All-trans retinoic acid downregulates ALDH1-mediated stemness and inhibits tumour formation in ovarian cancer cells

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Abstract

Aldehyde dehydrogenase 1 (ALDH1) is a cancer stem-like cell (CSC) marker in human cancers; however, the specific ALDH1-regulated function and its underlying signalling pathways have not been fully demonstrated. Here, we investigated the ALDH1-regulated function and its underlying signalling and tested whether all-trans retinoic acid (ATRA) can suppress ALDH1-regulated tumour behaviour in ovarian cancer cells. By modulating ALDH1 expression using flow cytometry enrichment and exogenous overexpression or knockdown, we showed that the ALDH1 activity is positively correlated with stemness in ovarian cancer cells according to measures such as sphere formation and CSC marker expression as well as tumourigenesis in a mouse xenograft model. The findings indicate that the ALDH1 directly regulates the functions of ovarian cancer cells. We also showed that ALDH1 can regulate the expression of FoxM1 and Notch 1, which are involved in the downstream signalling of ALDH1-mediated biofunctions. Inhibition of FoxM1 by Thiostrepton and of Notch1 by DAPT downregulated the sphere formation ability of cells. ATRA reduced ALDH1 expression, suppressed tumour formation and inhibited sphere formation, cell migration and invasion in ALDH1-abundant ovarian cancer cells. We conclude that ATRA downregulates ALDH1/FoxM1/Notch1 signalling and suppresses tumour formation in ovarian cancer cells.

Introduction

Ovarian cancer is the second most common gynaecological malignancy and a major cause of death from cancer in women (1,2). Epithelial ovarian cancer (EOC) is usually diagnosed at an advanced stage, and despite cytoreductive surgery followed by combination chemotherapy, many EOC patients eventually experience recurrence with the development of chemoresistant tumours and subsequently die of their disease (3). Recent studies have shown that a subpopulation of cancer cells, cancer stem-like cells (CSCs), are characterized by their enhanced ability for tumour formation and drug resistance, and that they share some CSC markers, such as Aldehyde dehydrogenase 1 (ALDH1) and Notch1 (4–7). ALDH1 converts retinol to retinoic acid, and functions as a modulator of cell proliferation, cellular detoxification and stem cell differentiation (8–12). Increased ALDH1 expression has been linked with the induction of chemoresistance in ovarian cancer cells (13). However, the results obtained from studies of ALDH1 expression and EOC patient survival have been controversial (14–19). In addition, little is known about the importance of ALDH1 in the regulation of ovarian cancer cells.

At present, anticancer approaches aimed at eliminating CSCs are not applicable to clinical situations (20). One way to treat cancer without removing CSCs is to induce their differentiation and cause the loss of their self-renewal capability (21,22). Drugs such as retinoic acid (RA, a vitamin A metabolite) can stimulate CSC differentiation. Modulating CSCs using all-trans retinoic
ellipsoid body [volume = (length/2) × (width) × (height)] measured using Vernier callipers by external measurement of the length and width.

observed mice for tumour formation every other day after cell inoculation to investigate the role of ALDH1 in the regulation of stemness and tumourigenic properties, and we test the effect of ATRA on the ALDH1-regulated oncogenic potential of ovarian cancer cells.

Materials and methods

Cell culture

The human ovarian cancer cell lines ES2, A2780 and its cisplatin-resistant derivative CP70 were obtained from the American Type Culture Collection (Manassas, VA). These cells were grown in RPMI-1640 medium with 10% foetal bovine serum. Cells were cultured and stored according to the supplier’s instructions and were used between passages 5 and 20. Once resuscitated, the cell lines were regularly authenticated through cell morphology monitoring, growth curve analysis, species verification and contamination checks.

Inhibitors

The ALDH inhibitor diethylaminobenzaldehyde (DEAB) was purchased from StemCell Technologies (Vancouver, BC, Canada). DAPT ([2S-[3,5-difluorophenyl]acetyl]-L-alanyl-2-phenyl-1,1-dimethylepy ester-glycine; Cayman Chemical, Ann Arbor, MI) dissolved in dimethyl sulfoxide, was used to test the effect of Notch signalling blockade. The FoxM1 inhibitor thioasteron and ATRA were purchased from Sigma (Sigma, St Louis, MO).

MTT cytotoxicity assay

The cell lines were cultured in a humidified incubator containing 95% air and 5% CO₂ at 37°C in 96-well flat-bottomed microtitre plates. After 72h of incubation, the in vitro cytotoxic effects of treatments were determined by MTT assay (at 570 nm).

Sphere formation assay

Standard sphere formation assays were performed according to Zhang et al. (4) with minor modification. The cells (1 × 10⁵) were resuspended in serum-free DMEM/F12 medium supplemented with 5 μg/ml insulin (Sigma), 20 ng/ml human recombinant epidermal growth factor (EGF; Invitrogen, Life Technologies, Carlsbad, CA) and 10 μg/ml basic fibroblast growth factor (bFGF; Invitrogen) in ultra-low attachment plates (Corning Costar, Corning, NY). Spheres that arose within 1–2 weeks were counted. Colony diameters >50 μm were counted as a single-positive colony. The middle field was chosen for counting of spheres, and two fields for each plate were counted under a dissecting microscope. For all sphere formation experiments, a minimum of eight wells was run for each condition. All data represent the mean ± SEM of three separate experiments and at least 24 different fields.

In vivo mouse xenografts

All animal studies adhered to protocols approved by the Institutional Animal Care and Use Committee of National Cheng Kung University Medical Centre. The mouse xenograft model was prepared as previously reported (32). Briefly, cells were implanted in 50% Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously into the left flanks of 4–6-week-old female NOD/SCID (NOD.CB17-PRKDC(Scid)) mice. We observed mice for tumour formation every other day after cell inoculation and measured tumour size when it became measurable. Tumour size was measured using Vernier callipers by external measurement of the length and width, and the tumour volume was computed from the formula for an ellipsoid body [volume = (length/2) × (width)²]. When the tumours reached ~2000 mm³ at approximately 28 days, the mice were sacrificed. To assess

acid (ATRA) could potentially shift the CSCs into a more differentiated state (23–26), thereby making them sensitive to chemotherapy and less aggressive (27–29). ALDH1 has been shown to be one of the target proteins of ATRA (30,31). In this study, we investigate the antitumour effect of ATRA, when tumours reached 100–200 mm³, mice received vehicle or ATRA (0.05 or 0.1 mg/kg) once every other day. All tumours were excised, fixed in 10% neutral buffered formalin, and embedded in paraffin for histological assessment or shock-frozen in liquid N₂ and stored at −80°C for further analysis.

Transwell migration and invasion assays

Cells (1 × 10⁵) were seeded on Transwell filters with a pore size of 8 μm (Corning Costar) and were allowed to migrate toward medium containing 10% FBS. After 8h, the cells on the upper surface of the Transwell membrane were removed with a cotton swab, and the migrated cells (on the underside of the Transwell) were fixed and stained with methyl blue and Giemsa staining dye (Merck, Darmstadt, Germany). The invasion assay was conducted in the same manner as the migration assay, except that Matrigel (BD Biosciences) was used, the incubation time differed (24h) and the number of cells added to the upper chamber was 2 × 10⁵ cells. Cell migration and invasion were quantified by counting the migrated cells in six random fields under a light microscope.

Separation of ALDH1-Low and -High cells

An Aldefluor® kit (StemCell Technologies) was used to assess ALDH activity in the ovarian cancer cell lines, as previously described (33). In brief, 1 × 10⁵ cells were incubated in Aldefluor® assay buffer containing a 1.5 μM ALDH substrate for 30 min at 37°C. Each sample was treated with 50 μM of DEAB, and used as a negative control. Prior to analysis, cells were stained with 1 mg/ml of propidium iodide to evaluate their viability. The fluorescence intensity of the stained cells was analysed using a FACSAria cell sorter Flow Cytometer (BD Biosciences). The reaction with DEAB was used to define the baseline for the assay. The ALDH activity of a sample was determined to be ‘high’ or ‘low’ based on the fluorescence intensity beyond or below the threshold defined by the reaction with DEAB. The purity of FACS-sorted ALDH1 ‘high’ or ‘low’ cells is shown in Supplementary Figure 1A, available at Carcinogenesis Online.

ALDH1 and FoxM1 overexpression/knockdown and transfection

We generated stable cell lines (A2780-ALDH1, A2780-FoxM1, CP70-shALDH1 and CP70-shFoxM1) from A2780 and CP70 cells with plasmid vectors encoding ALDH1 and shALDH1. ALDH1 short hairpin RNA was prepared and maintained according to the protocol provided by the National RNAi Core Facility, Academia Sinica, Taipei, Taiwan. To establish stable clones, the ALDH1 knockdown plasmids (NM-000689, National RNAi Core Facility) were transfected into CP70 cells, and the ALDH1 overexpression plasmid (ALDH1-pcDNA3.1, Addgene, Cambridge, MA) was transfected into A2780 cells using Lipofectamine (Invitrogen). Forty-eight hours after transfection, stable sh-ALDH1 and sh-FoxM1 transfectants were selected with puromycin (Sigma) at 0.3 μg/ml and stable ALDH1 and FoxM1 transfectants were selected in G418 (Sigma) at 600 μg/ml. After 2 weeks of selection in puromycin or G418, clones of resistant cells were isolated and allowed to grow in medium containing puromycin at 0.3 μg/ml or G418 at 600 μg/ml. The integration and quantification of transfected plasmid DNA was confirmed by reverse transcription–PCR and western blot analyses.

RNA isolation and quantitative reverse transcription–PCR

Total RNA was isolated using Trizol reagent (Invitrogen). Reverse transcription and real-time PCR experiments were performed using a High Capacity cDNA Reverse Transcription Kit (Promega, Madison, WI) and SYBR® Green PCR kit, respectively (Invitrogen). The following primers were used to amplify the various ALDH isozymes: ALDH1: 5′-TCCTGGTTATGGGCTTACAG-3′ (forward), 5′-CTGGCCCTGGTGTAGAATA-3′ (reverse); GAPDH: 5′-GACAGTTAGCCGATCTTCTT-3′ (forward), 5′-TTAAAAGCAGCCCTGGTGAC-3′ (reverse).

Western blotting

The cells were lysed and then harvested using a cell lifter (Corning Costar). Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were then separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Western blotting was performed using

Abbreviations:

ALDH1: aldehyde dehydrogenase 1
ATRA: all-trans retinoic acid
CSC: cancer stem-like cell
DEAB: diethylaminobenzaldehyde
EOC: epithelial ovarian cancer

[3,5-difluorophenyl]acetyl-L-alanyl-2-phenyl-1,1-dimethylpy ester-glycine; Cayman Chemical, Ann Arbor, MI) dissolved in dimethyl sulfoxide, was used to test the effect of Notch signalling blockade. The FoxM1 inhibitor thioasteron and ATRA were purchased from Sigma (Sigma, St Louis, MO).

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the following antibodies at the indicated dilutions: anti-ALDH1 (1:1000; BD Biosciences), anti-Oct4 (1:1000; BD Biosciences), anti-Nanog (1:1000; BD Biosciences), anti-Notch1 (1:1000; Abcam, Cambridge, UK), anti-FoxM1 (1:1000; Santa Cruz Biotec, Santa Cruz, CA) and anti-beta actin (1:5000; Sigma).

Immunohistochemical staining

The paraffin-embedded sections (5 μm thick) were placed on silane-coated slides and processed for immunohistochemistry. Immunohistochemical staining was performed on deparaffinized tissue sections of formalin-fixed materials after microwave-enhanced epitope retrieval, based on the standard automated immunohistochemical procedure (Ventana XT autostainer; Ventana Medical Systems, Tucson, AZ). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The slides were incubated with primary mouse monoclonal antibody against ALDH1 (clone 44/ALDH, 1:200 dilution; BD Biosciences, San Jose, CA) for 60 min at room temperature. The anti-ALDH1 antibody was located using a cocktail of horseradish peroxidase-labelled secondary antibodies (the ultraView Universal DAB Detection Kit), containing biotin-free reagents to eliminate biotin background staining and to optimize specificity. The complex was then visualized with hydrogen peroxide substrate, 3, 3′-diaminobenzidine tetrahydrochloride chromogen and hematoxylin II (Ventana) counterstain. A negative control was established by replacing the primary antibody with phosphate-buffered saline. Normal hepatic cells were used as positive controls.

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences, Version 17.0 for Windows (SPSS). Values in this study are represented as the means ± standard deviation. Student’s t-test and analysis of variance with Tukey’s post-hoc test were used to test for differences between two groups and multiple comparisons, respectively. P < 0.05 (two-sided) was considered significant.

Results

ALDH1 regulates stemness in ovarian cancer cells

To evaluate whether the manipulation of ALDH1 activity can alter stemness and tumour formation in vivo, we enriched the

![Figure 1](image_url)

**Figure 1.** ALDH1 regulates stemness and tumourigenic ability in ovarian cancer cells. (A) ALDH1-Low and ALDH1-High cells were stained with an Aldefluor® assay kit and separated with a FACSAria cell sorter. A total of 1×10^3 A2780 (upper panel) and CP70 (lower panel) cells were seeded into a 96-well plate to evaluate the cells’ sphere formation ability (scale bar = 200 μm). DEAB was used to inhibit the reaction of ALDH1 and also served as a negative control. (B) Western blotting was performed to evaluate ALDH1, FoxM1, Notch1, Oct-4 and Nanog expression. β-actin was used as a protein loading control. The histogram represents the relative fold change of protein expression (right panel). (C) The tumourigenic ability of A2780-Low and A2780-High cells was assessed by inoculating 1×10^3 cells into NOD/SCID mice. (D) A2780 cells were transfected with the ALDH1 overexpression plasmid (A2780-ALDH1), and CP70 cells were transfected with the shALDH1 plasmid (CP70-shALDH1). A2780-ALDH1 (upper panel) and CP70-shALDH1 (lower panel) cells were seeded into 96-well plates to evaluate their sphere formation ability (scale bar = 200 μm). (E) The expression of CSC markers was analysed by western blotting, and the quantitative analysis is shown (right panel). (F) The tumourigenic ability of A2780-ALDH1 and CP70-shALDH1 cells was assessed by inoculating 1×10^3 cells into NOD/SCID mice. Student’s t-test was used for statistical analyses (*P < 0.05).
endogenous population of cells expressing high ALDH1 activity by flow cytometry. Cells with high or low ALDH1 activity were termed 'ALDH1-High' or 'ALDH1-Low'. The sphere formation efficiency of ALDH1-High A2780 or CP70 cells was significantly higher than that of their ALDH1-Low counterparts (Figure 1A). DEAB, an ALDH1 activity inhibitor, suppressed sphere formation in ALDH1-High cells. The expression of CSC marker proteins such as FoxM1, Notch1, Oct 4 and Nanog was also inhibited by DEAB treatment (Figure 1B). Moreover, DEAB treatment did not affect cell proliferation in these cells (Supplementary Figure 2A, available at Carcinogenesis Online). We then tested in vivo tumourigenesis by inoculating $1 \times 10^3$ cells subcutaneously into the mice.

Figure 2. ALDH1 regulates stemness and tumourigenesis through the FoxM1 and Notch1 signalling pathways. (A) A2780, CP70, CP70-vector and CP70-shALDH1 cells were treated with a FoxM1 inhibitor (1 µM Thiostrepton) or a Notch1 inhibitor (10 µM DAPT) and were then evaluated in sphere formation assays. Representative images show spheres generated from single-cell cultures after 10 days. The lower panel depicts the relative sphere formation ratio (scale bar = 200 µm). (B) The ALDH1, FoxM1 and Notch-1 expression levels were evaluated by western blotting and the relative fold changes in expression are also shown. (C) The sphere formation ability of CP70-shFoxM1 (clones 1 and 2) and A2780-FoxM1 (clones 1 and 2) cells treated with or without DAPT (scale bar = 200 µm), and protein expression analysed by western blotting are shown in (D). V: vector. Student’s t-test was used for statistical analyses (* $P < 0.05$).
and we found that A2780-High cells had accelerated tumour formation ability compared with the A2780-Low cells (Figure 1C). We then developed A2780-ALDH1 and CP70-shALDH1 cell lines that were stably transfected with ALDH1 and sh-ALDH1 complementary DNA. The ALDH1 RNA and protein levels in the aforementioned cells were highly correlated with the ALDH1 activity as measured by the Aldefluor® assay (Supplementary Figure 3, available at Carcinogenesis Online). The overexpression of ALDH1 resulting from transfection increased sphere formation efficiency (Figure 1D, upper panel), induced upregulated expression of CSC markers (Figure 1E) and enhanced tumour formation in a mouse xenograft model (Figure 1F, upper panel). In contrast, ALDH1 silencing decreased sphere formation (Figure 1D, lower panel), induced downregulation of CSC markers (Figure 1E) and suppressed tumour formation (Figure 1F, lower panel and Supplementary Figure 4, available at Carcinogenesis Online). Collectively, our results indicate that ALDH1 contributes to stemness and tumourigenic ability in ovarian cancer cells.

FoxM1 and Notch1 are involved in the regulation of ALDH1-mediated stemness

FoxM1 and Notch1 signalling have been reported to play a role in the biology of ovarian CSCs and to be involved in the pathophysiology of ovarian cancer (34). Because the expression levels of FoxM1 and Notch1 are concordant with that of ALDH1 (Figure 1B and E), we tested whether FoxM1 and Notch1 signalling are involved in ALDH1-mediated stemness. As shown in Figure 2A, sphere formation was decreased in shALDH1 compared with control cells. FoxM1 and Notch1 inhibition by Thiotrepton and DAPT decreased sphere formation efficiency in control and shALDH1 cells. DEAB downregulated the expression levels of both FoxM1 and Notch1 (Figure 2B and E), whereas Thiotrepton and DAPT did not affect ALDH1 expression (Figure 2B). DAPT treatment did not affect proliferation of these cells. Thiotrepton at 1 mM induced slight toxicity in A2780 and CP70-shALDH1 cells, but the effect was not significant (Supplementary Figure 2B, available at Carcinogenesis Online). This finding indicated that ALDH1 regulates stemness through downstream FoxM1 and Notch1 signalling. Similar to the results observed in A2780 and CP70 cells, Thiotrepton reduced the expression not only of FoxM1 but also of Notch1, while DAPT decreased Notch1 expression as well as FoxM1 expression, but to a lesser extent (Figure 2B). To further clarify the interaction between FoxM1 and Notch1 in ALDH1-regulated signalling, we generated CP70-shFoxM1 and A2780-FoxM1 cells for further analysis. FoxM1 silencing reduced sphere formation, whereas overexpression of FoxM1 increased sphere formation activity (Figure 2C). In contrast to the results shown in Figure 2B, immunoblot analysis showed that DAPT failed to attenuate FoxM1 expression in FoxM1-overexpressing cells (Figure 2D).

The antitumour effect of ATRA in ovarian cancer cells

After pretreatment with 10 µM ATRA for 28 days, ALDH1, FoxM1 and Notch1 expression (Figure 3A), ALDH1 activity (Figure 3B), sphere formation ability (Figure 3C) and cell migration and invasion abilities (Figure 3D and E) were significantly reduced in A2780-High and CP70-High cells. ATRA treatment did not affect proliferation of these cells (Figure 3F).

We then inoculated 1 × 10⁴ ATRA-pretreated A2780-High or CP70-High cells into mice. Tumour formation ability was almost completely abrogated in A2780-High cells, and a significant reduction of tumour size was also observed in CP70-High-inoculated mice (Figure 3G). Immunostaining further confirmed that ATRA reduced ALDH1 expression in tumour cells (Figure 3H). ATRA treatment decreased the proportion of ALDH1-High ovarian cancer cells (Figure 3B and I); this finding is in contrast to the increased proportion of ALDH1-High cells resulting from treatment with chemotherapeutic agents such as paclitaxel (Figure 3I).

Similarly, Thiotrepton and DAPT decreased sphere formation in ES2-High cells in a dose-dependent manner (Figure 4A). Thiotrepton and DAPT downregulated the expression levels of both FoxM1 and Notch1 in ES2-High cells in a dose-dependent manner, but did not affect ALDH1 expression (Figure 4B). However, these findings were not observed in ES2-Low cells (Figure 4A and B). ATRA inhibited sphere formation ability (Figure 4C), ALDH1, FoxM1 and Notch1 expression (Figure 4D) and cell migration ability (Figure 4E) in ES2-High cells. ATRA treatment did not affect proliferation of these cells (Figure 4F).

Antitumour efficacy of ATRA in mouse xenografts

Cell suspensions of 1 × 10⁶ A2780-High or CP70-High cells were inoculated subcutaneously into mice. ATRA (0.05 or 0.1 mg) was injected into the peritoneal cavity three times per week, as illustrated in Figure 5A. ATRA treatment inhibited A2780-High tumour growth in a dose-dependent manner and significantly suppressed CP70-High tumour growth at 22 days after tumour cell inoculation (Figure 5B). ALDH1 expression in tumour cells from tissue sections was downregulated in ATRA-treated mice compared with control mice (Figure 5C). These results further confirmed that ATRA can target ALDH1 and reduce the oncogenic potential of ALDH1-abundant cells.

Discussion

In this study, we investigated the importance of ALDH1 and the therapeutic role of ATRA in ovarian cancer cells. The principal finding of our study was that ALDH1 is a key player in regulating stemness and tumour formation in ovarian cancer cells; this regulation occurs through the downstream signalling of FoxM1/Notch1. In addition, ATRA downregulates ALDH1/FoxM1/Notch1 signalling and suppresses sphere formation ability, cell migration and invasion and tumourigenesis.

ALDH1 is regarded as a CSC marker in several human malignancies. However, the mechanisms and signalling pathways underlying its biologic effects in cancer cells and CSCs remain unclear. Studies of ovarian cancer have demonstrated that ALDH1 alone may or may not act as an ovarian CSC marker (35–37). In addition, conflicting results have been obtained regarding the prognostic impact of ALDH1 in EOC patients (14–19). Here, we demonstrated that ALDH1 is not only a stem cell marker but also directly regulates the functions of ovarian cancer cells. ALDH1 expression was closely associated with tumourigenic potential in various ovarian cancer cell lines (Table 1), and FoxM1 and Notch1 were found to be important downstream effectors for ALDH1-regulated cancer stemness in ovarian cancer cells.

FoxM1 affects the expression and function of a variety of genes that are critical to cell proliferation and survival, invasion, angiogenesis, and self-renewal of cancer stem cells (38,39). Genome-wide gene expression profiling of cancers has identified FoxM1 as one of the most commonly overexpressed genes in solid tumours (40,41). Importantly, its expression is often correlated with poor prognosis and resistance to chemotherapy (42). FoxM1 has also been reported to induce human epithelial stem/progenitor cell expansion and to be required for maintenance of the pluripotency of embryonic carcinoma cells (43,44). The
only known mechanism by which FoxM1 can regulate stemness involves cross-talk between FoxM1 and β-catenin in glioma stem cells (45). Recent reports have also implicated the importance of Notch signalling in maintaining CSC properties as well as in conferring resistance to chemotherapy. Our results indicate the involvement of Notch and FoxM1 signalling in ALDH1-mediated stemness and tumour behaviour, consistent with findings showing that Notch and FoxM1 signalling are involved in the pathophysiology of ovarian cancer (34). Regarding the interaction between Notch and FoxM1, Thiostrepton suppressed the expression of FoxM1 and Notch1, and the Notch1 inhibitor DAPT suppressed FoxM1 expression in addition to that of Notch1 (Figures 2B and 4B). Thiostrepton has been shown to inhibit FoxM1 and Notch1 activity; combined with our results, this suggests that the downregulation of Notch by DAPT inhibited FoxM1 expression, which is in agreement with findings in prostatic cancer cells (46). However, in FoxM1-overexpressing A2780 cells, upregulated expression of Notch was also observed.

Figure 3. ATRA treatment reduces ALDH1 expression, sphere formation ability, cell migration and invasion, and tumour growth in ovarian cancer cells. (A) Cells were treated with ATRA (10 µM) for 28 days, and cell lysates were collected for western blotting. The quantitative analysis is shown in the right panel. (B) ALDH1 enzyme activity was detected using an Aldefluor® assay kit. (C) Changes in sphere formation ability in response to 10 µM ATRA treatment in A2780 (upper panel) and CP70 (lower panel) cells (scale bar = 200 µm). (D) Representative images of migrating cells treated with or without ATRA in a Transwell assay. The histogram shows the number of migrated cells (scale bar = 100 µm). (E) Representative images of invading cells treated with or without ATRA in a Transwell assay. The histogram shows the number of invading cells (scale bar = 100 µm). (F) The cell doubling time was measured using an MTT cytotoxicity assay after treatment with or without ATRA. (G) ALDH1-High cells (1 × 10^4) were pretreated with vehicle (DMSO) or ATRA (10 µM) for 28 days, and cells were collected for tumourigenesis analysis using a xenograft model. N.D.: not detected. (H) ALDH1 immunohistochemical analysis of tumour tissue sections shows the downregulation of ALDH1 expression after ATRA pretreatment. (I) A2780-Low and CP70-Low cells were treated with DMSO, ATRA (10 µM), or paclitaxel (60 ng/ml) for 4 days. The effect of ATRA and paclitaxel treatments on the percentage of ALDH1-High cells is shown. Student’s t-test was used for statistical analyses (*P < 0.05).
and DAPT inhibited FoxM1-mediated sphere formation but did not suppress FoxM1 protein expression (Figure 2C and D). This suggests that FoxM1 regulates Notch expression. These conflicting results might be due to cell type specificity or differences between experimental settings. Further studies should be conducted to elucidate the precise cross-talk between FoxM1 and Notch1 in ALDH1-mediated biofunctions.

One limitation of this study is that only two cell types were evaluated. EOC is a highly heterogeneous disease that consists of four major histologic subtypes: serous, endometrioid, clear cell and mucinous carcinoma. In this study, we used A2780 (endometrioid) and ES2 (clear cell) cell lines to investigate the role of ALDH1 in the regulation of stemness and tumorigenic properties. The role of ALDH1 in other cell types requires further evaluation.

Retinol is oxidized into retinaldehyde by alcohol dehydrogenase, after which it can be oxidized into RA by ALDH1 (47). RA and its derivatives are involved in many critical physiological processes, including the regulation of gene expression, morphogenesis and development (48). ATRA has been used to treat hematologic cancers through its ability to induce cancer cell differentiation (49). However, its therapeutic role in solid tumours is currently under investigation in brain, kidney and lung cancers (50). In addition to ATRA's known ability to promote cell differentiation in leukaemia, in this study we identified a novel role of ATRA for inhibition of stemness via ALDH1-regulated signalling in ovarian cancer. ATRA treatment decreases the proportion of ALDH1-positive cancer cells; this result implies that ATRA can target the stem-like ALDH1-positive cell population. This finding is in contrast to the known tendency of chemotherapeutic agents, such as paclitaxel, to target the non-stem-like ALDH1-negative cell population; paclitaxel treatment thus increases the proportion of ALDH1-positive CSCs. The antitumour effect of ATRA, achieved by targeting the self-renewal pathways (ALDH1/FoxM1/Notch1) of ovarian cancer cells, indicates that ATRA might have therapeutic applications via inhibition of tumour behaviour in ALDH1-expressing cancer cells or CSCs. Our findings also implicate the involvement of FoxM1/Notch1, further suggesting
Figure 5. ATRA inhibits tumour growth in NOD/SCID mice. (A) Flowchart of ATRA treatment in a mouse xenograft model. Cell suspensions of $1 \times 10^6$ A2780-High or CP70-High cells were inoculated subcutaneously into mice. (B) Representative tumours and tumour volumes in ALDH1-High A2780 or CP70 cells. Student’s *t*-test was used for statistical analyses (*P < 0.05). (C) Immunohistochemical staining for ALDH1 in tumour tissue sections (scale bar = 100 µm) (D) Model depicting ALDH1 as a mediator of ATRA-induced tumour growth suppression in ovarian cancer cells.

Table 1. Correlation between ALDH1 expression and tumourigenic potential in ovarian cancer cells

<table>
<thead>
<tr>
<th>No. of cell injected</th>
<th>Number of tumours/number of injections (average tumour volume, mm$^3$)</th>
<th>Tumour-initiating cell frequency (95% confidence interval)</th>
</tr>
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<tbody>
<tr>
<td>A2780</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>A2780-vector</td>
<td>0/4 (0)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>A2780-ALDH1</td>
<td>4/5 (1910)</td>
<td>1/1 (1479)</td>
</tr>
<tr>
<td>A2780-Low</td>
<td>0/5 (0)</td>
<td>2/2 (1002)</td>
</tr>
<tr>
<td>A2780-High</td>
<td>5/5 (1980)</td>
<td>8/9 (1115)</td>
</tr>
<tr>
<td>A2780-High-ATRA</td>
<td>4/9 (109)</td>
<td>2/2 (1400)</td>
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<tr>
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<tr>
<td>CP70-High-ATRA</td>
<td>4/5 (545)</td>
<td></td>
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</tbody>
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*The frequency of ovarian-initiating cells was calculated by uploading the data into the web-based ELDA (Extreme Limiting Dilution Analysis) statistical software at http://bioinf.wehi.edu.au/software/elda/index.html.
that the inhibition of the ALDH1/FoxM1/Notch1 signalling pathways by ATRA or other agents might provide new opportunities for therapeutic intervention.

**Supplementary material**

Supplementary Figures 1–4 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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Conflict of Interest Statement: None declared.

**References**
