibition of calcium-independent phospholipase A_2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells

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PLA_2 (phospholipase A_2) enzymes play critical roles in membrane phospholipid homoeostasis and in generation of lysophospholipid growth factors. In the present study, we show that the activity of the cytosolic $iPLA_2$ (calcium-independent PLA_2), but not that of the calcium-dependent $cPLA_2$ (cytosolic PLA_2), is required for growth-factor-independent, autonomous replication of ovarian carcinoma cells. Blocking $iPLA_2$ activity with the pharmacological inhibitor BEL (bromoenol lactone) induces cell cycle arrest in S- and G_2/M-phases independently of the status of the p53 tumour suppressor. Inhibition of $iPLA_2$ activity also leads to modest increases in apoptosis of ovarian cancer cells. The S- and G_2/M-phase accumulation is accompanied by increased levels of the cell cycle regulators cyclins B and E. Interestingly, the S-phase arrest is released by supplementing the growth factors LPA (lysophosphatidic acid) or EGF (epidermal growth factor). However, inhibition of $iPLA_2$ activity with BEL remains effective in repressing growth-factor- or serum-stimulated proliferation of ovarian cancer cells through G_2/M-phase arrest. Down-regulation of $iPLA_2\beta$ expression with lentivirus-mediated RNA interference inhibited cell proliferation in culture and tumorigenicity of ovarian cancer cell lines in nude mice. These results indicate an essential role for $iPLA_2$ in cell cycle progression and tumorigenesis of ovarian carcinoma cells.

Key words: calcium-independent phospholipase A_2, cell cycle, growth autonomy, lysophosphatidic acid, ovarian cancer cell, tumorigenesis.

INTRODUCTION

Cancer cells exhibit reduced dependence on exogenous growth factors for continuous proliferation and survival [1,2]. The development of growth advantages is a critical step towards oncogenic and metastatic phenotype of tumour cells. Human ovarian cancer cells represent a prototype model of growth autonomy [3–6]. In culture, most ovarian cancer cell lines proliferate in the absence of exogenous growth factors, leading to a slow yet steady increase in cell populations [3–6]. The mechanism for the growth factor-independent replication of ovarian cancer cells is poorly understood. It has been suggested that the growth autonomy of ovarian cancer cells is mediated by constitutive production of LPA (lysophosphatidic acid) and other autocrine growth factors or by genetic alterations that bypass the requirement of exogenous stimuli for growth [1–6].

PLA_2 (phospholipase A_2) enzymes have been implicated in activation of cell migration as well as in production of LPA in ovarian carcinoma cells [7–11]. PLA_2 catalyses the hydrolysis of glycerophospholipids at the *sn*-2 ester bond, producing unesterified fatty acids such as arachidonic acid and 2-lysophospholipids [12]. The PLA_2 enzymes are classified into three major categories: the $sPLA_2$ (secreted PLA_2), the Group IV calcium-dependent $cPLA_2$ (cytosolic PLA_2) and the Group VI cytosolic $iPLA_2$ (calcium-independent PLA_2) [12]. In the present study, we examined the potential involvement of PLA_2 enzymes in growth regulation of ovarian cancer cells. The autonomous replication and growth-factor-stimulated proliferation of ovarian cancer cells are highly sensitive to inhibition of $iPLA_2$ but are refractory to inhibition of $cPLA_2$. When ovarian cancer cells were grown under growth-factor-independent conditions, suppression of $iPLA_2$ activity led to accumulation of cell populations in both S- and G_2/M-phases. Supplementation of exogenous growth factors such as LPA, EGF (epidermal growth factor) or serum to culture released the S-phase arrest but did not affect the G_2/M arrest associated with inhibition of $iPLA_2$. In addition to the prominent effect on cell cycle, inhibition of $iPLA_2$ also induced weak to modest increases in apoptosis. Down-regulation of $iPLA_2\beta$ with lentivirus-mediated RNAi (RNA interference) targeting $iPLA_2\beta$ expression inhibited cell proliferation in culture and decreased tumorigenicity of ovarian cancer cell lines in athymic nude mice. These results revealed a pivotal role for $iPLA_2$ in driving cell cycle progression and tumorigenesis of ovarian cancer cells.

EXPERIMENTAL

Reagents

BEL (bromoenol lactone) [13] was purchased from Cayman Chemicals, and 1 mM stock solution was made in DMSO. The $cPLA_2$ inhibitor pyrrolidine *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]-acrylamide, HCl [14] was obtained from Calbiochem and dissolved as 1 mM in water. LPA (18:1, oleoyl) was obtained from Avanti Polar Lipids and dissolved as 21.8 mM stock solution in chloroform. Before use, chloroform was evaporated in a tissue culture hood and LPA was resuspended in PBS containing 0.5 %

Abbreviations used: BEL, bromoenol lactone; EGF, epidermal growth factor; FBS, foetal bovine serum; GFP, green fluorescent protein; HEK-293 cells, human embryonic kidney cells; LPA, lysophosphatidic acid; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; shRNA, small-hairpin RNA; siRNA, short interfering RNA.

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fatty-acid-free BSA. EGF (tissue culture grade) was obtained from Sigma. Antibodies against $iPLA_2\beta$, cyclin B, cyclin E and GFP (green fluorescent protein) were purchased from Santa Cruz Biotechnology. Anti-c-Myc, $cPLA_2$ and tubulin antibodies were obtained from Cell Signaling Technology.

Cells

The sources of ovarian cancer cell lines have been described previously [15,16]. They were maintained in culture in RPMI 1640 medium supplemented with 10 % (v/v) FBS (foetal bovine serum). All lines were frozen at early passages and used for less than 10 weeks in continuous culture.

$iPLA_2$ activity assay

$iPLA_2$ activity was assayed according to the method described previously [17]. Briefly, cell homogenates were prepared in 10 mM Hepes (pH 7.4), 0.34 M sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF, and were lysed by sonication on ice. The cell lysates were incubated for 2 h at 40 °C with a substrate consisting of 100 μM DPPC (dipalmitoyl phosphatidylcholine) and 400 μM Triton X-100, with 0.02 μCi L-α-dipalmitoyl[2-palmitoyl-1-^{14}C]phosphatidylcholine added as a tracer. The released palmitic acid was quantified as described by Dole [18]. The assays were performed under conditions where substrate hydrolysis was linear with respect to protein and time.

Western blot

Cells in culture plates were washed with PBS and lysed with SDS sample buffer. After boiling for 5 min, cell lysates were resolved by SDS/PAGE, transferred on to Immobilon (PVDF) and immunoblotted with antibodies following the protocols of the manufacturers. Immunocomplexes were visualized with an ECL® (enhanced chemiluminescence) detection kit (GE Healthcare) by using appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology).

Growth assay

The propagation of ovarian cancer cell lines in culture was analysed by colorimetric staining with Crystal Violet or by quantification of cell numbers with a Coulter counter. For Crystal Violet staining, the cells were cultured in 6- or 12-well plates, washed with PBS and incubated with 0.5 % Crystal Violet at room temperature (22 °C) for 20 min. The staining solution was removed and the cells were washed four times with deionized water. After the plates were air-dried, the Crystal Violet was solubilized with the Sorenson's Buffer [0.1 M sodium citrate, pH 4.2, and 50 % (v/v) ethanol] and the D_{570} was measured using a spectrophotometric plate reader (BioTek Instruments).

Apoptosis assay

Both floating and attached cells were harvested and washed twice with PBS before staining with the Vybrant Apoptosis Assay kit #3 (Invitrogen). Briefly, cells (2×10^5) were resuspended in 0.2 ml of staining buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM CaCl) and incubated for 20 min at room temperature with 10 μl of FITC-conjugated Annexin V. After the incubation, 200 μl of the staining buffer was added. The percentages of Annexin V-positive cells were determined with a fluorescence microscope by counting four randomly selected fields totalling 200 or more cells. The results are the means $\pm$ S.D. for triplicate assays, representative of three independent experiments.

Cell cycle analysis

Cells were plated and cultured in complete medium in 100 mm culture dishes. At approx. 40 % confluence, the cells were incubated with RPMI 1640 medium supplemented with or without growth factors in the presence of indicated concentrations of BEL. The attached cells were harvested by trypsinization, washed twice with PBS and resuspended at a concentration of 1×10^6 cells/ml in a fluorochrome staining solution (3.8 mM sodium citrate, 0.05 mg/ml propidium iodide, 0.1 % Triton X-100 and 7 Kunitz units/ml RNase B) and incubated on ice for 3 h or kept at 4 °C for up to 2 weeks before flow cytometric analysis.

siRNA (short interfering RNA) knockdown of $cPLA_2$

$cPLA_2\alpha$ siRNA or non-target control siRNA (2.25 μg) (Dharmacon RNA Technologies) was transfected into ovarian cancer cell lines with Amaxa nucleofector II (Kit V/program V-05 for SKOV-3 and Kit T/program T-30 for Dov-13). The electroporated cells were cultured in 12-well plates in complete medium. After 24 h of recovery, the cells were fed serum-free RPMI 1640 medium and incubated for 1–3 days for growth assay and for Western blot analysis of $cPLA_2$ protein expression.

Lentivirus-mediated shRNA (small-hairpin RNA)

Lentivirus-based U6-promoted $iPLA_2\beta$ shRNA constructs were generated by cloning PCR products carrying sense and antisense oligonucleotides of $iPLA_2\beta$ into the pLL3.7 vector as described previously [19,20]. Three $iPLA_2$ shRNA constructs were made that target the nucleotide sequence 40–60 (construct 1), 810–831 (construct 2) and 1673–1693 (construct 3) of the human $iPLA_2\beta$ cDNA (GenBank® accession number NM_003560) [21]. The viral construct carrying a non-target shRNA sequence (CTTGTTAACGCGCGGTGACCC) was used as a control. The viruses were packaged in HEK-293 (human embryonic kidney) FT cells following the method of Rubinson et al. [19].

Ovarian cancer cell lines in six-well plates at approx. 50 % confluence were incubated with 1 ml of the viral supernatants in the presence of polybrene (8 μg/ml). The infected cells were then kept in regular complete medium for 48 h. Inspection with fluorescence microscopy confirmed the presence of more than 70 % of GFP-positive cells after viral infection. Efficient down-regulation of $iPLA_2\beta$ expression in infected cells was also confirmed by immunoblotting for $iPLA_2\beta$ protein and by enzymatic analysis of cellular $iPLA_2$ activity. Both construct 1 and construct 2 strongly inhibited $iPLA_2\beta$ expression in testing experiments, and construct 1 was used for further experiments in the study.

Tumorigenesis assay in nude mice

Uninfected and lentivirus-infected cells were cultured in 100-mm dishes. The cells in exponential growth stage were trypsinized, washed twice with serum-free RPMI 1640 medium and resuspended in the serum-free medium. The cells (1×10^6 of SKOV-3 and 2×10^6 of OVCAR-3) were injected subcutaneously on the right flank of Balb/c nude mice (Nu/Nu, female, 5–6 weeks old). The formation of subcutaneous tumours was monitored and measured with a digital calliper. The tumour volumes were calculated based on the formula $lw^2/2$ where l is the length and w is the shortest width of the tumour. All procedures for animal studies were conducted in compliance with the policies and regulations of Virginia Commonwealth University Institutional Animal Care and Use Committee.

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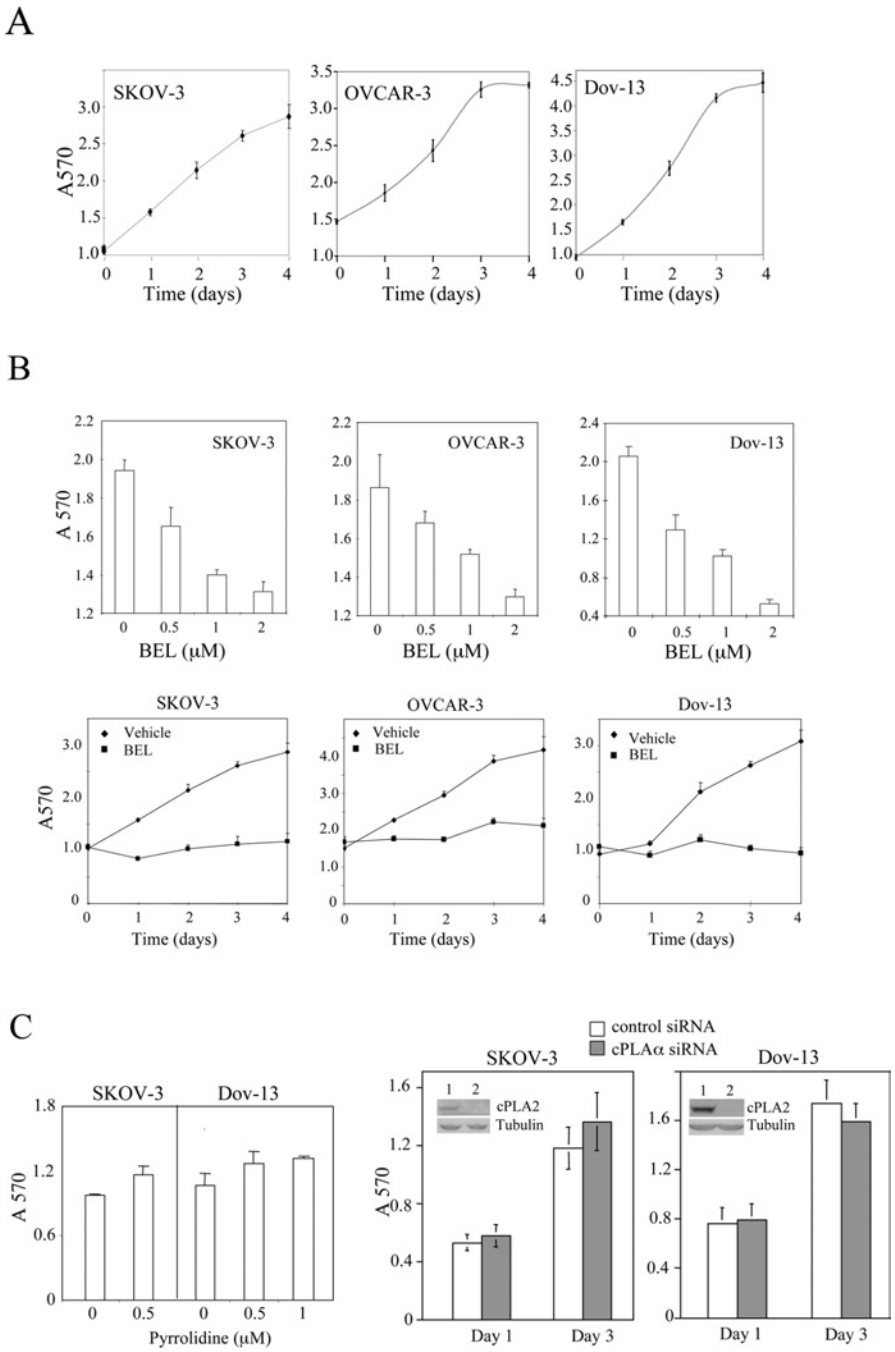

Figure 1 Suppression of growth-factor-independent replication of ovarian cancer cells by inhibition of cellular $iPLA_2$ activity

(**A**) Ovarian cancer cell lines proliferate independently of exogenous growth factors. SKOV-3, OVCAR-3 and Dov-13 cells were plated on to six-well plates with the complete medium. At approx. 40 % confluence, the cells were washed and incubated with serum-free medium for the indicated periods of time (days). Cells were stained with Crystal Violet and the staining was quantified by measuring the D_{570} (arbitrary units). (**B**) The $iPLA_2$ inhibitor BEL suppressed the replication of ovarian cancer cell lines. SKOV-3, OVCAR-3 and Dov-13 cells were incubated for 2 days in serum-free medium with the indicated concentrations of BEL (upper panel) or the cells were incubated in serum-free medium with 2 μM BEL or DMSO (vehicle) for the indicated periods of time (days) (lower panel). (**C**) The $cPLA_2$ inhibitor pyrrolidine (left panel) or siRNA knockdown of $cPLA_2$ expression (right panel) did not inhibit autonomous growth of ovarian cancer cell lines. At 1 day after transfection with control siRNA or $cPLA_2\alpha$-specific siRNA, the cells were switched to serum-free medium and maintained for 1–3 days for growth assay with Crystal Violet staining as described in (**A**). Knockdown of cPLAα expression by $cPLA_2\alpha$ siRNA (lane 2) compared with control siRNA-transfected cells (lane 1) was confirmed by Western blotting (insets of right panels). All numerical results are means $\pm$ S.D. for triplicates, representative of three independent experiments.

Statistics

All numerical results are presented as means $\pm$ S.D. The statistical significance of differences was analysed using Student's t test, where $P < 0.05$ was considered to be statistically significant.

RESULTS

Growth autonomy of ovarian cancer cells requires cellular $iPLA_2$ activity

Most ovarian carcinoma cell lines are capable of multiplying autonomously under serum-free conditions, leading to a

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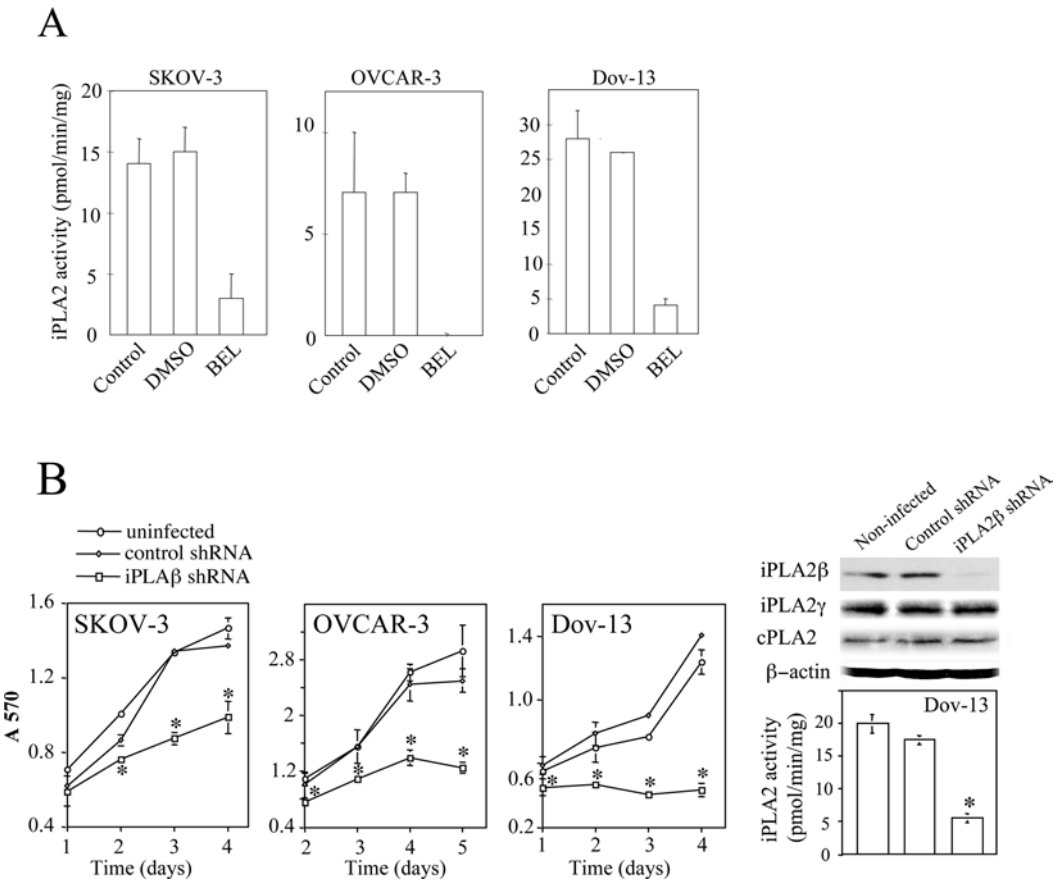

Figure 2 Inhibition of $iPLA_2$ enzymatic activity by BEL and down-regulation of $iPLA_2\beta$ expression by lentivirus-mediated shRNA

(**A**) SKOV-3, OVCAR-3 and Dov-13 cells exhibited $iPLA_2$ activity that was inhibited by BEL. The $iPLA_2$ enzymatic activity in cell lysates was assayed in the absence or presence of BEL or DMSO as detailed in the Experimental section. (**B**) siRNA down-regulation of $iPLA_2\beta$ expression caused growth inhibition. SKOV-3, OVCAR-3 and Dov-13 cells were infected with no virus (uninfected), control virus or with $iPLA_2\beta$-specific shRNA virus. At 3 days after infection, cell lysates were analysed for expression of $iPLA_2\beta$ protein and other PLA_2 isoforms ($iPLA_2\gamma$ and $cPLA_2$). The enzymatic activity of $iPLA_2$ in uninfected, control virus and $iPLA_2$ shRNA virus-infected cells was measured as detailed under the Experimental section. Parallel cultures were incubated in serum-free medium for the indicated periods of time for growth analysis as described in Figure 1. The statistical significance of differences of data in Figures 2–6 was determined by Student's *t* test, where $P < 0.05$ was considered statistically significant and marked with an asterisk.

continuous increase in cell numbers (Figure 1A). In the absence of exogenous growth factors, ovarian cancer cell lines displayed a majority of cells in G_1 and low percentages of populations in S- and G_2/M-phases (results not shown, also see Figure 3), indicating that the cell number increases result from slow yet continuous cell cycling. To explore the mechanism of the uncontrolled proliferation of ovarian cancer cells and potential therapeutic approaches for intervention, we examined whether cellular PLA_2 activities are required for the growth-factor-independent propagation of ovarian cancer cells. PLA_2 enzymes are potentially involved in migration of ovarian cancer cells and in generation of lipid growth factors that may constitute an autocrine stimulation loop to drive proliferation of ovarian cancer cells [2,7–11].

We examined the effect of specific pharmacological inhibitors of $iPLA_2$ and $cPLA_2$ on the proliferation of ovarian cancer cells under serum-free conditions. The $iPLA_2$ inhibitor BEL [13] strongly blocked the growth of various ovarian cancer cell lines including OVCAR-3, SKOV-3 and Dov-13 (Figure 1B). The inhibitory effect of BEL in ovarian cancer cell lines was dose dependent. A significant effect was detected when as low as 0.5 μM BEL was applied (Figure 1B). Treatment of ovarian cancer cell lines with BEL at 2 μM, a concentration 5–10-fold lower than the doses used by others in previous publications [22–24], strongly inhibited cell growth during a 4 day incubation time (Figure 1B). The results suggest that cellular $iPLA_2$ activity is critical for growth-factor-independent replication of ovarian cancer cells.

In contrast with BEL, the $cPLA_2$ inhibitor pyrrolidine [14] at pharmacologically relevant concentrations (0.5–1.0 μM) did not have an inhibitory effect on these cells as analysed by the colorimetric staining assay (Figure 1C). Consistent with this, knockdown of $cPLA\alpha$ expression with specific siRNA did not affect the growth-factor-independent proliferation of these cells (Figure 1C).

To confirm that BEL indeed exerts its effect through targeting $iPLA_2$, we measured cellular $iPLA_2$ activity using *in vitro* enzymatic assay. All ovarian cancer cell lines examined showed significant $iPLA_2$ activity which was blocked by addition of BEL to cell lysates (Figure 2A). Although BEL does not affect other PLA_2s, it has been shown to inhibit phosphatidic acid phosphohydrolase [25]. Therefore, to confirm the involvement of $iPLA_2$ in cell proliferation, we used lentivirus-mediated shRNA to down-regulate expression of $iPLA_2\beta$, the major $iPLA_2$ isoform potentially involved in regulation of proliferation of other types of cell [26,27]. As demonstrated in Figure 2(B), $iPLA_2\beta$ knockdown led to strong growth inhibition in OVCAR-3, SKOV-3 and Dov-13 cells compared with uninfected or non-target control virus-infected cells. In Dov-13 and OVCAR-3 cells, significant decrease

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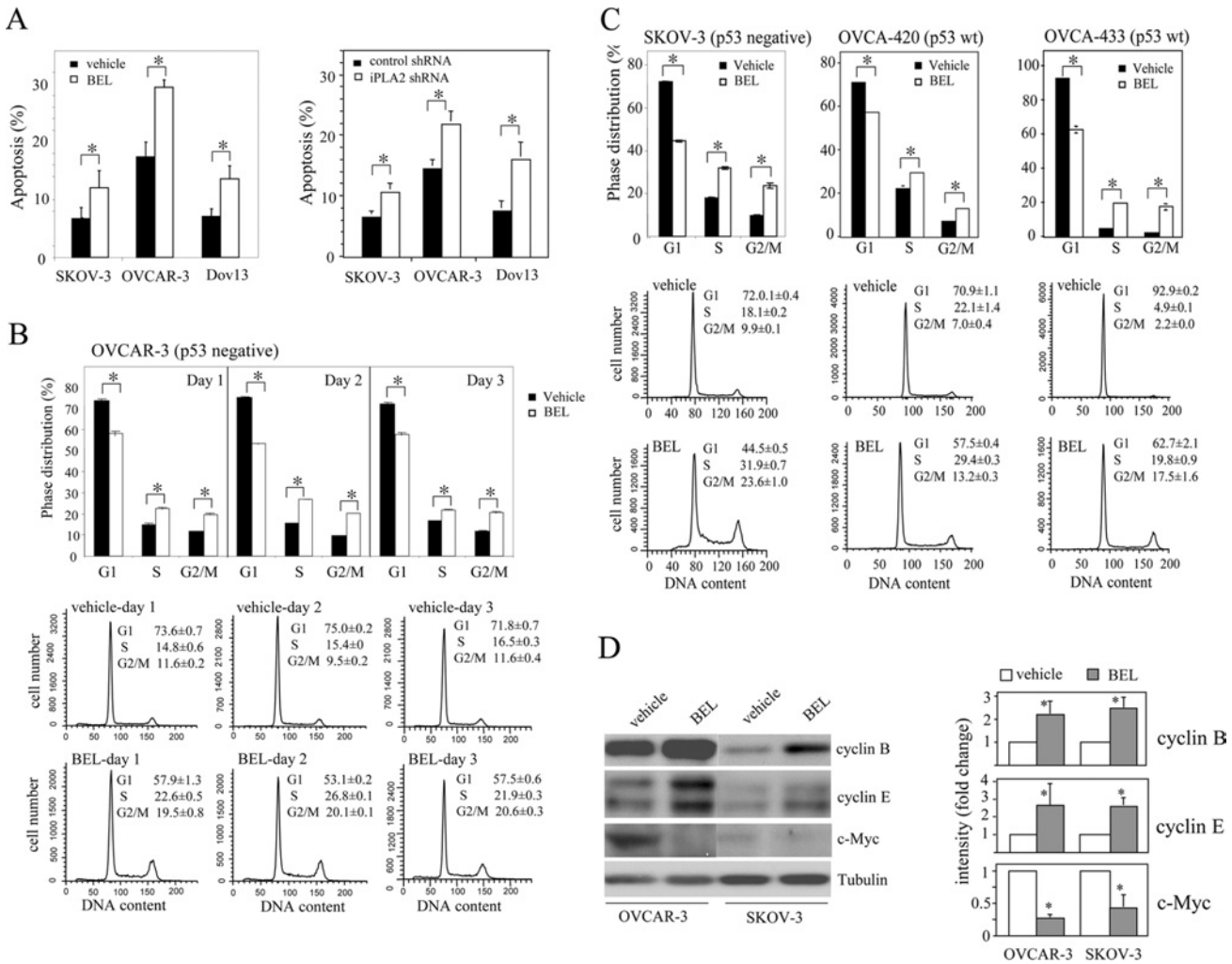

Figure 3 The effect of $iPLA_2$ inhibition on viability and cell cycle progression of ovarian cancer cells

(**A**) Treatment of ovarian cancer cell lines with BEL or knockdown of $iPLA_2\beta$ expression with lentivirus-mediated shRNA led to modest increases in apoptotic cell death. Ovarian cancer cell lines were incubated for 2 days with BEL (2 μM) or DMSO (vehicle) in serum-free medium (left panel). Alternatively, the cells were infected with control virus or $iPLA_2\beta$ shRNA virus and then switched to serum-free conditions for two more days before analysis of apoptosis (right panel). Both floating and attached cells were harvested and stained with FITC-conjugated Annexin V as described in the Experimental section. Shown are percentages of Annexin V-positive, apoptotic cells (mean $\pm$ S.D. for triplicates), representative of three independent assays. (**B**, **C**) Inhibition of $iPLA_2$ with BEL caused cell cycle arrest in S- and G_2/M-phases in both p53-positive and p53-negative ovarian carcinoma cell lines. The p53-deficient cell lines OVCAR-3 (**B**) and SKOV-3 (**C**) and the p53-wild-type cell lines OVCA-420 (**C**) and OVCA-433 (**C**) were incubated in serum-free medium with DMSO (vehicle) or BEL (2 μM) for 1–3 days in (**B**) or for 2 days in (**C**). The cells were harvested by trypsinization, washed with PBS and stained with propidium iodide for flow cytometric analysis. Upper panel: means $\pm$ S.D. for three determinations. Lower panel: flow cytogram, representative of three independent assays. The statistical significance ($P < 0.05$) of differences between vehicle and BEL-treated cells is indicated with an asterisk. (**D**) Cell cycle arrest in S- and G_2/M-phases caused by $iPLA_2$ inhibition was associated with increased abundance of cyclins B and E and with down-regulation of c-Myc. The cells were incubated with vehicle (DMSO) or BEL (2 μM) for 2 days before immunoblotting analysis of the cyclins and c-Myc. The band intensities from three independent experiments were determined by densitometry and presented as relative values with that of vehicle-treated cells (control) defined as 1 arbitrary unit. The statistical significance ($P < 0.05$) of differences in band intensities is indicated with an asterisk.

in colorimetric staining of shRNA-treated cells was detected 1 day after culturing under serum-free conditions. Significant growth decreases in SKOV-3 cells were observed after 2 days in culture.

Inhibition of $iPLA_2$ activity results in a modest increase in cell apoptosis

A decrease in cell viability could potentially contribute to the observed growth suppression associated with inhibition of $iPLA_2$ activity. To evaluate this possibility, we determined whether inhibition of $iPLA_2$ with BEL induces apoptotic cell death. Most ovarian cancer cell lines are largely resistant to serum deprivation-triggered apoptosis [2,16]. After 2 days of incubation under serum-free conditions, only low percentages of OVCAR-3, SKOV-3 or Dov-13 cells underwent apoptosis as analysed by fluorescence-conjugated Annexin V staining (Figure 3A). Inclusion of 2 μM BEL in culture medium to inhibit cellular $iPLA_2$ led to modest yet consistent increases in percentages of apoptotic cells in each of these cell lines (Figure 3A). Knockdown of $iPLA_2\beta$ expression with shRNA also modestly increased apoptosis of ovarian cancer cell lines cultured under serum-free conditions (Figure 3A).

Inhibition of $iPLA_2$ activity induces cell cycle arrest in S- and G_2/M-phases in a p53-independent manner

The cellular $iPLA_2$ activity is regulated in a cell cycle-dependent manner [17,22]. The highest $iPLA_2$ activity is observed at the G_2/M- and late S-phase in proliferating T-cells and CHO-K1

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(Chinese-hamster ovary K1) cells [17,22], suggesting that appropriate regulation of $iPLA_2$ activity is necessary for cell cycle progression. Previous studies in the INS-1 insulinoma cells indicated that disruption of G_1-phase phospholipid turnover by inhibition of $iPLA_2$ induced G_1-phase arrest in a p53 tumour suppressor-dependent manner [23]. We examined whether growth suppression in ovarian cancer cells by inhibition of $iPLA_2$ was also mediated by cell cycle arrest. Ovarian cancer cell lines were incubated with 2 μM BEL or vehicle in serum-free medium for 1–3 days. The cells were harvested on a daily basis for cell cycle analysis by flow cytometry. In contrast with previous studies in INS-1 cells [23], BEL treatment did not induce G_1 arrest in these ovarian cancer cell lines. Instead, we observed marked accumulation of cells in S- and G_2/M-phases accompanied by a concordant decrease in G_1 populations in OVCAR-3 (Figure 3B), SKOV-3 (Figure 3C) and Dov-13 (results not shown). In the absence of BEL, OVCAR-3 and SKOV-3 showed a majority of cells in G_1-phase (70–75 %) and low percentages of populations in S-phase (15–20 %) and G_2/M-phase (approx. 10 %) after 2–3 days in culture (Figures 3B and 3C). The results demonstrate that $iPLA_2$ activity is required for the growth-factor-independent cell cycle progression through S- and G_2/M-phases.

The G_1 cell cycle arrest induced by inhibition of $iPLA_2$ in the INS-1 insulinoma cells is dependent on the function of the p53 tumour suppressor gene [23]. OVCAR-3 and SKOV-3 are deficient in p53 [28,29], which may explain the lack of G_1 arrest in these cells treated with BEL. We therefore extended to examine other ovarian cancer cell lines known to have normal p53 such as OVCA-420 and OVCA-433 [28,29]. Cell cycle analysis indicated that treatment of these two p53-positive ovarian cancer cell lines with BEL also led to cell cycle arrest in S- and G_2/M-phases, but not in G_1 (Figure 3C), confirming a p53-independent effect of $iPLA_2$ on cell cycle transit through S- and G_2/M-phases.

Consistent with the significant accumulation of cells in S- and G_2/M-phases in BEL-treated cells, immunoblotting analysis showed increases in expression levels of S-phase and G_2/M-phase-associated cell cycle regulators such as cyclin B and cyclin E (Figure 3D) [30,31]. SKOV-3 and OVCAR-3 cells showed low levels of cyclin D1 that were not altered by treatment with BEL (results not shown). We also observed down-regulation of c-Myc levels in BEL-treated cells (Figure 3D). Since c-Myc expression is highest at late G_1 promoting G_1-to S-transition [30], the decreased expression of c-Myc seen in BEL-treated cells may reflect the partial loss of G_1 cells following inhibition of $iPLA_2$.

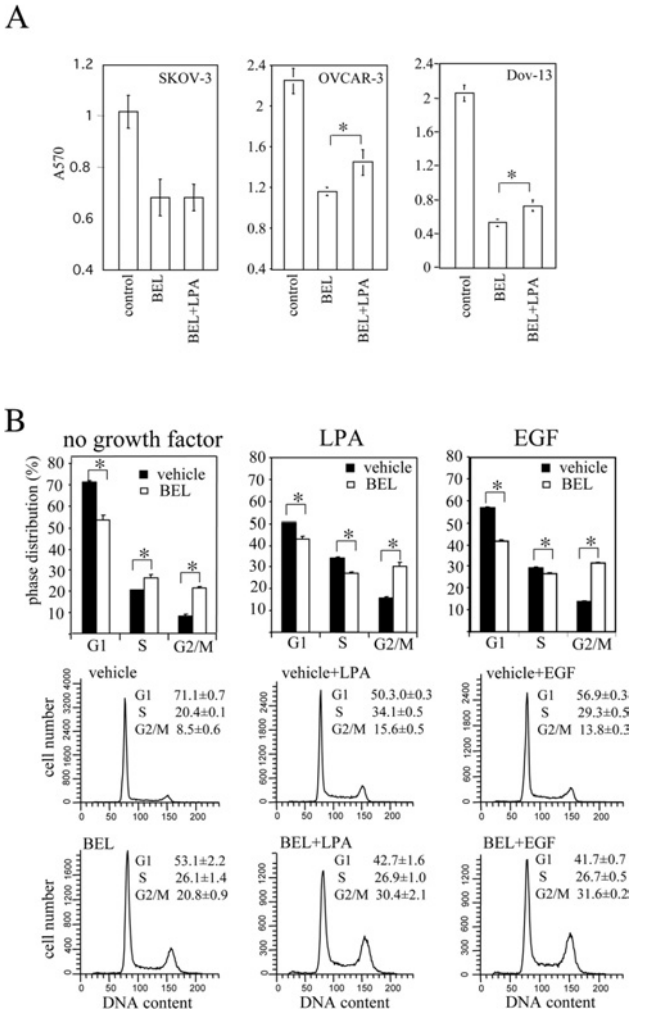

Figure 4 The effect of $iPLA_2$ inhibition on growth and cell cycle progression of ovarian cancer cells maintained with exogenous growth stimuli

(**A**) Exogenous LPA failed to reverse the inhibitory effect of BEL on cell division. SKOV-3, OVCAR-3 and Dov-13 cells were incubated for 2 days with BEL (2 μM) in serum-free medium supplemented with 10 μM LPA (BEL+LPA) or without LPA (BEL). The growth of SKOV-3 and OVCAR-3 cells under these experimental conditions was assayed as described in Figure 1. (**B**) The S-phase arrest, but not the G_2/M arrest was released by LPA or EGF. OVCAR-3 cells were treated with or without BEL (2 μM) for 24 h before addition of LPA (10 μM), EGF (50 ng/ml) or no exogenous growth factor. After a further incubation for 42 h, the cells were stained with propidium iodide for flow cytometric analysis.

The S-phase arrest is released by supplying exogenous growth factors

In addition to the membrane phospholipid turnover, $iPLA_2$ may also participate in generating lysophospholipids, particularly LPA, which is a potent growth factor towards ovarian cancer cells [2,7–11]. Inhibition of $iPLA_2$ activity may block autocrine production of LPA, leading to loss of replicative stimulation and growth arrest at S- and G_2/M-phases. To assess this possibility, we added LPA to cultures to determine whether LPA could rescue ovarian carcinoma cells from BEL-induced growth arrest. As shown in Figure 4(A), LPA had a weak reversal effect on BEL-mediated growth suppression in OVCAR-3 and Dov-13 cells but had no effect in SKOV-3 cells, suggesting that potential inhibition of LPA production by BEL is not likely to be the major determinant of BEL-induced growth repression of ovarian cancer cells.

We next asked whether BEL inhibited cell cycle progression at S and G_2/M transit when exogenous growth factors such as LPA or EGF were present. To this end, we added LPA or EGF to OVCAR-3 cells that had been arrested in S- and G_2/M-phases by BEL (Figure 4B). Cell cycle parameters were analysed 42 h after addition of EGF or LPA. As shown in Figure 4(B), LPA and EGF were each able to release the S-phase accumulation in BEL-treated cells. However, BEL remained effective in arresting cells at G_2/M in cultures supplemented with LPA or EGF. The control cells maintained without exogenous growth factor were arrested by BEL in both S- and G_2/M-phases (Figure 4B).

Inhibition of $iPLA_2$ suppresses growth factor- or serum-stimulated mitogenesis through cell cycle arrest in G_2/M-phase

To address whether the G_2/M arrest enforced by inhibition of $iPLA_2$ also suppresses the growth factor-driven proliferation of

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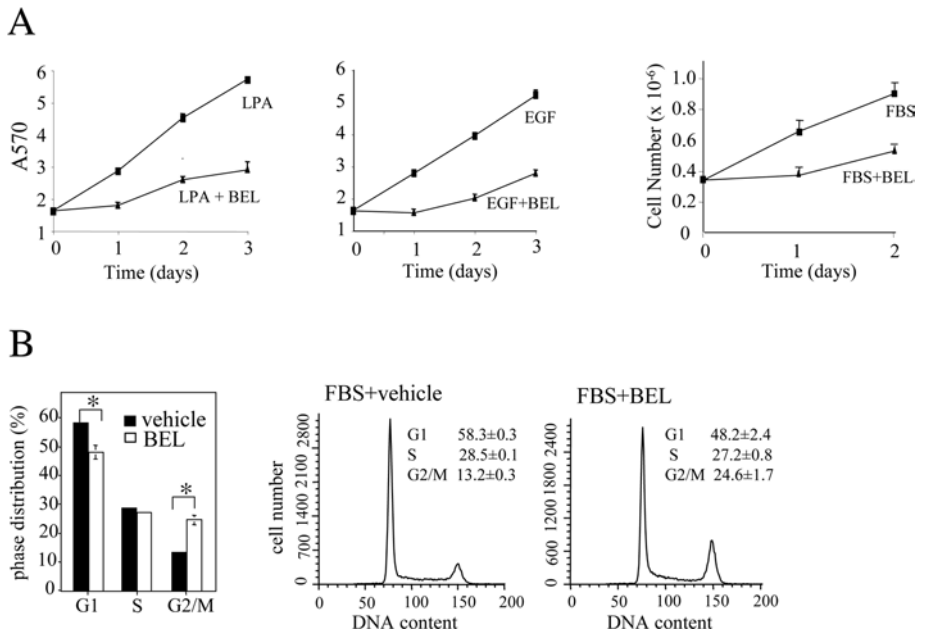

Figure 5 BEL suppression of growth factor- and serum-stimulated proliferation of ovarian cancer cells through cell cycle arrest in the G_2/M-phase

(**A**) Inhibition of $iPLA_2$ with BEL suppressed LPA, EGF or FBS-stimulated growth. OVCAR-3 cells were incubated in medium supplemented with LPA (10 μM), EGF (50 ng/ml) or FBS (10 %) in the presence or absence of indicated concentrations of BEL (2 μM for LPA and EGF and 15 μM for FBS). The growth of cells was assayed with Crystal Violet staining as described in Figure 1 or by quantification of cell numbers with a Coulter counter. (**B**) Inhibition of $iPLA_2$ with BEL induced cell cycle arrest at G_2/M in OVCAR-3 cells grown with serum. The cells were incubated in the complete medium containing 10 % FBS with BEL (15 μM) or vehicle for 42 h before cell cycle analysis.

ovarian cancer cells, we incubated OVCAR-3 cells in medium supplemented with EGF or LPA. As shown in Figure 5(A), EGF- and LPA-induced proliferation of OVCAR-3 cells was strongly decreased by addition of 2 μM BEL (Figure 5A). Cell cycle analysis indicated that in the presence of EGF or LPA, OVCAR-3 cells were growth arrested at G_2/M by treatment with BEL (results not shown), similar to the results presented in Figure 4(B).

We next determined whether inhibition of $iPLA_2$ suppresses the growth of ovarian cancer cells maintained in the complete medium containing 10 % FBS. BEL, which at 1–2 μM strongly inhibited division of ovarian cancer cell lines under serum-free conditions or in LPA- or EGF-supplemented medium, had little or minimum effect in ovarian cancer cell lines maintained in the complete medium (results not shown), consistent with reduced stability and/or efficacy of BEL in the presence of serum. However, 10–20 μM BEL, a concentration range similar to that used by others in previous studies [22–24], strongly inhibited the proliferation of OVCAR-3 (Figure 5A) and other ovarian cancer cell lines. Similar to the cells grown with LPA or EGF, OVCAR-3 cells supported by serum were arrested at G_2/M by the high doses of BEL (Figure 5B). Thus inhibition of cellular $iPLA_2$ activity suppresses growth factor or serum-stimulated propagation of ovarian cancer cells through a G_2/M checkpoint mechanism.

siRNA knockdown of $iPLA_2\beta$ expression decreases tumorigenicity of ovarian cancer cells in nude mice

To explore further the biological significance of $iPLA_2$ in regulation of proliferation of ovarian cancer cells *in vivo*, we examined the effect of down-regulation of $iPLA_2$ on tumorigenicity of SKOV-3 and OVCAR-3 cells. Dov-13 cells do not grow in nude mice [32], precluding evaluation of the *in vivo* effect of $iPLA_2$ down-regulation on this line. Expression of $iPLA_2\beta$ was stably decreased by lentivirus-mediated shRNA as demonstrated in Figure 2(B). As demonstrated in Figure 6(A), uninfected SKOV-3 cells and SKOV-3 cells infected with control virus carrying a non-targeting sequence were highly tumorigenic in nude mice after subcutaneous injection. However, the cells infected with $iPLA_2\beta$ shRNA virus became less tumorigenic as reflected by reduced tumour volumes and tumour growth rate (Figure 6). $iPLA_2\beta$ knockdown also significantly decreased the tumorigenicity of OVCAR-3 cells in nude mice (Figure 6A). The co-expressed GFP was present in the tumours derived from virus-infected cells as demonstrated by Western blot analysis (Figure 6B) and fluorescence microscopy (Figure 6C), confirming the long-term stable expression of the shRNA *in vivo*. These results indicate that inhibition of $iPLA_2$ could offer a novel strategy to control tumour growth and progression *in vivo*.

DISCUSSION

In the present study, we demonstrated that $iPLA_2$ activity is required for cell cycle progression and tumorigenesis of ovarian cancer cells. Inhibition of $iPLA_2$ activity with the irreversible and mechanism-based specific inhibitor BEL [13] strongly blocked the growth-factor-independent and stimulated proliferation of ovarian cancer cells. In the absence of exogenous stimuli, inhibition of $iPLA_2$ induced cell cycle arrest in S- and G_2/M-phases. However, the S-phase accumulation could be reversed by providing a growth factor such as LPA or EGF in culture. When ovarian cancer cells are maintained with the support of a defined growth factor or serum, inhibition of $iPLA_2$ remained effective in arresting cells in G_2/M-phase, leading to efficient suppression of cell division. Under these experimental conditions, we noticed that inhibition of $iPLA_2$ also induced apoptotic cell death albeit modestly, which may contribute to the observed growth suppression associated with $iPLA_2$ inhibition. Furthermore, intact $iPLA_2$ activity seems to be crucial in maintaining proliferation and survival of ovarian cancer cells *in vivo* as stable siRNA

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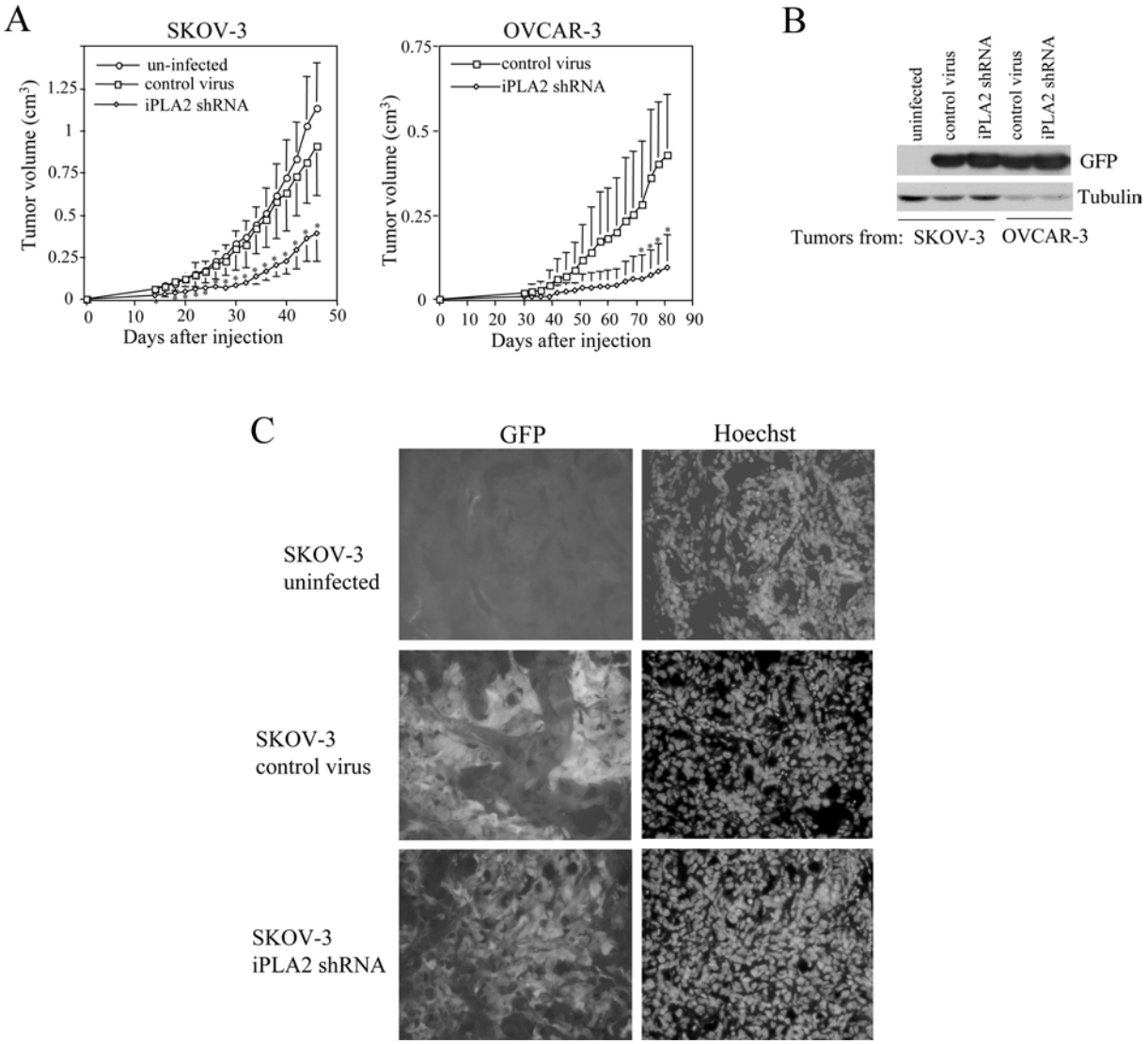

Figure 6 Inhibition of tumorigenicity of ovarian cancer cell lines by shRNA knockdown of iPLA$_2\beta$ expression

SKOV-3 and OVCAR-3 cells were infected with lentivirus carrying non-target control or iPLA$_2\beta$-specific shRNA. These infected cells and uninfected SKOV-3 cells were injected subcutaneously on the right flank of Balb/c nude mice (four mice/group). Shown are volumes of tumours (mean $\pm$ S.D.) grown from uninfected SKOV-3 cells, control virus-infected cells and the iPLA$_2\beta$ shRNA virus-infected cells (**A**). The statistical significances of differences in tumorigenicity between iPLA$_2\beta$ knockdown cells and control virus-infected cells are indicated with an asterisk if $P < 0.05$. The expression of GFP in tumours derived from the virus-infected cells was confirmed by Western blot analysis (**B**) and by fluorescence microscopy (**C**).

down-regulation of iPLA$_2$ expression dramatically decreased tumorigenicity of ovarian cancer cell lines in athymic nude mice.

Phosphatidylcholine is a major membrane phospholipid in mammalian cells [33]. During the cell cycle, cells must double their phospholipid mass and reassemble the membrane to form daughter cells. Therefore the metabolism of phospholipids must be co-ordinately regulated throughout the cell cycle, which is achieved by the opposing actions of CTP:phosphocholine cytidylyltransferase and iPLA$_2$ [34,35]. The phospholipid turnover rate is high in the G_1-phase and decreases during the S-phase, which coincides with the doubling of PC mass [17]. Recently, Zhang et al. [23] reported that iPLA$_2$ activity was essential for the entry of G_1-phase cells into the S-phase. Blockade of iPLA$_2$-mediated phospholipid turnover induced cell cycle arrest in G_1 in the INS-1 insulinoma cells in a p53-dependent manner [23]. In ovarian cancer cell lines, however, we consistently observed suppression of cell cycle in G_2/M- and S-phases, but not in G_1-phase upon inhibition of iPLA$_2$ activity in both p53-positive and p53-negative cell lines. It is apparent that the p53 status is an unlikely determinant of the unique pattern of cell cycle arrest in our model system. We cannot exclude the possibility that the mode of iPLA$_2$ regulation of the cell cycle is cell type-specific. Indeed, Atsumi et al. [36] reported that iPLA$_2$ inhibitors suppress Fas- and TNF (tumour necrosis factor)-induced apoptosis of the U937 human monocytic leukaemia cells, while overexpression of a highly active variant of iPLA$_2$ suppresses proliferation of HEK-293 cells.

We tested the effect of BEL on replication of ovarian cancer cell lines maintained in a medium supplemented with FBS. It required >5-fold higher concentrations of BEL to achieve a similar magnitude of growth inhibition compared with cells cultured under serum-free conditions or in a medium chemically defined with growth factors such as EGF or LPA. The requirement of high levels of BEL for growth inhibition may reflect a decreased stability and/or efficacy of BEL in the presence of serum components. In addition, serum contains

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high levels of lysophospholipids such as lysophosphatidylcholine that can be utilized as a substrate for PC synthesis to affect membrane phospholipid metabolism as seen in cells cultured in choline-deficient medium or treated with CTP:phosphocholine cytidylyltransferase inhibitors [37,38]. Furthermore, activity or expression of enzymes involved in phospholipid homoeostasis, such as CTP:phosphocholine cytidylyltransferase and other isoforms of PLA_2, may be modulated by sterol or diverse serum factors in compensation for enforced inhibition of $iPLA_2$ activity [24,39].

The findings described in our study suggest that $iPLA_2$ activity is especially required for S- and G_2/M-phase transition, which is consistent with the fact that $iPLA_2$ activity is low at the G_1/S border and reaches a peak at late S- and G_2/M-phases [17,22]. Our results of experiments in nude mice suggest that $iPLA_2$ activity is also necessary for cell cycle progression *in vivo*. It is yet to be determined how inhibition of $iPLA_2$ activity culminates in cell cycle arrest in G_2/M- or S-phases. Since $iPLA_2$ is involved in generation of LPA, a potent lysophospholipid growth/survival factor in ovarian cancer [2,7–11], inhibition of $iPLA_2$ may cause discontinuation of autocrine LPA production in ovarian cancer cells. Other investigators reported that BEL suppresses LPA production in ovarian cancer cells [7,8]. However, exogenous LPA was incapable of rescuing ovarian cancer cell lines from BEL-mediated growth inhibition in spite of the prominent effect on the S-phase transit. Taken together, these results indicate that the role of $iPLA_2$ in cell division is more than the generation of lysophospholipid growth factors. Most likely, inhibition of $iPLA_2$ may induce alterations in the contents and function of membrane phospholipids that could trigger activation of intracellular signalling pathways to halt cell cycle progression.

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