Targeting estrogen receptor subtypes (ERα and ERβ) with selective ER modulators in ovarian cancer

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Abstract

Ovarian cancer cells express both estrogen receptor α (ERα) and ERβ, and hormonal therapy is an attractive treatment option because of its relatively few side effects. However, estrogen was previously shown to have opposite effects in tumors expressing ERα compared with ERβ, indicating that the two receptor subtypes may have opposing effects. This may explain the modest response to nonselective estrogen inhibition in clinical practice. In this study, we aimed to investigate the effect of selectively targeting each ER subtype on ovarian cancer growth. Ovarian cancer cell lines SKOV3 and OV2008, expressing both ER subtypes, were treated with highly selective ER modulators. Sodium 3′-(1-(phenylaminocarbonyl)-3,4-tetrazolium)-*bis*(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay revealed that treatment with 1,3-*bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) (ERα antagonist) or 2,3-*bis*(4-hydroxy-phenyl)-propionitrile (DPN) (ERβ agonist) significantly suppressed cell growth in both cell lines. In contrast, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (ERα agonist) or 4-[2-phenyl-5,7-*bis* (trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP) (ERβ antagonist) significantly enhanced cell growth. These results were confirmed on a xenograft model where SKOV3 cells were injected s.c. into ovariectomized mice. We observed that the average size of xenografts in both the DPN-treated group and the MPP-treated group was significantly smaller than that for the vehicle-treated group. In addition, we found that phospho-AKT expressions in SKOV3 cells were reduced by 80% after treatment with MPP and DPN, indicating that the AKT pathway was involved. The combined treatment with MPP and DPN had a synergistic effect in suppressing ovarian cancer cell growth. Our findings indicate that targeting ER subtypes may enhance the response to hormonal treatment in women with ovarian cancer.

Key Words
- estrogen receptors
- SERMS
- ovarian cancer
- hormonal treatment

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Introduction

Ovarian cancer is the fifth commonest cancer in women in western countries (Siegel *et al*. 2011). Primary treatment largely consists of cytoreductive surgery followed by adjuvant chemotherapy. However, despite optimal treatment, recurrences are common and the overall prognosis is poor. A number of second-line chemotherapy regimes

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are available, achieving an overall response rate of about 20–30%, while producing significant side effects. Hormonal therapy would be an attractive treatment option because of its minimal side effects and relative ease of administration. Theoretically, ovarian cancer is a hormone-sensitive tumor. Previous studies have shown that the expression of estrogen receptors (ERα and ERβ) was detectable in 60–100% of ovarian cases and estrogen was shown to increase tumor cell proliferation in ovarian cancer cell lines (Lindgren *et al.* 2004, De Stefano *et al.* 2011). However, tamoxifen, a well-established selective ER modulator (SERM) for treatment of breast cancer, produces only a modest response rate of 10–15% in ovarian cancer (Perez-Gracia & Carrasco 2002). One of the key issues is how to improve its effectiveness. For many years, estrogen has been known to act through ERs. In 1996, a new ER (ERβ) was discovered and was found to be genetically distinct from the classical ER (ERα) (Kuiper *et al.* 1996). Since then, ERβ has been found in most estrogen-target tissues such as the prostate (Weihua *et al.* 2001) and the ovary (Brandenberger *et al.* 1998). Reduced levels of ERβ mRNA expression were found in malignant tissues compared with normal tissues in various estrogen-dependent tumors such as breast, prostate, and ovarian cancers (Iwao *et al.* 2000, Horvath *et al.* 2001, Skliris *et al.* 2003, Chan *et al.* 2008), indicating that the loss of ERβ expression may be involved in carcinogenesis. This is further supported by the findings that ectopic expression of ERβ in breast, prostate, and ovarian cancer cells inhibits motility and cell invasion and leads to increased apoptosis (Lazennec *et al.* 2001, Cheng *et al.* 2004). In breast cancer cells, estrogen increased cell proliferation in the presence of ERα but in the presence of ERβ it inhibited proliferation (Strom *et al.* 2004). As the activation of each subtype may lead to opposing effects, the overall effect may be compromised by the relative expression of the ER subtypes in individual tumors. In this study, we explored the effects of estradiol (E_2), 4-hydroxytamoxifen (4-OH Tam), a SERMs, and fulvestrant (Ful), a pure estrogen antagonist, on cell proliferation after individually knocking down each receptor subtype in two ovarian cancer cell lines that express both receptor subtypes. In addition, we also attempted to explore the possibility of improving hormonal response by using highly selective agonists and antagonists that only bind to one subtype. We treated ovarian cancer cells with highly selective ERα and ERβ agonists and antagonists and determined their effects on cell growth. We then attempted to confirm these findings using *in vivo* nude mice model and elucidate the molecular pathway involved.

Materials and methods

Chemicals

The SERMs 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), 1,3-*bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), 2,3-*bis*(4-hydroxy-phenyl)-propionitrile (DPN), 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP), and Ful (ICI 187 280) were purchased from Tocris Bioscience (Bristol, UK). E_2 and 4-OH Tam were purchased from Sigma–Aldrich.

Cell culture

The ovarian epithelial cancer cell lines OV2008, C13, A2780S, A2780CP (gifts from Prof. B K Tsang, Department of Obstetrics and Gynaecology, University of Ottawa), SKOV3, and OV326 and the breast cancer cell lines MCF7 and T47D (purchased from the American Type Cell Collection, Rockville, MD, USA) were used in this study (Sasaki *et al.* 2000, Asselin *et al.* 2001). Authentication of the cell lines was carried out by short tandem repeat profiling to confirm the cell of origin. Cells were cultured in MEM medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Gibco). Four days before the experiment, the cultures were switched to phenol-red-free MEM medium (Gibco) supplemented with 10% charcoal–dextran-treated fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics. All cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were routinely subcultured when they reached 80% confluency.

Cell proliferation assay

Cells were plated at an initial concentration of 1200 cells/well of 96-well plate in full medium or phenol-red-free medium. The next day, cells were washed with PBS and then subjected to treatment with various concentrations of SERMs for 96 h. Cell proliferation was measured every 24 h using sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay (Roche Diagnostics) according to the manufacturer's protocol. Briefly, XTT labeling mixture was prepared by mixing XTT solution (1 mg/ml) with electron coupling reagent at a ratio of 50:1. Culture medium was discarded and cells were washed with PBS. About 100 μl PBS and 50 μl XTT labeling mixture were added to each

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well and the 96-well plate was incubated at 37 °C for 4 h. Mitochondrial enzymes in living cells cleave the yellow XTT salt to give water-soluble orange formazan. After incubation at 37 °C, the amount of the orange formazan was quantified using an Infinite 200 Microplate Reader at 492 nm (Tecan Group Ltd, Männedorf, Switzerland).

Quantitative real-time PCR

Total RNA of cells was isolated with TRIzol reagent according to the manufacturer's protocol (Invitrogen). cDNA was then synthesized from 1 μg of total RNA by High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Quantitative real-time PCR was carried out using Applied Biosystems TaqMan system (ABI Prism 7500, Applied Biosystems). Specific primers (ERα-forward, AGGTGCCCTACTACCTGGAGAAC; ERα-reverse, GGTGGCTGGACACATATAGTCGTT and ERβ-forward, AAGAGCTGCCAGGCCTGCC; ERβ-reverse, GCGCACTGGGGCGGCTGATCA) and specific probes (ERα, CGCCGGCATTCTACAGGCCAAA and ERβ, CTCACCCTCCTGGAGGCTGAGCCGC) were employed for the measurement of mRNA expressions of ERα and ERβ. Expression of TBP mRNA was used as an internal control. A standard curve was constructed with three tenfold dilutions of plasmid DNAs of TBP, ERα, and ERβ for the determination of absolute copy number of TBP, ERα, and ERβ respectively. The copy number of ERα and ERβ was calculated as the number of copies of ERα and ERβ per copy of TBP. The experiments were conducted in duplicates.

Gene-silencing experiment

SKOV3 and OV2008 cells were plated in a six-well plate at the density of 5×10^5 cells/well in phenol-red free medium and were transfected with either negative control (NTC) siRNA, ERα-targeting siRNAs (siRNA1, ACATCATCTCGGTTCCGCA and siRNA2, CAGGCACATGAGTAACAAA), and/or ERβ-targeting siRNAs (siRNA1, AGTGTACAATCGATAAAAA and siRNA2, CCTTACCTGTAAACAGAGA) (Ambion, Applied Biosystems) using lipofectamine reagent (Invitrogen). Twenty-four hours after transfection, cells were reseeded into a 96-well plate for cell viability assay. Cells were plated at 2000 cells/well in a 96-well plate. The next day, cells were washed with PBS and then subjected to treatment with DMSO, E_2, 4-OH Tam, Ful, DPN, or MPP for 48 h. Cell viability was accessed by XTT assay. Cell lysates were also collected for the detection of ERs by western blot. The experiments were conducted in duplicates.

Nude mice assay

A group of 4-week-old female BALB/c nude mice were anesthetized and bilaterally ovariectomized. SKOV3 cells were harvested and resuspended in matrix gel (Matrigel, BD BioSciences, San Jose, CA, USA). Cells (5×10^6) were inoculated s.c. into the right flank of the ovariectomized mice. Mice bearing xenografts of around 5 mm diameter were divided randomly into three groups of five mice each. Each group was treated with a vehicle solution, DPN (1 mg/kg per day) or MPP (1 mg/kg per day), by an i.p. injection everyday. Tumor size was monitored every 2 days by measuring the largest and smallest diameters of the tumor and estimated according to the following formula: volume $=1/2\times$(largest diameter)$\times$(smallest diameter)^2. This experiment was carried out following the Animals (Control of Experiments) Ordinance (Hong Kong) and the Institute's guidance on animal experiments.

Western blotting

For the examination of AKT activity, ovarian cancer cells (SKOV3) treated with vehicle solution DMSO, MPP (100 pM), DPN (10 nM), or a combination of MPP and DPN at different time points (0, 4, and 24 h) were collected using a cell scraper and washed in cold PBS. To examine the expression of estrogen-responsive genes, SKOV3 cells were treated with DMSO, MPP (100 pM), DPN (10 nM), or a combination of MPP (100 pM) and DPN (10 nM) in the absence or presence of E_2 (10 nM) for 24 h. Collected cells were lysed with NET lysis buffer containing 0.2% NP40, 10 μg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Proteins were analyzed by western blotting using antibodies including anti-ERα (sc-8002, Santa Cruz), anti--ERβ (sc-8974, Santa Cruz), AKT, p-AKT (Ser473) (Cell Signaling, Danvers, MA, USA), cyclin D1 (Santa Cruz), HER2 (Epitomics, Inc., Burlingame, CA, USA), p21 (Santa Cruz), EGFR (Cell Signaling), FBLN-1 (Santa Cruz), and HRP-conjugated secondary antibody, and detected with ECL. Total cell lysates were also blotted with β-actin (Sigma) or GAPDH (Sigma) antibodies as a loading control. Experiments were carried out at least twice to confirm the results.

Statistical analysis

The Prism Software Package (GraphPad Software, Inc., San Diego, CA, USA) was utilized for statistical analysis. For the XTT assay, data are expressed as optical density and are mean ± S.D. of determinations from a single

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experiment that was repeated three times. The results, whenever applicable, were analyzed by two-tailed Student's *t*-test to determine if significant differences ($P<0.05$) had been observed.

Results

Conventional hormonal treatment of with E_2, 4-OH Tam, and Ful mainly acts via ERα in ovarian cancer cell lines

Western blotting was carried out to evaluate the protein expressions of ERα and ERβ. We confirmed that SKOV3 and OV2008 cells expressed both ERα and ERβ proteins (Fig. 1A). Since the sensitivity of specific antibodies might affect the band intensity in western blotting, the results obtained might not reflect the protein abundance for ERα and ERβ expression. We then evaluated the actual copy number of ERα and ERβ by quantitative real-time PCR in SKOV3 and OV2008 cells. We found that the copy number of ERα after normalization with the internal control gene TBP was 401 and 101 in SKOV3 and OV2008 respectively (Fig. 1B). The copy number of ERβ was seven in both SKOV3 and OV2008. Therefore, the ERα:ERβ ratio of SKOV3 is 57:1 and that of OV2008 is 14:1.

We investigated the effective dose of E_2, 4-OH TAM, and Ful on SKOV3 cell proliferation by XTT assay with various dosages of hormonal drugs. We found that 10 nM of E_2 significantly increased cell proliferation ($P<0.001$, Student's *t*-test) while both 4-OH Tam ($P<0.05$, Student's *t*-test) and Ful ($P<0.01$, Student's *t*-test) reduced cell proliferation at the minimum dosages of 100 nM (Fig. 1C). We then examined cell proliferation with the same dosages of E_2, 4-OH Tam, and Ful in OV2008 (Fig. 1D). Similar results were obtained. These results indicated that both OV2008 and SKOV3 responded to conventional hormonal drug action. To investigate whether the hormonal drugs exerted their action through ERs, SKOV3 cells were then transfected with negative control siRNA (NTC), ERα-targeting siRNAs, or ERβ-targeting siRNAs in order to knockdown the expression of individual receptor subtype. Expressions of ERα and ERβ were evaluated by western blot analysis after siRNA transfection and the band intensity was quantified using a densitometer. ERα and ERβ siRNAs profoundly reduced ERα and ERβ expression respectively by ~70 and 90% in SKOV3 cells relative to the control (NTC) siRNA and nontransfected cell (−ve) (Fig. 2A and B). To determine the effect of these drugs in the absence of ERα subtype, cells transfected with ERα siRNAs were treated with E_2, 4-OH Tam, and Ful. The stimulatory effect of E_2 on cell growth was significantly abolished after ERα silencing (Fig. 2C, $P=0.0044$, Student's *t*-test). The inhibitory effects of 4-OH Tam (Fig. 2D, $P=0.0152$, Student's *t*-test) and Ful were significantly abolished (Fig. 2E, $P=0.0023$, Student's *t*-test). The effects of these drugs were then investigated in the absence of ERβ in cells transfected with ERβ siRNAs. The stimulatory effect of E_2 on cell growth remained unchanged (Fig. 2C) while the inhibitory effects of 4-OH Tam and Ful were slightly reduced in ERβ-silenced cells (Fig. 2D and E). These results indicated that the effects of E_2, 4-OH Tam, and Ful were mainly mediated via ERα.

The effects of highly selective SERM treatment on cell growth in ovarian cancer cell lines

We first examined the effects of highly selective SERMs on cell growth of ovarian cancer cells with ERα and ERβ expression in normal MEM medium supplemented with 10% fetal bovine serum. SKOV3 cells were treated with different dosages of SERMs and the effects on cell growth were evaluated by XTT assay. We found that 100 pM of PPT (ERα agonist, $P<0.02$, Student's *t*-test) and PHTPP (ERβ antagonist, $P<0.01$, Student's *t*-test) significantly increased cell proliferation while both MPP (ERα antagonist, $P<0.02$, Student's *t*-test) and DPN (ERβ agonist, $P<0.004$, Student's *t*-test) significantly reduced cell proliferation at the minimum dose of 100 pM and 10 nM respectively (Fig. 3A). In addition, to test whether these SERMs are effective in the absence of ERα and ERβ expression, cell proliferation of ovarian cancer cell line A2780S (with no ERα and ERβ expression) was also examined with SERM treatment under the same condition. Notably, no significant stimulatory or inhibitory effect on cell proliferation was observed after SERM treatment (Fig. 3B). These results indicated that SERMs are only effective in ERα- and ERβ-expressing cell lines. To examine whether the effects of SERMs were also valid in media with reduced levels of hormones, SKOV3 was then cultured in phenol-red-free MEM medium (Gibco) supplemented with 10% charcoal–dextran-treated fetal bovine serum before the experiment. Cell proliferation was evaluated with or without SERM treatment in SKOV3. XTT assay revealed that treatments with MPP or DPN also significantly suppressed growth of the cells. Furthermore, treatment with PPT or PHTPP significantly enhanced ovarian cancer cell growth in SKOV3 cells (Fig. 4A). These results indicated that SERMs exert their stimulatory or inhibitory effects even under very low levels of hormones in ovarian cancer cells. To confirm that the effects of SERMs were not cell-line-specific, another ovarian cancer cell line OV2008, expressing both ERα and ERβ, was chosen for

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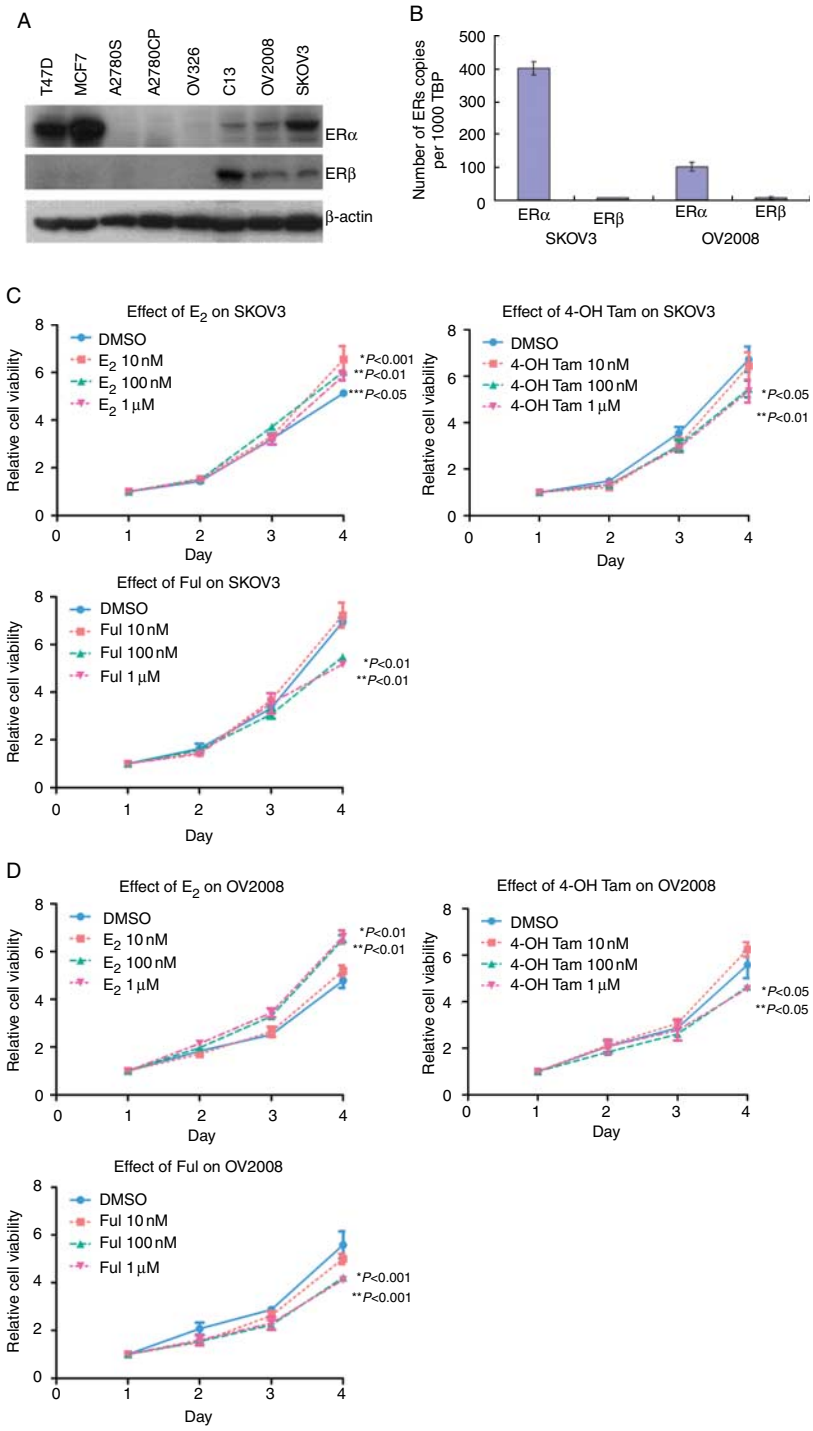

Figure 1

(A) Protein expression of ERα and ERβ in breast cancer and ovarian cancer cell lines was examined by western blot. (B) The mRNA expression of ERα and ERβ was examined in SKOV3 and OV2008 by quantitative real-time PCR. (C) Effects of different dosages of E_2, 4-hydroxytamoxifen (4-OH Tam), and fulvestrant on cell growth of SKOV3 cells. Cells were treated with different dosages of E_2 (10 nM, 100 nM, and 1 μM), 4-OH Tam (10 nM, 100 nM, and 1 μM), or fulvestrant (10 nM, 100 nM, and 1 μM) and the cell viability (normalized to day 1) was measured by XTT assay for 4 days. (D) The effects of different dosages of E_2, 4-OH Tam, and fulvestrant on cell growth of OV2008 cells. A full colour version of this paper is available at http://dx.doi.org/10.1530/JOE-13-0500

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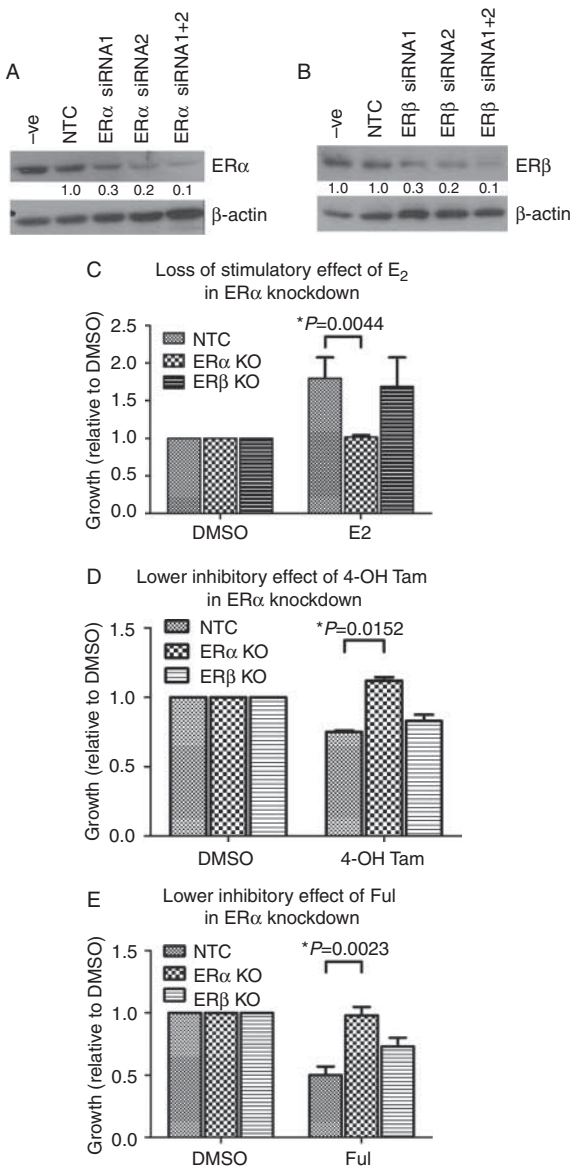

Figure 2
Effect of estrogen receptor status on drug efficacy. SKOV3 cells were transfected with negative control (NTC) siRNA, ERα- or ERβ-targeting siRNA and treated with drugs for 48 h. siRNA1 and siRNA2 are two different siRNAs targeting the same gene. The expression of (A) ERα and (B) ERβ was confirmed by western blot using anti-ERα and anti-ERβ antibodies 72 h after siRNA transfection respectively. The values below the blots represent the change in protein expressions of the siRNA-transfected cells normalized to the expression in the NTC siRNA-transfected cells. After siRNA transfection, the cell viability of SKOV3 cells was measured by XTT assay after treatment with (C) E_2 (100 nM), (D) 4-hydroxytamoxifen (100 nM), and (E) fulvestrant (100 nM) for 48 h. Growth was compared with the mock-treated controls (DMSO).

further investigation in phenol-red-free MEM medium (Gibco) supplemented with 10% charcoal–dextran-treated fetal bovine serum. Similar effects of SERMs on cell proliferation were observed in OV2008 cells (Fig. 4B).

The suppressive effects of MPP and DPN treatment are mediated by ERs

In order to determine if the effects of highly selective SERM treatment were mediated by the particular ERs, we employed siRNA specifically targeting ERs in SKOV3 cells. After siRNA transfection, the cell viability assay was carried out to evaluate the effects of SERMs on cell growth in ERα- or ERβ-silenced cells. We found that knockdown of ERα significantly reduced the response to MPP (Fig. 4C, $P<0.05$, Student's *t*-test) and showed higher cell viability as compared with NTC siRNA-transfected cells even with MPP treatment. Also, knockdown of ERβ also significantly diminished the suppressive effect of DPN on cell growth (Fig. 4D, $P<0.05$, Student's *t*-test). These results implied that the inhibition of ovarian cancer cell growth by MPP or DPN was regulated by targeting to their specific ERs.

MPP and DPN treatments reduce *in vivo* tumor cell growth in mice

By *in vitro* XTT assay, we observed a drastic effect on cell growth for MPP and DPN. Next, we sought to investigate whether MPP and DPN exhibit the same effect *in vivo*. We established a xenograft model by s.c. injection of SKOV3 cell into ovariectomized mice. Mice bearing xenografts of 5 mm diameter received vehicle solution, MPP (1 mg/kg per day) or DPN (1 mg/kg per day), by an i.p. injection. We observed that the average size of xenografts in both the MPP-treated group ($P<0.0001$, Wilcoxon's rank-sum test) and the DPN-treated group ($P=0.0005$, Wilcoxon's rank-sum test) was significantly smaller than that for the vehicle-treated group (Fig. 5A). These results indicated that MPP and DPN also exert an inhibitory effect on ovarian cancer growth *in vivo*.

Combined treatment with MPP and DPN synergistically suppress ovarian cancer cell growth

To see whether the combination of MPP and DPN could enhance the inhibitory effects on ovarian cancer cell growth compared with single-SERM treatment, we carried out the XTT assay on SKOV3 treated with DMSO (control), MPP alone (100 pM), DPN alone (10 nM), and MPP (10 pM)+DPN (10 pM). As expected, the growth rate was inhibited following either MPP or DPN single treatment. Combined treatment with MMP and DPN showed greater growth inhibition than either treatment alone. In addition, lower concentrations of each SERM were required to produce the inhibitory effect (Fig. 5B). The result implied

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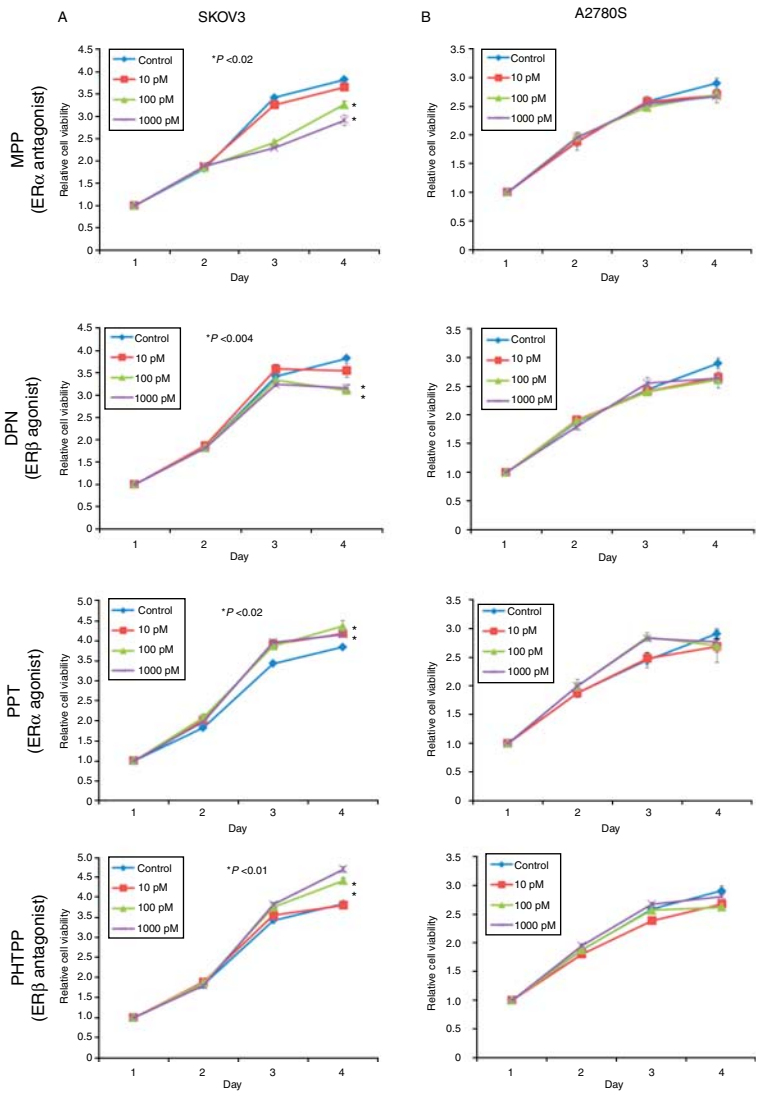

Figure 3

(A) Effects of selective estrogen receptor modulators (SERMs) on cell growth in ERα- and ERβ-positive ovarian cancer cell line SKOV3 in normal MEM medium supplemented with 10% fetal bovine serum. (B) Effects of SERMs on cell growth in ERα- and ERβ-negative ovarian cancer cell line A2780S in normal MEM medium supplemented with 10% fetal bovine serum. Cells were treated with different dosages of SERMs and the cell viability (normalized to day 1) was measured by XTT assay for 4 days. Values are means ± S.D., $n=3$. Means with (*) are significantly higher or lower than those for the control culture. A full colour version of this paper is available at http://dx.doi.org/10.1530/JOE-13-0500

that combined treatment with MPP and DPN enhanced the inhibitory effect on ovarian cancer cell growth.

MPP and DPN treatment alter the expression of estrogen-responsive genes

To examine the effects of SERM treatment on the expression of estrogen-responsive genes, the protein expressions of cyclin D1, HER2, p21, EGFR, and FBLN-1 were examined by western blot. We found that two of the examined genes EGFR and FBLN-1 showed altered protein expression in SERMs-treated cells in the presence of E_2. In vehicle-treated SKOV3 cells, the addition of E_2 induced the expression of EGFR and FBLN-1 by 2.1- and 9.8-fold respectively (Fig. 6). The addition of MPP, DPN, or combined SERM treatment (MPP+DPN) suppressed the expressions of EGFR and FBLN-1 induced by E_2 (Fig. 6). No observable alterations were found in the protein expressions of cyclin D1, HER2, and p21 in E_2- and SERM-treated cells.

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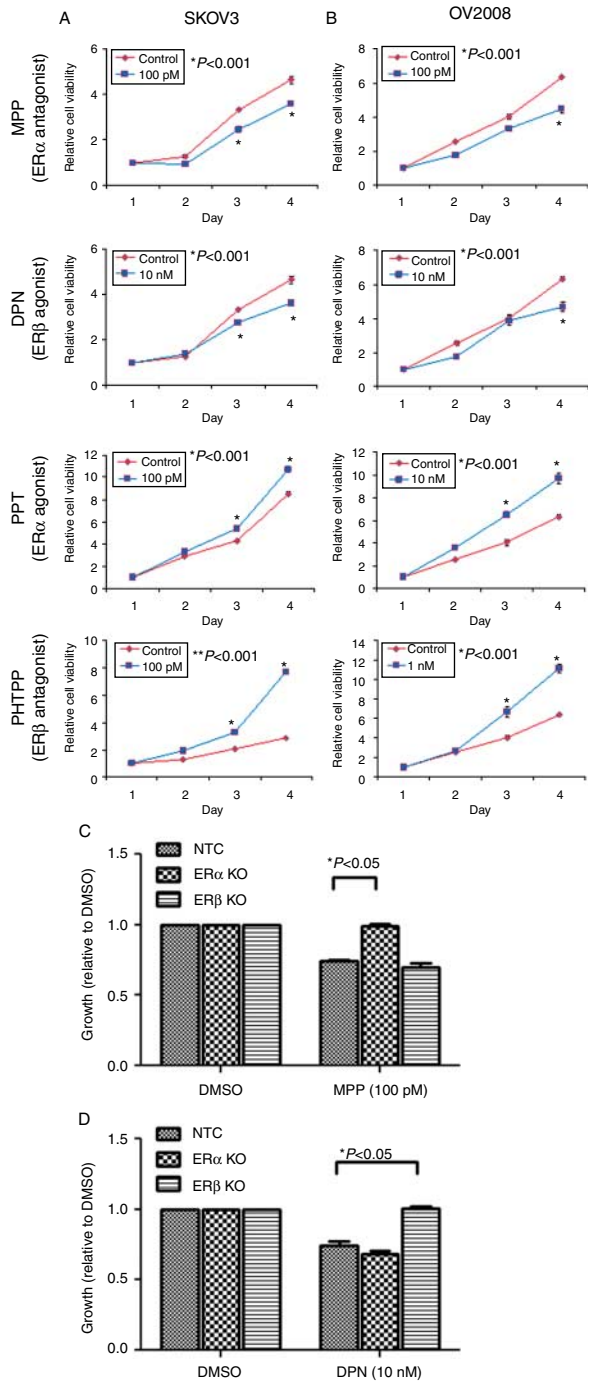

Figure 4
Effects of SERMs on cell growth in (A) SKOV3 and (B) OV2008 in phenol-red-free MEM medium supplemented with 10% charcoal–dextran-treated fetal bovine serum. Cells were treated with indicated concentrations of SERMs and the cell viability (normalized to day 1) was measured by XTT assay for 4 days. (C and D) Inhibition of cell growth by SERMs is mediated through ERs. SKOV3 cells were transfected with negative control (NTC) siRNA, ERα- or ERβ-targeting siRNA and then treated with MPP (100 pM) or DPN (10 nM) for 48 h. Cell viability of SKOV3 cells was measured by XTT assay after treatment. Growth was compared with the mock-treated controls (DMSO). A full colour version of this paper is available at http://dx.doi.org/10.1530/JOE-13-0500

MPP and DPN suppress AKT activity in SKOV3 cells

To test whether ERs mediate nongenomic pathway in ovarian cancer cells, SKOV3 was treated with vehicle solution (DMSO), MPP, DPN, or MPP+DPN at different time points (0, 4, and 24 h). We investigated whether treatment of SERMs on ERs involved the inactivation of the AKT signaling pathway. Treatment with SERMs suppressed p-AKT expression without modifying the levels of total AKT (Fig. 7). The level of p-AKT expression was reduced by 20 and 80% after treatment with MPP for 4 and 24 h respectively. Similar observations were found in DPN-treated SKOV3 cells. DPN treatment reduced p-AKT expression by 10% (4 h) and 80% (24 h). Notably, reductions of 20% (4 h) and 90% (24 h) of p-AKT expression were detected with combined MPP and DPN treatment. No obvious alteration was observed in vehicle-solution-treated cells after 24 h. As the phosphorylation of serine 473 in AKT is an activating phosphorylation of the AKT protein, these results indicated that the AKT activity in SKOV3 cells was suppressed by MPP and DPN.

Discussion

With about 60% of tumors expressing ERs, ovarian cancer was believed to be a hormone-responsive tumor. The initial findings showed that estrogen enhances cell proliferation while tamoxifen, an antiestrogen, was shown to suppress tumor cell proliferation. Tamoxifen has been in clinical use in the treatment of ovarian cancer for over 15 years. However, the overall response rate was only about 10% (Williams *et al.* 2010). These initial findings had not taken into account the presence of ER subtypes. Both ERα and ERβ were found to be present in both normal ovarian tissues and ovarian cancer tissues, with a possible reduction of ERβ expression as tumors progress (Bardin *et al.* 2004*a*, Chan *et al.* 2008). The loss of ERβ expression indicates that ERβ exerts tumor-suppressive functions and may have a protective role. Similar findings were reported in other hormone-responsive tissues such as breast, colon, and prostate (Skliris *et al.* 2003, Bardin *et al.* 2004*b*). In this study, we first attempted to confirm the previously reported effects of E_2 and 4-OH Tam in two ovarian cancer cell lines, SKOV3 and OV2008, which express both ERα and ERβ. Receptor subtype status of SKOV3 had been controversial in the literature. SKOV3 was reported to be ERα-negative and ERβ-positive (Treeck *et al.* 2006) and it had also been described as estrogen-unresponsive because it expresses a dysfunctional ERα and very low levels of ERβ (Jones *et al.* 1994, Lau *et al.* 1999).

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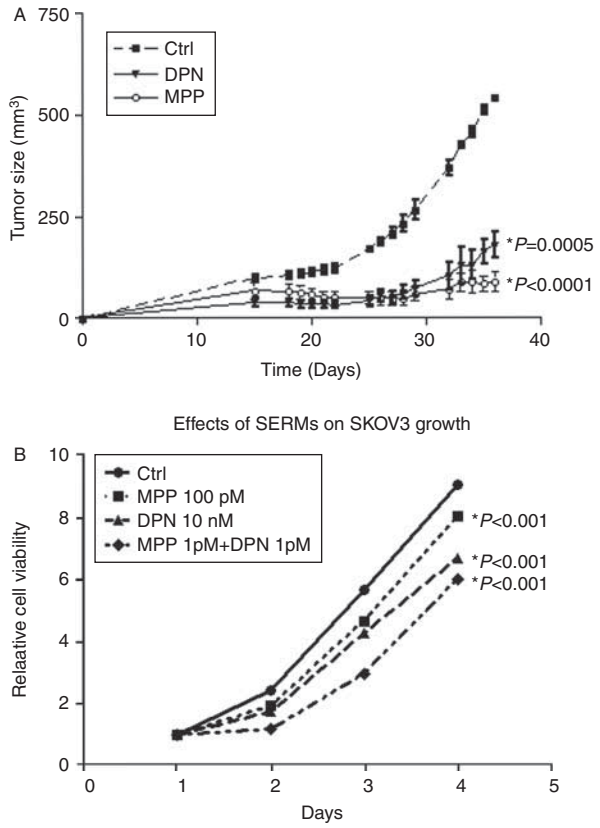

Figure 5
(A) Ovariectomized mice received s.c. injections of SKOV3 cells. Drug treatments began after the tumors were around 5 mm in diameter. Animals with established tumors were treated with DPN (1 mg/kg per day) or MPP (1 mg/kg per day) by an i.p. injection everyday for 5 weeks. Tumor size was monitored every 2 days by measuring the largest and smallest diameters of tumor. Tumor size was compared between groups at various time points. (B) Cells were treated with MPP (100 pM), DPN (10 nM), or a combination of MPP (10 pM) and DPN (10 pM). The cell viability (normalized to day 1) was measured by XTT assay for 4 days. The combination of MPP and DPN synergistically suppressed cell growth of SKOV3 cells.

However, we demonstrated by western blotting that SKOV3 expressed both receptors, although the expression of ERα was higher than that of ERβ in the ratio of 57:1 by quantitative real-time PCR, which is compatible with the findings of O'Donnell *et al.* (2005) and we demonstrated that this cell line was responsive to estrogen. The use of a cell line that has high ERα:ERβ ratio has the advantage that it may better reflect the clinical situation where ERβ expression is lower than that of ERα in ovarian cancer tissue samples. Nonetheless, it also has the disadvantage that it is difficult to completely isolate the effects of individual receptor subtypes by siRNA gene-silencing experiments because it is not possible to achieve a 100% knockdown rate.

Our findings confirmed that E_2 stimulated- while 4-OH Tam inhibited ovarian cancer cell growth in these two cell lines as seen in previous reports (Langdon *et al.* 1990, 1994). Our initial gene-silencing experiment results also agreed with previous reports that the action of estrogen and 4-OH Tam was mainly via ERα (O'Donnell *et al.* 2005). In addition, our results also indicated that Ful also mainly acted via ERα. However, the apparent lack of effect from ERβ in the gene-silencing experiments maybe due to the incomplete knockdown of ERβ. With the knockdown rate of about 80% in a cell line SKOV3 with a 57:1 ERα:ERβ ratio, even when 80% of ERα is knocked down, the final effect may still be a result of both receptor subtypes.

In order to circumvent this issue, we used highly selective agonists and antagonists for each receptor subtype and we found that ERβ activation led to inhibition of ovarian cancer cell growth in SKOV3. This agrees with previous findings where the overexpression of ERβ1 ligand was associated with antitumoral effects on ovarian cancer cells (Treeck *et al.* 2007). In order to show that these results are not cell-line-specific, we repeated the experiment with another ovarian cancer cell line OV2008 where there was a ERα:ERβ ratio of 14:1. Even with this very low relative ratio of ERβ, the inhibitory effect of ERβ agonist was still evident. A previous report showed that DPN did not produce any effect on cell proliferation until it reached a high concentration of 1 μM where it might start to cross-react with ERα (O'Donnell *et al.* 2005). In our experiments, however, we found that even at a concentration as low as 10 nM, DPN produced significant growth inhibition and the effect appeared to be dose-dependent. Furthermore, gene silencing with siRNAs for individual receptor subtypes confirmed that these effects were mediated via

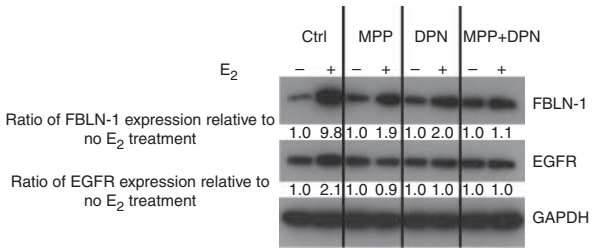

Figure 6
Protein expressions of estrogen-responsive genes in SERM-treated ovarian cancer cells. SKOV3 cells were treated with vehicle or SERMs in the absence or presence of E_2 (10 nM). Western blotting was carried out to examine the expression levels of EGFR and FBLN-1 before and after SERM treatment. Relative expression of protein bands was evaluated by densitometric analysis. The values below the blots represent the change in protein expressions of the E_2-treated cells normalized to the vehicle-solution-treated cells. GAPDH was probed as a loading control.

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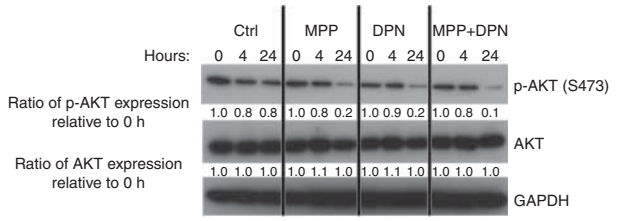

Figure 7
Western blotting with SERM treatment on ovarian cancer cells. SKOV3 cells were treated with vehicle solution (DMSO), DPN, or MPP for different times (0, 4, and 24 h). Expression of AKT and p-AKT (S473) was evaluated by western blotting. GAPDH was probed as a loading control. Relative expression of protein bands was evaluated by densitometric analysis. The values below the blots represent the change in protein expressions with treatment at 4 or 24 h normalized to 0 h.

the respective receptors, without significant cross-reaction with the other subtype.

The molecular pathways involved in the activation of ERα are well reported but the pathways involved in ERβ activation are much less well established (Drummond & Fuller 2010). Therefore in this study, we also attempted to elucidate the molecular pathways involved. Both genomic and nongenomic pathways are known to mediate the effects of ERα and we anticipate that these pathways would also be involved in the activation of ERβ. We evaluated the effect of SERM treatment in regulating gene transcription of estrogen-responsive genes. Expressions of EGFR and FLBN-1 were increased in E_2-treated SKOV3 cells. Addition of MPP, DPN, or a combination of MPP and DPN suppressed the protein expressions of EGFR and FBLN-1 induced by E_2. Our result indicated that SERM treatment of ovarian cancer cells might also be involved in the regulation of transcription of estrogen-responsive genes.

In addition, we also investigated the effects of SERM treatment on the nongenomic pathway. As AKT mediates a variety of cellular responses and plays a central role in oncogenic transformation by blocking apoptosis and stimulating cell growth, we attempted to examine whether binding of ERα antagonist and ERβ agonist could also suppress the phosphorylation of AKT in ovarian cancer cells expressing ERs. We found that suppression of ERα by MPP or stimulation of ERβ by DPN treatment dramatically suppressed the expression of p-AKT in SKOV3 cells. With the role of AKT in activating cell growth, the suppression of p-AKT expression by MPP or DPN treatment may contribute to the inhibition of cell proliferation of ovarian cancer cells. Involvement of other nongenomic pathways and the upstream and downstream targets should be further explored in a subsequent study.

Apart from ovarian cancer, ERβ has been implicated in the carcinogenesis of various other malignancies including prostate cancer (Attia & Ederveen 2012), colorectal cancer (Castiglione *et al.* 2008), and cholangiocarcinoma (Marzioni *et al.* 2012). The development of very selective ERβ agonists has been very promising in recent years (Mohler *et al.* 2010). There is increasing evidence in recent years that ERβ may have a tumor suppressor function in different tumors. In breast cancer, ERβ agonist was found to arrest angiogenesis and tumor growth in mouse xenograft models (Hartman *et al.* 2006). Selective ERβ agonists have also been shown to reduce hepatoma, gliomas, and medulloblastoma growth (Zhou *et al.* 2010, Mancuso *et al.* 2011, Sareddy *et al.* 2012). Each cell type has different cofactors and coregulators and the effect of receptor activation might be different and hence tissue-specific. The exact ERβ agonistic effects on ovarian tumors have not been reported, but previous studies have indicated a role for ERβ in ovarian cancer. Zhu *et al.* (2011) reported that reexpression of ERβ inhibits the proliferation of ovarian clear cell adenocarcinoma cells. Apart from its potential use in tumor suppression, the use of selective ERβ agonist has also been investigated in different diseases. ERβ agonist alleviated the chronic inflammatory pain state and was suggested to represent a novel way of addressing neuropathies (Gardell *et al.* 2008). Its anti-inflammatory effect may even attenuate atherogenesis (Sun *et al.* 2011). ERβ has also been implicated in brain function and aging (Foster 2012, Handa *et al.* 2012). It was shown to have anxiolytic and antidepressant effects, which may be useful in affective disorders (Hughes *et al.* 2008). ERβ agonist was also shown to improve survival in animal models of severe sepsis due to its protective effects on gastrointestinal barrier function (Cristofaro *et al.* 2006). Furthermore, a selective ERβ modulator, MF101, has been shown to reduce menopausal symptoms in phase 2 clinical trials (Grady *et al.* 2009, Stovall & Pinkerton 2009).

Our findings indicate that there may be a role for these highly selective SERMs in the treatment or prevention of ovarian cancer. Our previous study showed that ERβ expression in clinical samples decreased from normal tissue to borderline tumors to malignant tumors. The tumor-suppressor effects in ovarian cancer may be beneficial in various clinical situations. Its clinical effectiveness in reducing hot flushes in menopausal symptoms together with its tumor-suppressive effects in ovarian cancer may provide a safe option for 'hormone replacement therapy' in younger women who have undergone hysterectomy and bilateral oophorectomy for the treatment of ovarian cancer. The abundance of ERβ

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expression in borderline tumors may indicate that selective ERβ agonists could be a potential treatment option in borderline ovarian tumors, which are typically chemoresistant. In malignant ovarian clinical samples, especially at the advanced stage, ERβ expression is reduced. Nonetheless, we demonstrated a significant effect on reduction of cell proliferation in our cell lines where there was a very low ERα:ERβ ratio. This indicated that ERβ agonist may still be beneficial even in the presence of relatively low ERβ expression.

Optimizing the response to hormonal treatment may offer a good treatment option. Various strategies had been explored. Blockage of production of E_2 by blocking the aromatase or sulfatase pathways by aromatase inhibitors was tried but only with limited success (Papadimitriou *et al*. 2004). This may be partially explained by the intratumoral production of E_2 that can enhance tumor growth (Suzuki *et al*. 2008). Blocking the E_2 receptors would also counteract the intratumoral production, but the use of antiestrogens such as 4-OH Tam and Ful (Argenta *et al*. 2009) only showed a poor response. Targeting the ER subtypes by using an ERβ agonist with or without an ERα antagonist in women with known ER subtype status may offer a new option. The response may be further enhanced when combined with aromatase inhibitors. Future studies on animal models and the molecular mechanisms involved would be required to further explore this possibility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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