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Full Length Research paper

Differential induction of LRP16 by liganded and unliganded estrogen receptor α in SKOV3 ovarian carcinoma cells

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Previously, we investigated the induction effect of LRP16 expression by estrogen (E2) and established a feed-forward mechanism that activated ERα transactivation in estrogen-dependent epithelial cancer cells. LRP16 is required for ERα signaling transduction by functioning as an ERα coactivator. In this study, we demonstrated that LRP16 expression was up-regulated in E2-responsive BG-1 ovarian cancer cells, but was down-regulated in estrogen-resistant SKOV3 ovarian cancer cells. Pure estrogen antagonist ICI 182 780 did not affect LRP16 expression in SKOV3 cell. The unliganded ERα up-regulated LRP16 expression and enhanced LRP16 promoter activity in SKOV3 cells; however, this induction was blocked by estrogen stimulation. Results from chromatin immunoprecipitation experiment revealed a strong recruitment of the unliganded ERα at LRP16 promoter in the absence of estrogen; however, ERα was largely released from the DNA upon E2 stimulation. Although LRP16 did not significantly change the proliferation rate of SKOV3 cells, it seemed to slightly modulate the growth responsiveness of cells to E2. Knockdown of LRP16 by RNA interference in SKOV3 cells markedly attenuated estrogen response element-dependent ERα reporter gene activity and E2-induced c-myc expression. Our study suggests a novel mechanism of estrogen resistance of ovarian cancer by which estrogen-repressed signaling pathway antagonizes estrogen-activated signaling transduction.

Key words: LRP16, estrogen, estrogen receptor α, SKOV3.

INTRODUCTION

Estrogen plays a crucial role in the control of development, sexual behavior, and reproductive functions. Its effects have been linked to the onset and progression of gynaecological malignancies including breast cancer and endometrial cancer (Eliassen and Hankinson, 2008; Yager and Davidson, 2006; Shang, 2007). Estrogen, a major steroidal product of the ovary, has been also associated with increased ovarian cancer risk (Bai et al., 2000; Rodrigez et al., 2001; Riman et al., 2002). The biological effects of estrogens are mediated by two forms of estrogen receptor, ERα and ERβ.

Classically, ERα is activated by estrogen binding, which leads to receptor phosphorylation, dimerization, and recruitment of coactivators to the estrogen-bound receptor complex. This complex then binds promoter regions of target genes via direct interaction with DNA binding sites refereed to as estrogen response elements (ERE) and initiate transcriptional activity (McDonnell and Norris, 2002). Estrogen-bound ERα can also transactivate additional target genes through interacting with other transcriptional factors such as Ap1, Sp1 or nuclear factor κB (DeNardo et al., 2002; Safe, 2001; Shang and Brown, 2002). The activation of an estrogen receptor results in an altered expression of its direct transcriptional targets, thereby affecting downstream secondary biological activities. Estrogen regulation of protein expression has been well-documented in breast cancer models but to date little

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is known about estrogen-regulated gene expression in ovarian cancer.

Epithelial ovarian carcinoma, which represents about 90% of ovarian cancer (Auersperg et al., 2001), is one of the most frequently occurring cancers among women and the leading cause of gynecological cancer deaths (Boente et al., 1993; Greenlee et al., 2000). Approximately two-thirds of all ovarian cancers express ERα at the time of diagnosis (Slotman and Rao, 1998). The significance of estrogen in the etiology of ovarian carcinoma has been emphasized by the fact that anti-estrogenic intervention will inhibit the growth of ovarian carcinoma *in vivo* and *in vitro* (Langdon et al., 1990, 1994a), and that estrogen replacement therapy induces ovarian cancer (Gompel and Plu-Bureau, 2007; Zhou et al., 2008). Cell-based studies have shown that estrogen-driven growth of epithelial ovarian carcinoma is mediated by activation of ERα-mediated but not ERβ - mediated transcription (O'Donnell et al., 2005). Although estrogens are believed to be major regulators of growth in the development and progression of ovarian carcinoma, ERα-positive ovarian cancer is often unresponsive to estrogen and refractile to antiestrogen therapy (Smyth et al., 2007; Wagner et al., 2007). ERα signaling pathway in estrogen-resistant ovarian cancer cells is poorly understood. So, characterizing ERα mediated gene expression in estrogen-insensitive ovarian cancer cells might underlie the unresponsiveness of ovarian cancer to estrogen and resistance to hormonal therapy. The SKOV3 human ovarian carcinoma cells, which have functional ERα but are growth-resistant to estrogen and antiestrogens (Hua et al., 1995; Langdon et al., 1994b), were usually used as an *in vitro* model for estrogen and antiestrogen resistant ovarian cancer (Havrilesky et al., 2001; O'Donnell et al., 2005).

LRP16 was previously identified as an estrogen-responsive target gene. Estrogen-induced up-regulation of LRP16 expression is mediated by ERα, but not by ERβ (Han et al., 2003). In cell culture, it has been shown that the expression level of LRP16 is strongly dependent on the estrogen actions in ERα-positive breast cancer cell lines (Han et al., 2007; Meng et al., 2007). A proximal region of -676 to -24 bp of the human LRP16 promoter, in which a 1/2ERE/Sp1 site and multiple GC-rich elements that confer estrogen responsiveness have been recognized, is essential for estrogen action (Zhao et al., 2005; Han et al., 2008). Expression level of LRP16 was positively linked to the proliferation and invasion of the ERα-positive breast cancer and endometrial cancer cell lines and to the progression of primary breast cancers (Han et al., 2003, 2007; Liao et al., 2005; Meng et al., 2007). Importantly, estrogenically regulated LRP16 can interact with ERα and enhance the receptor's transcriptional activity in a ligand-dependent manner, by which LRP16 promotes proliferation of the estrogen-responsive human breast cancer cells (Han et al., 2007). Given that LRP16 acting as ERα target and its coactivator, characterizing the estrogen induction and the functional significance of LRP16 gene in estrogen-unresponsive epithelial ovarian cancer cells might be helpful to understand the mechanism of hormone therapy resistance of ovarian carcinoma.

In this study, we investigated the regulatory effects of estrogen, estrogen antagonist, and the unliganded-ERα on LRP16 gene expression and LRP16 gene promoter activity in SKOV3 human ovarian cancer cells, with the aim to determine whether LRP16 is an estrogen-responsive gene in estrogen-unresponsive SKOV3 ovarian cancer cells. We also surveyed the effect of LRP16 expression on ERα-mediated transcriptional activity by ERE-based reporter assay and the proliferation of SKOV3 cells to determine whether disruption of the ERα-LRP16 feed-forward pathway in estrogen-resistant ovarian cancer cells can change the cell response to estrogen.

MATERIALS AND METHODS

Chemicals and cell lines

17β-Estradiol (E2) was purchased from Sigma (St. Louis, MO, USA). Pure estrogen antagonist ICI 182780 was provided by Dr. Qinong Ye at the College of Military Medicine Scientific Institute of China. Human ovarian epithelial adenocarcinoma cell line SKOV-3 was originally purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and maintained as monolayer cultures in RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Utah, USA). BG-1 human ovarian epithelial cancer cell line was cultured as described previously (Geisinger et al., 1989). Steroid-deprived serum was prepared as described previously (Zhao et al., 2005).

Plasmids

The pGL3-Basic and pRL-SV40 were originally purchased from Promega (Madison, WI, USA). The pcDNA3.1-LRP16 expression vector containing human LRP16 full-length cDNA was constructed previously **Error! Bookmark not defined.**. Mammalian expression plasmid for ERα (pS5G-hERα was provided by Prof. Hajime Nawata at Kyushu University, and the reporter 3×ERE-TATA-*Luc* was provided by Prof. Donald P McDonnell at Duke University Medical Center (Norris et al., 1998). The luciferase reporter constructs pGL3-S_0, pGL3-S_2, pGL3-S_4, pGL3-S_5, pGL3-S_{B1}, containing the fragment of -2623 to -24 bp, -1775 to -24 bp, -1064 to -24 bp, -676 to -24 bp and -213 to -24 bp of the LRP16 upstream regulatory region, respectively, have been described previously (Zhao et al., 2005, Han et al., 2008).

Cell transfection

SKOV3 cells were seeded in 60 mm culture dishes before transfection. When the cell confluence reached to 40 - 60%, 5 μg of pcDNA3.1-LRP16 was stably transfected using the Superfect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The empty vector was used as a negative control. Two days post-transfection, the SKOV3 cells were treated with 1 mg/ml G418 (Gibco, New York, USA) for 10 - 14 d and then were continuously cultured with 400 μg/ml G418.

For siRNA transfection, SKOV3 cells were seeded in 60 mm culture dishes and grown to 80% confluence before transfection. SiRNA duplexes were transfected using Lipofectamine 2000 accor-

ding to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). SiRNA oligonucleotides were chemically synthesized by Shanghai GeneChem Co., Ltd (Shanghai, China). The sequences of LRP16-siRNA374 and LRP16-siRNA668 were reported previously (Han et al., 2007). The siRNA sequence against ERα was referred as previously described (Cheng et al., 2007). The unrelated siRNA sequence (sense strand, 5'-ttctccgaacgtgcacgt-3') was used as a control. The siRNA duplex was transfected in each dish with a final concentration of 50 nM.

Luciferase reporter assays

SKOV3 cells were cultured with RPMI-1640 supplemented with 5% steroid-deprived FBS for at least 3 days, then were plated in 35 mm culture dishes. Cells that had reached a 50% confluency rate were transiently cotransfected using Superfect reagent. 0.5 μg of luciferase reporters was cotransfected with or without 0.5 μg of ERα expression vector into cells. pRL-SV40 (10 ng), a renilla luciferase control vector, was added to each dish as an internal control to assess the transfection efficiency. The total DNA was adjusted to 2 μg/dish with pBSK + empty plasmid. 30 h after transfection, cells were treated with E2 (10^{-8} M) or dimethyl sulfoxide (DMSO) for an additional 12 h. The cells were lysed and harvested using the dual-luciferase reporter assay system. Luciferase activity was measured using TD-20/20^{n} Luminometry System (Promega). All experiments were performed in triplicate and repeated at least for three times.

Cell proliferation assay

A total of 1 x 10^4 viable cells were seeded in 24-well plates. After cell attachment, the medium was replaced with 1 ml of fresh RPMI 1640 supplemented with 1% steroid-stripped FBS and were treated with E2 (10^{-7} or 10^{-8} M/l) or DMSO in the same fresh medium and the medium was changed every 2 days. Cell number was counted by Trypan Blue exclusion method using a hemocytometer. Cell proliferation rate was quantified using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). Each experiment was performed in triplicate and repeated on three occasions.

RNA isolaton and northern analysis

Total RNA was isolated by the acid guanidium thiocyanate phenol chloroform method using Triblue reagent (Biotec Co., Beijing, China). The procedure of northern blot analysis was described previously (Han et al., 2007). Briefly, 20 μg of total RNA was electrophoresed through a 1% agrose gel containing formaldehyde and was transferred to a Hybond N$^+$ membrane (Amersham, Buckinghamshire, UK). The membranes were hybridized using the following probes labeled with [α-^{32}P] dCTP by random priming: 550 bp fragment of LRP16 (432 - 981 bp, NM_014067) and 515 bp of β-actin (457 to 971 bp, NM_001101).

Western blot and antibodies

The expression of LRP16, ERα and β-actin proteins were examined by western blot analysis as described previously (Han et al., 2007). Briefly, cell lysates were electrophoresed by SDS-PAGE using 12% acrylamide gels and blotted onto PVDF membranes (Amersham). Blots were probed with the primary antibodies, washed and then incubated with horseradish peroxidase-labeled secondary antibodies (Santa Cruz, CA, USA), and binding was detected using enhanced chemiluminesence. The rabbit anti-LRP16 antibody was used as described previously (Han et al., 2007). Rabbit anti-ERα, c-myc and β-actin were purchased from Santa Cruz.

Chromatin immunoprecipitation (ChIP) assays

SKOV3 cells (1 × 10^6) were grown in 10 cm tissue culture plates in RPMI 1640 supplemented with 10% steroid-depleted FBS. After 24 h, the cells were transfected with 10 μg of plasmid DNA mixture (1:1 for pGL3-S_5 and pS5G-hERα) using Superfect reagent. Forty hours later, the cells were treated with E2 (10^{-8} M/l) for 3 h. Chromatin immunoprecipitation (ChIP) assays were performed according to the protocol for the ChIP assay kit (Upstate Biology, NY, USA). Immunoprecipitation was carried out overnight at 4℃ with ERα (Santa Cruz) antibody or non-specific IgG. DNA fragments were purified with a QIAquick Spin Kit (Qiagen). The presence of the target gene promoter sequences in both the input and the recovered DNA immunocomplexes was detected by PCR. The proximal promoter (-476 to -241 bp) of LRP16 was amplified using the following primer set: forward primer, 5'-GCGCCAGGCTCTCCCAGCTCG -3', and reverse primer, 5'-CCCAGTGTCGCGGATGGAGC-3'.

Statistical analysis

Experiments were performed in triplicate, and the results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Statview software. All data were evaluated for paired variables to compare two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

LRP16 expression is up-regulated by E2 in BG-1 cells, but is down-regulated in SKOV3 cells

Previously published work from our laboratory demonstrated that E2 up-regulated the level of the human LRP16 gene through ERα activation in several E2-responsive human breast and endometrial cancer cells (Han et al., 2007; Meng et al., 2007). Here, to address whether LRP16 can be induced by E2 in ERα-positive human ovarian cancer BG-1 and SKOV3 cells, we performed northern blot analysis. As seen in Figure 1A, the mRNA expression level of LRP16 was up-regulated by E2 (10^{-8} M/l) in BG-1 cells as it was in MCF-7 and Ishikawa cells (Han et al., 2003; Meng et al., 2007). However, LRP16 expression was not up-regulated by E2 treatment; conversely, it was down-regulated by E2 in a dose-dependent fashion (Figure 1B). Comparing with the LRP16 expression level in cells cultured under normal conditions (Figure 1B, lane 6), a dramatic increase was observed in cells under steroid-deprived culture conditions (Figure 1B lane 1), indicating that the endogenous estrogen with much low concentration in culture medium is enough to suppress LRP16 expression to a larger extent after a long-term exposure of cells to it. To confirm that the LRP16 mRNA levels were indicative of protein levels, the expression level of LRP16 protein was examined in E2-treated SKOV3 cells by using western blot analysis and the results showed the consistent change with that observed at the mRNA level (Figure 1B). Next, we performed western blot analysis to determine the time course for the effect of E2 (10^{-8} M/l) on the expression level of LRP16 protein over a 72 h time period (Figure 1C). A more than

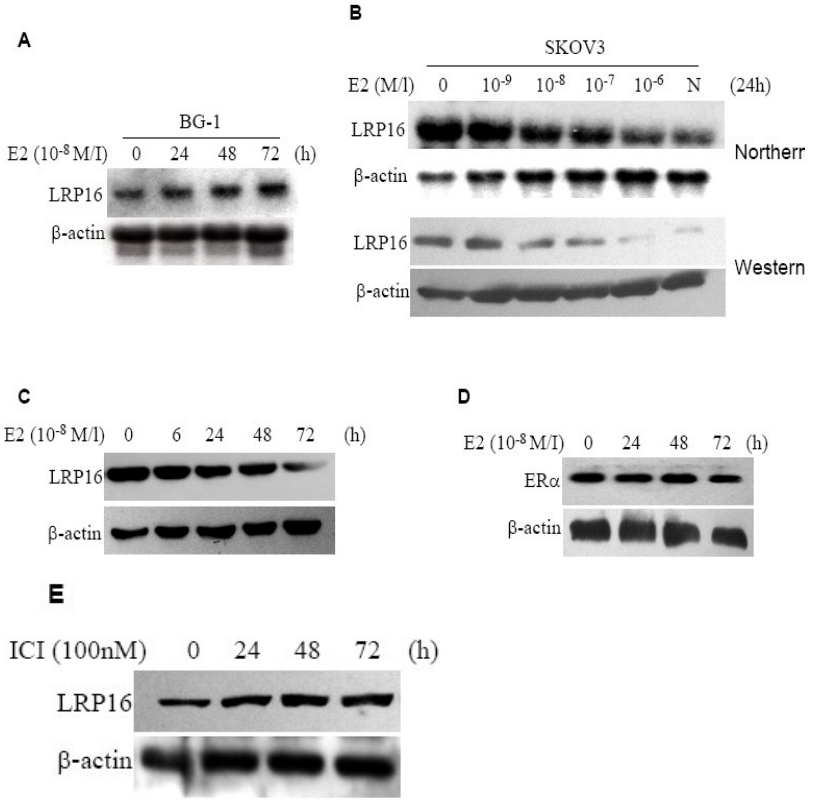

Figure 1. E2 regulation of LRP16 expression in BG-1 and SKOV3 cells.
A) BG-1 cells were cultured in medium containing steroid-stripped FBS for at least 3 days, and then were treated with E2 at 10^{-8} M/l for the indicated time points. The total RNA was extracted and the expression levels of LRP16 mRNA and β-actin were determined by northern blot analysis. **B)** SKOV3 cells were cultured in medium containing steroid-stripped FBS for at least 3 days, and then were treated with various concentrations of E2 from 10^{-9} to 10^{-6} M/l for 24 h. Northern and western blots were probed for LRP16 and β-actin. N, cells were cultured in routine culture medium. **(C, D)** SKOV3 cells were cultured in medium containing steroid-stripped FBS for at least 3 days, and then were treated with E2 at 10^{-8} M/l for the indicated time points. Immunoblots were probed for LRP16, ERα and β-actin. **(E)** SKOV3 cells were cultured in routine culture medium and were treated with ICI 182 780 for the indicated times. Immunoblots were probed for LRP16 and β-actin. These results shown in A to E are representatives of two independent experiments.

2-fold decrease in LRP16 protein level was observed as early as 6 h after addition of E2, and this was continued to 48 h time point. At 72 h time point after treatment of E2, the expression of LRP16 protein decreased to a much low level. To rule out the possibility that E2-induced LRP16 decrease resulted from altered ERα expression, we measured the ERα protein levels in E2-treated cells. As shown in Figure 1D, E2 treatment (10^{-8} M/l) did not significantly change the expression of ERα protein during the 72 h time course. These results demonstrated that LRP16 is not an estrogen up-regulated target gene in estrogen-insensitive SKOV3 ovarian carcinoma cells as it is in estrogen-responsive epithelial cancer cells, but is an estrogen down-regulated gene which exhibits a sensitive and continuous response for E2 treatment.

To assess the effect of anti-estrogen on the expression level of LRP16 in SKOV3 cells, the selective estrogen antagonist, ICI 182 780, was used to treat proliferating cells with the concentration of 100 nM. Total protein over a 72 h time course was extracted and western blot analysis was used to determine the protein level. As shown in Figure 1E, there was no significant increase of LRP16 protein level in cells observed after ICI 182 780 additions, indicating that anti-estrogen treatment can not effectively antagonize the E2 suppression of LRP16 expression in estrogen-resistant ovarian cancer cells.

Unliganded ERα up-regulates LRP16 gene expression/gene promoter activity in SKOV3 cells

To determine whether the inhibitory effect of LRP16 expression by E2 in SKOV3 cells is mediated by ERα, we

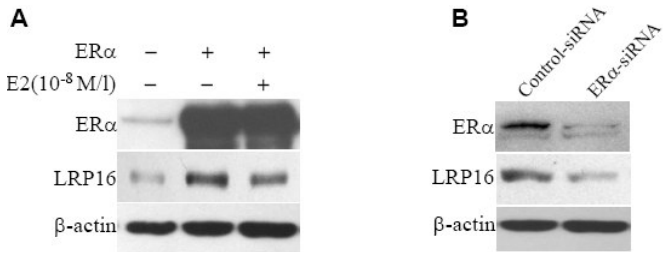

Figure 2. ERα regulation of LRP16 expression in SKOV3 cells. **A)** SKOV3 cells were first cultured in steroid-stripped medium for at least 3 days, and then were transiently transfected with ERα expression vector or empty vector. 36 h after transfection, cells were treated with or without E2 (10^{-8} M/l) and cultured for an additional 12 h. Immunoblots were probed for ERα, LRP16 and β-actin. **B)** SKOV3 cells were cultured in steroid-deprived medium for at least 3 days, and then were transfected with the ERα-specific siRNA or the control-siRNA oligonucleotides. 48 h after transfection, total protein was extracted and subjected to immunobloting analysis using the indicated antibodies. The results shown in A and B are representatives of two independent experiments.

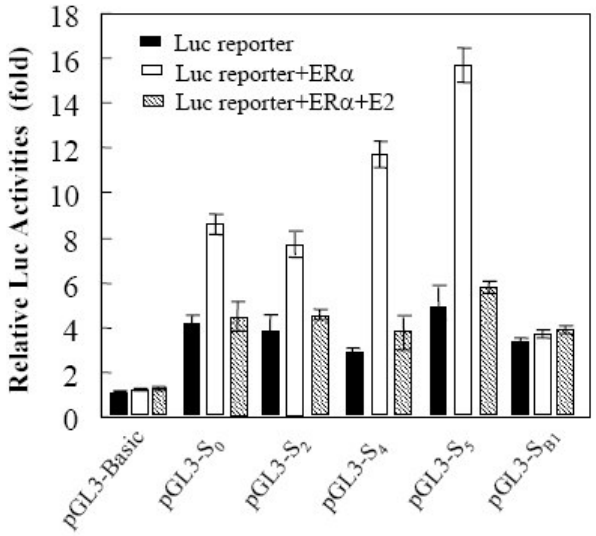

Figure 3. Unliganded and liganded ER□ regulation of LRP16 gene promoter activity in SKOV3 cells. SKOV3 cells were cultured in steroid-deprived medium for at least 3 days, and then were cotransfected with the indicated vectors. pRL-SV40 was also transfected to assess the transfection efficiency. 30 h after transfection, cells were treated with E2 (10^{-8}M) or dimethyl sulfoxide (DMSO) for an additional 12 h and underwent luciferase assay. The relative luciferase activity levels were normalized in all cases by mock effector transfection and arbitrarily assigned a value of 1. All experiments were performed in triplicate and were repeated at least three times; results are expressed as mean ± SEM.

investigated the induction effects of unliganded ERα on LRP16 expression by performing western blot analysis in ERα-transfected SKOV3 cells. As illustrated in Figure 2A, ERα protein level dramatically increased in ERα-transfected cells. The protein level of LRP16, but not β-actin, was markedly increased by the ectopic ERα expression in cells; however, this elevation was reduced by E2 treatment (Figure 2A). To further address the regulatory effect of unliganded ERα on LRP16 expression, we also measured the LRP16 expression in ERα -inhibited SKOV3 cells. Results from immunoboltting analysis showed that not only the endogenous ERα was largely repressed by ERα-specific siRNA transfection, but also the endogenous LRP16 (Figure 2B). These findings confirmed that the non-E2 bound ERα efficiently induces LRP16 expression in SKOV3 cells, but this induction can be blocked by E2 binding.

To further address the regulatory roles of liganded and unliganded ERα on the transcriptional activities of LRP16 gene in SKOV3 cells, we performed luciferase reporter assays with a series of LRP16 promoter-driving luciferase constructs. Transfection of SKOV3 cells with reporter alone revealed background luciferase activities (Figure 3). Cotransfection of ERα did not significantly change the luciferase activities for pGL3-S_{B1} and the control vector pGL3-Basic, but indeed resulted in a 2- to 4-fold increase of reporter gene activities for pGL3-S_0, pGL3-S_2, pGL3-S_4 and pGL3-S_5 constructs (Figure 3). However, the unliganded ERα activation of LRP16 promoter constructs was effectively blocked by E2 treatment (10^{-8} M/l). These findings further confirmed the distinct regulation of unliganded and liganded ERα on the transcriptional activity of LRP16 gene and suggested that this regulation may be mainly conferred by the fragment from -676 to -214 bp of LRP16 upstream region.

E2 inhibits the recruitment of ERα to LRP16 gene promoter

To analyze whether the increased promoter activity of LRP16 by unliganded ERα is the direct result of recruitment of ERα, we performed ChIP assays. The LRP16 promoter containing construct pGL3-S_5, which was authenticated to be activated by ERα in the absence of E2 as illustrated in Figure 3, together with ERα expression vector was cotransfected into SKOV3 cells. The cells were then treated with or without E2 (10^{-8} M/l) for 3 h, and the recruitment of ERα was analyzed by ChIP (Figure 4). In the absence of E2 treatment, we repeatedly detected a high level of ERα binding at the promoter of LRP16. However, ERα was apparently lost from the LRP16 promoter after estrogen treatment. As a control experiment, the fragment of -476 to -241 bp was not detected in the nonspecific IgG group. These results indicated that LRP16 is a primary target gene of ERα in SKOV3 cells.

LRP16 slightly modulates the growth responsiveness of SKOV3 cells to E2

Previous reports from our laboratory demonstrated that over-expression of LRP16 promotes proliferation of estro-

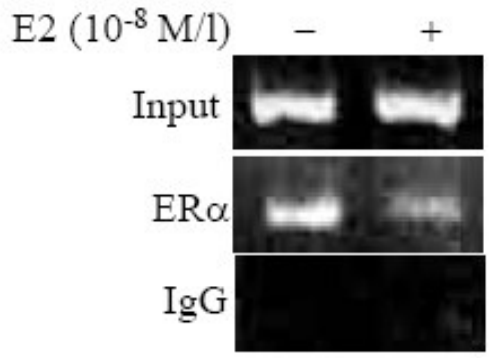

Figure 4. E2 inhibition of ERα recruitment to LRP16 gene promoter in SKOV3 cells. SKOV3 cells were transiently cotransfected with ERα and pGL3-S_5. 40 h after transfection, the cells were treated with E2 (10^{-8} M/l) or DMSO for 3 h and were subjected to immunoprecipitation and PCR as described in "Material and Methods". The representative results of repeated experiments are shown here.

gen sensitive MCF-7 breast cancer cells (Han et al., 2003). To investigate the effect of LRP16 on SKOV3 cell growth, we stably transfected pcDNA3.1-LRP16 or pcDNA3.1 empty vector into SKOV-3 cells and performed cell proliferation assays. After 10 to 14-day G418 screening, the drug-resistant clones appeared and were mixed for amplified culture. All of the parental cells were killed by G418 within this period. The expression level of LRP 16 was measured within 30 d after transfection by western blot analysis and the results showed that the ectopic transfection markedly increased the LRP16 expression (Figure 5A). Although LRP16 over-expression did not significantly give rise to alteration in proliferation of SKOV3 cells cultured without E2, it seemed to slightly promote cell growth in the presence of E2 (Figure 5B). Next, we transiently transfected LRP16 specific siRNAs or control-siRNA into SKOV3 cells to evaluate the effect of LRP16 knock down on cell proliferation. Compared with the control-siRNA, both LRP16-siRNA374 and LRP16-siRNA668 caused a specific reduction of LRP16 expression at protein level, but not change the expression level of the β-*actin* gene (Figure 6A). Consistent with our previous report (Han et al., 2007), LRP16-siRNA374 was reproducibly better than LRP16-siRNA668 and was used more frequently in later experiments. Results from cell proliferation assays showed that knock down of LRP16 slightly inhibited the proliferative rate of SKOV3 cells when the cells were treated with E2 (Figure 6B). By immunoblotting analysis, we observed that the endogenous LRP16 in SKOV3 cells still can be partially inhibited by LRP16-siRNA 374 at day 7 after transfection (Figure 6C). These findings suggested that LRP16 may slightly modulate the growth responsiveness of the estrogen-resistant ovarian cancer cells to estrogen.

LRP16 modulates ERα-mediated signaling transduction in SKOV3 cells

Although the growth of SKOV3 cells is insensitive to E2 stimulation, the normal estrogen regulation of an ERE-driven reporter gene activity and a few of ERα target genes such as c-myc and c-fos in E2-sensitive breast cancer cells can still be observed in SKOV3 cells (Hua et al., 1995). To ask whether LRP16 functions as an active ERα coactivator in SKOV3 cells as it does in estrogen-dependent ERα-positive epithelia cancer cells, we test the effect of LRP16 expression on ERα-mediated transcription by using 3xERE-TATA-*Luc* reporter construct. SKOV3 cells were cultured in steroid-stripped medium for at least 3 days, and then were cotransfected with 3xERE-TATA-*Luc*, ERα and LRP16-siRNA374, LRP16-siRNA668 or control-siRNA. As shown in Figure 7A, E2 (10^{-8} M/l) stimulation elicited a 2-fold increase of ERα-mediated reporter gene activity in control-siRNA expressing cells, which was in agreement with the previous report (Hua et al., 1995). However, the E2-activated reporter gene activity was markedly attenuated by LRP16-siRNA668 and LRP16-siRNA374 transfected cells. Next, we measured the E2 induction of c-myc protein in LRP16-inhibited SKOV3 cells. As illustrated in Figure 7B, E2 induced an increase of c-myc protein expression in control-siRNA expressing SKOV3 cells, which is consistent with that in MCF-7 cells as reported previously (Han et al., 2007); however, this induction was blocked by LRP16 knockdown. These results suggested that LRP16 is required for E2-stimulated ERα signaling transduction and inhibition of LRP16 expression can efficiently suppress ERα-mediated transcription activity and target gene expression.

DISCUSSION

The mechanisms underlying the estrogen-independent, antiestrogen-resistant ovarian cancer are poorly understood despite being a major problem in endocrine therapy. In estrogen-sensitive BG-1 and estrogen-insensitive SKOV3 ovarian carcinoma cells, we demonstrate the inverse regulation of LRP16 expression by E2. Consistent with our previous report (Han et al., 2007), E2 can induce LRP16 expression in estrogen-sensitive BG-1 cells, whereas LRP16 was repressed in SKOV3 cells. As a functioning ERα coactivator, decreased expression of LRP16 in SKOV3 cells is capable to attenuate estrogen-activated ERE-dependent reporter gene activity and gene expression such as c-myc. Our observations suggest an estrogen-repressed signaling pathway in estrogen-resistant ovarian cancer cells, which in turn antagonizes the "classical" ERα-activated signaling transduction. The antagonism between these two parallel estrogen signaling pathways underscores a novel mechanism of estrogen unresponsiveness of ovarian cancer.

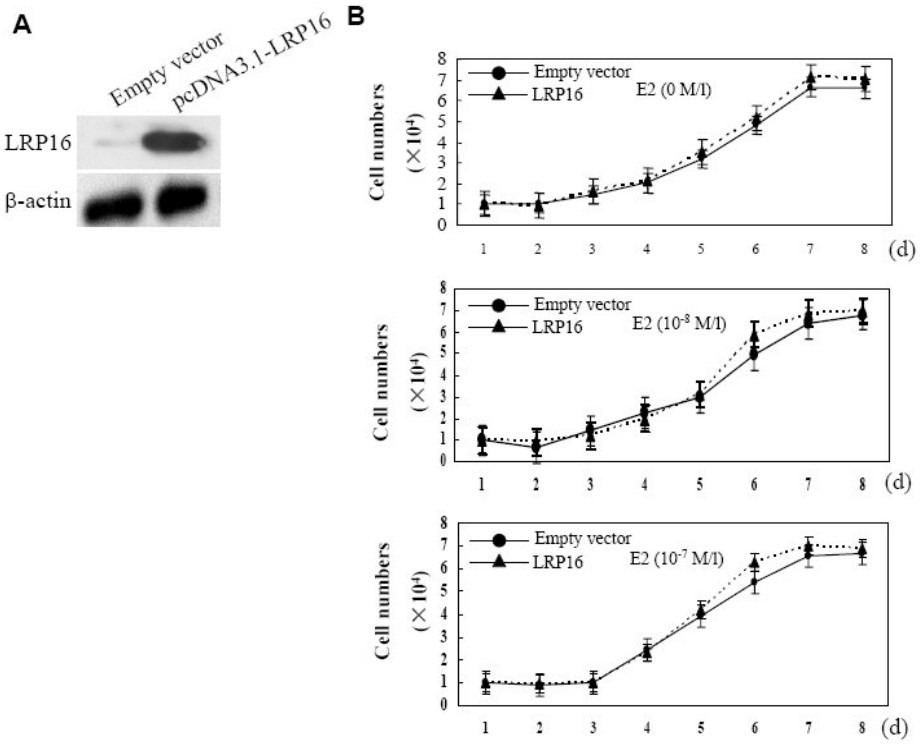

Figure 5. Effect of overexpression of LRP16 in SKOV3 cells on cell proliferation. **A)** SKOV3 cells were stably transfected with LRP16 or pcDNA3.1 empty vector. Immunoblots were probed for LRP16 and β-actin within 30 d after transfection. **B)** SKOV3 cells expressing ectopic LRP16 or empty vector were cultured in steroid-deprived medium for at least 3 days and treated with E2 (10^{-7} or 10^{-8} M/l) or DMSO. The cell number was determined by Trypan Blue exclusion method. Each data point represents the mean ± SEM number of cells counted in triplicate dishes.

Recently, it has been revealed that estrogen action is mediated by complex signaling pathways. Although it is believed that estrogen exerts most of its effects through direct activation of ER-regulated gene expression-this is called the genomic or classical action of ERα (Cheskis et al., 2007; Heldring et al., 2007), several lines of evidence demonstrated the existence of an estrogen-repressed signaling pathway in various estrogen target cells and it is possibly linked to the pathogenesis of some diseases (Cheskis et al., 2007; Zubairy and Oesterreich, 2005). For example, transcriptional activation of proliferative genes by estrogen is associated with breast cancer (Foster et al., 2001) and transcriptional repression of cytokine genes by estrogen underlines an important mec-hanism whereby estrogen prevents inflammatory disea-ses associated with menopause (Ammann et al., 1997; Pai et al., 2004; Pfeilschifter et al., 2002). Even in estrogen-sensitive MCF-7 breast cancer cells and PEO-1 ovarian cancer cells, the number of estrogen down-regulated genes is nearly equal to that of estrogen up-regulated genes (Charpentier et al., 2000; O'Donnell et al., 2005). The observation of estrogen repression of LRP16 gene expression in SKOV3 cells suggests the existence of an estrogen-repressed signaling pathway in estrogen-resistant ovarian cancer cells. Moreover, the involvement of LRP16 in the classic estrogen-activated signaling suggests the crosstalk between estrogen-repressed and estrogen-activated pathways in estrogen-insensitive ovarian cancer cells. The crosstalk between different ERα-mediated signaling pathways also was observed in other cases. For instance, results from the analysis of a nonclassical ERα knock-in mice model suggested the crosstalk between ERE-dependent and independent ERα signaling pathways, and their alterations can result in a markedly aberrant response to estrogen (Syed et al., 2005, 2007).

Some genes, identified as being estrogen-regulated in estrogen-sensitive cells, have previously shown to be estrogen targets in estrogen-insensitive cells. For example, estrogen can induce expression of the early growth response genes c-myc and c-fos in both MCF-7 and SKOV3 cells (Hua et al., 1995; Prall et al., 1998). However, several genes are differentially regulated by estrogen in different cell context. For instance, Cyr61 is up-regulated by estrogen in PEO-1 ovarian cancer cells (O'Donnell et al., 2005), but is down-regulated in MCF-7 cells (Sampath et al., 2001). FN1 is down-regulated in PEO-1 cells (O'Donnell et al., 2005) yet is up-regulated in other cell types (Woodward et al., 2001; Mercier et al.,

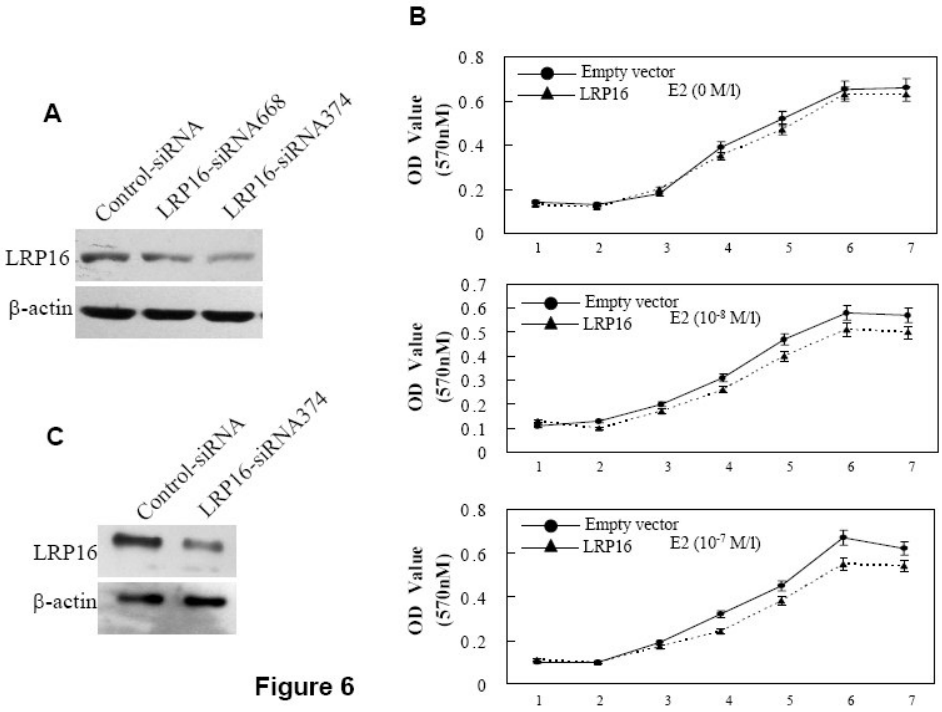

Figure 6. Effect of LRP16 knockdown in SKOV3 cells on cell proliferation. **A)** SKOV3 cells were cultured in steroid-deprived medium for at least 3 days and transfected with LRP16-siRNA 374, LRP16-siRNA668 or control-siRNA. 48 h after transfection, total protein was extracted and subjected to immunobloting analysis using the indicated antibodies. **B)** SKOV3 cells were cultured in medium supplemented with steroid-stripped FBS (5%) for 3 days and then transiently transfected with LRP16-siRNA374 or control-siRNA. 48 h after transfection, cells were treated with E2 at different concentrations for the indicated times. Cell proliferation rate was quantified by CellTiter 96 AQueous assay. Each data point represents the mean ± SEM of at least three independent experiments. **C)** SKOV3 cells transfected with the indicated siRNAs were cultured in steroid-deprived medium for 7 d. Immunoblots were probed for LRP16 and β-actin.

2002). In this study, the effect of estrogen on LRP16 expression observed in estrogen-sensitive cancer cells opposed the effect observed in estrogen-insensitive SKOV3 cells. The differential induction of LRP16 expression by liganded and unliganded ERα in SKOV3 cells revealed completely different regulatory mechanism compared with that in estrogen-sensitive cancer cells. A proximal region of -676 to -24 bp of the human LRP16 promoter was previously identified to be essential for estrogen induction of LRP16 expression in MCF-7 cells (Han et al., 2008). Estrogen induces LRP16 gene transactivation by stimulating the interaction and recruitment of ERα and Sp1 transcription factor at a 1/2ERE/GC-rich site and multiple GC-rich sites present in -676 to -24 bp of the upstream regulatory region of LRP16 gene (Zhao et al., 2005; Han et al., 2008). By promoter analysis, we demonstrate that the fragment from -676 to -214 bp of the LRP16 upstream regulatory region mainly confers estrogen-repressed effect of LRP16 expression (Figure 3). The observation that ERα is able to bind to this region in the absence of estrogen stimulation by ChIP analysis revealed that LRP16 is a primary target of ERα. Similarly, estrogen-repressed *Cyclin G2*, *TNFα* and *E-cadherin* genes are also ERα primary target genes (Stossi et al., 2006; Cvoro et al., 2006; Oesterreich et al., 2003). As ERα removal from LRP16 promoter region upon estrogen stimulation (Figure 4), apparent loss of ERα after estrogen treatment from TNFα promoter region was also observed in a previous report (Cvoro et al., 2006). A 1/2ERE/SP1 site of *cyclin G2* promoter, the most proxymal region of the *E-cadherin* promoter, which does not contain any classical ERE but three E-boxes, or an AP1/NF-κB element of *TNFα* promoter provides ERα binding sites and confers estrogen-repressed responsiveness (Stossi et al., 2006; Cvoro et al., 2006; Oesterreich et al., 2003). By computer-based analysis, three sites including a 1/2ERE/GC-rich Sp1 site, a NF-κB response element and an E-box site within the fragment from -676 to -214 bp of LRP16 upstream regulatory region were found, which may be the possible estrogen response sites. The detailed molecular mechanism of estrogen repression of LRP16 expression in SKOV3 cells is under investigation in our laboratory.

Increasing evidence revealed the existence of self feed

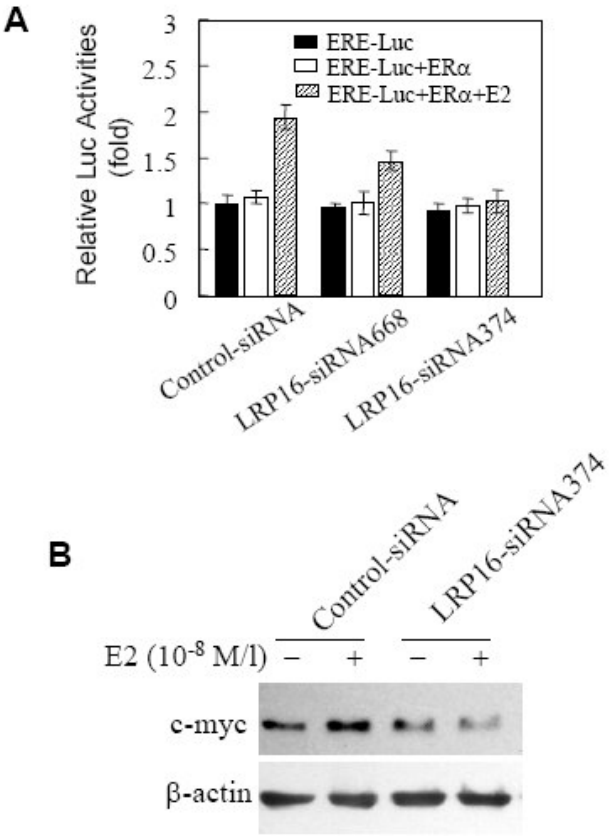

Figure 7. Effect of LRP16 knockdown on ERα-activated ERE-dependent transactivation and E2-induced c-myc expression in SKOV3 cells. **A)** SKOV3 cells were grown in media stripped of steroids for at least 3 days, then cotransfected with 3xERE-TATA-*Luc* reporter and the effector molecule ERα and LRP16-siRNAs or control-siRNA oligonucleotides. The relative normalized luciferase activity level for control-siRNA transfections were arbitrarily assigned a value of 1. All experiments were performed in triplicate and were repeated at least three times; results are expressed as mean ± SEM. **B)** SKOV3 cells were cultured in steroid-stripped medium for at least 3 days, then transiently transfected with the indicated siRNA oligonucleotides. 48 h after transfection, cells were treated with E2 or DMSO for an additional 3 h. Immunoblots were probed for c-myc and β-actin.

back regulation loop in steroid nuclear receptor-mediated signaling pathway (Zwijsen et al., 1997; Shi et al., 2001; Lauritsen et al., 2002; Hong et al., 2005). LRP16 is up-regulated by estrogen in estrogen-sensitive epithelial cancer cells, and in turn, it enhances ERα-activated signaling transduction in a ligand-dependent manner by interacting with the receptor (Han et al., 2007). As a coactivator, we have previously demonstrated that LRP16 is required for ERα-mediated transactivation and involved in proliferation of estrogen-responsive breast cancer cells (Han et al., 2007). This feed-forward mechanism for ERα activation may reflect the self-maintaining nature of ERα signaling in estrogen-sensitive target cells and may be involved in the progression of estrogen-dependent cancers. So, disruption of ERα feed-forward activation pathway such as by blocking estrogen-induced LRP16 up-regulation may predispose estrogen-sensitive cells to insensitive ones. This opinion was supported by our previous observation that knockdown of LRP16 in estrogen-dependent MCF-7 impaired estrogen-stimulated growth of cells (Han et al., 2007). In this study, modulation of LRP16 expression in SKOV3 cells did not change cell proliferation in the absence of estrogen stimulation, but slightly modulated the proliferative response of cells in the presence of estrogen treatment. This observation emphasized the functional contribution of estrogen repression of LRP16 expression in SKOV3 cells to estrogen resistance.

In general, these findings clearly verified that LRP16 is an estrogen-repressed target in estrogen-resistant SKOV3 human ovarian cancer cells. As we previously demonstrated, LRP16 can also exert its enhanced effect on the classical ERα-mediated transcription by functioning as an ERα coactivator in SKOV3 cells.

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