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

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Abstract

Previously, we investigated the induction effect of LRP16 expression by estrogen (17 β -estradiol, E₂) and established a feed-forward mechanism that activated estrogen receptor α (ER α) transactivation in estrogen-dependent epithelial cancer cells. LRP16 is required for ER α signaling transduction by functioning as an ER α coactivator. In this study, we demonstrated that LRP16 expression was upregulated in E₂-responsive BG-1 ovarian cancer cells, but was downregulated in estrogen-resistant SKOV3 ovarian cancer cells. Pure estrogen antagonist ICI 182 780 did not affect LRP16 expression in SKOV3 cell. The unliganded ER α upregulated 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 ER α at LRP16 promoter in the absence of estrogen; however, ER α was largely released from the DNA upon E₂ stimulation. Modulation in LRP16 expression level did not significantly change the proliferation rate of SKOV3 cells and the growth responsiveness of cells to E₂. Knockdown of LRP16 by RNA interference in SKOV3 cells markedly attenuated estrogen response element-dependent ER α 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.

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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 gynecological malignancies including breast cancer and endometrial cancer (Shang 2006, Yager & Davidson 2006, Eliassen & Hankinson 2008). Estrogen, a major steroidal product of the ovary, has also been associated with increased ovarian cancer risk (Bai *et al.* 2000, Rodriguez *et al.* 2001, Riman *et al.* 2002). The biological effects of estrogens are mediated by two forms of estrogen receptor, ER α and ER β . Classically, ER α 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 referred to as estrogen response elements (ERE) and initiate transcriptional activity (McDonnell & Norris 2002). Estrogen-bound ER α can also transactivate additional target genes through interacting with other transcriptional factors such as Ap1, Sp1 or nuclear factor κ B (Safe 2001, Shang & Brown 2002,

DeNardo *et al.* 2005). The activation of an ER 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 till date little 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 ER α at the time of diagnosis (Slotman & Rao 1988). 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 & 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 ER α -mediated but not ER β -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, ER α -positive ovarian cancer is often unresponsive to estrogen and refractory to antiestrogen therapy (Smyth *et al.* 2007, Wagner *et al.* 2007). The ER α signaling pathway in estrogen-resistant ovarian cancer cells is poorly understood. Hence, characterizing ER α -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 ER α but are growth-resistant to estrogen and antiestrogens (Langdon *et al.* 1994b, Hua *et al.* 1995), were commonly used as an *in vitro* model for estrogen and antiestrogen resistant ovarian cancer (Havrilesky *et al.* 2001, O'Donnell *et al.* 2005).

LRP16 is a special member of macro domain superfamily, the structure of which is simple in contrast to other macro domain protein members, composed of only a stand-alone macro module at its C-terminal region (Han *et al.* 2002, Aguiar *et al.* 2005). LRP16 was previously identified as an estrogen-responsive target gene. Estrogen-induced upregulation of LRP16 expression is mediated by ER α , but not by ER β (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 ER α -positive breast and endometrial 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 1/2 ERE/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). Interestingly, estrogen-upregulated LRP16 can interact with ER α and enhance the receptor's transcriptional activity in a ligand-dependent manner, thus establishing a positive feedback regulatory loop between LRP16 and ER α signal transduction in estrogen-responsive breast cancer cells (Han *et al.* 2007). Overexpression of LRP16 can stimulate the proliferation of MCF-7 human breast cancer cells by enhancing estrogen-activated ER α transcriptional function (Han *et al.* 2007). In addition, inhibition of LRP16 gene expression significantly suppresses the invasive capacity of estrogen-responsive breast and endometrial cancer cells by upregulating E-cadherin expression via ER α mediation (Meng *et al.* 2007). Consistent with the findings from cell culture, the mRNA level of LRP16 was observed to be positively linked to the progression of primary breast cancers (Liao *et al.* 2006). These data implied that LRP16 may play an important role in carcinogenesis and/or progression of hormone-dependent cancers by a feed-forward mechanism that activated ER α transactivation. In addition, we recently demonstrated that LRP16 can be upregulated by androgen in the androgen-sensitive LNCaP prostate cancer cells and that LRP16 serves as an essential coactivator of androgen receptor (Yang *et al.* 2009). Although the regulatory mechanism of LRP16 expression by estrogen and the functional role of LRP16 in estrogen-sensitive epithelial tumor cells are relatively well

documented, the estrogen induction and the functional significance of LRP16 gene in estrogen-unresponsive epithelial ovarian cancer cells are not clear so far.

In this study, we investigated the regulatory effects of estrogen, estrogen antagonist, and the unliganded-ER α on LRP16 gene expression and 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 ER α -mediated transcriptional activity by ERE-based reporter assay and the proliferation of SKOV3 cells to determine whether disruption of the ER α -LRP16 feed-forward pathway in estrogen-resistant ovarian cancer cells can change the cell response to estrogen.

Materials and Methods

Chemicals and cell lines

17 β -Estradiol (E₂) was purchased from Sigma. Pure estrogen antagonist ICI 182 780 was provided by Dr Qinong Ye at the College of Military Medicine Scientific Institute of China. Human ovarian epithelial adenocarcinoma cell line SKOV3 was originally purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained as monolayer cultures in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA). BG-1 human ovarian epithelial cancer cell line was cultured as previously described (Geisinger *et al.* 1989). Steroid-deprived serum was prepared as previously described (Zhao *et al.* 2005).

Plasmids

The pGL3-Basic and pRL-SV40 were originally purchased from Promega. The pcDNA3.1-LRP16 expression vector containing human LRP16 full-length cDNA was previously constructed (Han *et al.* 2003). Mammalian expression plasmid for ER α (pS5G-hER α) was provided by Prof. Hajime Nawata at Kyushu University, and the reporter 3 $\times$ ERE-TATA-Luc was provided by Prof. Donald P McDonnell at Duke University Medical Center (Norris *et al.* 1998). The luciferase reporter constructs pGL3-S₀, pGL3-S₂, pGL3-S₄, pGL3-S₅, and pGL3-S_{B1}, containing the fragment of -2623 to -24 bp, -1775 to -24 bp, -1064 to -24 bp, -676 to -24 bp, -213 to -24 bp of the LRP16 upstream regulatory region respectively, have been previously described (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 40–60%, 5 μ g pcDNA3.1-LRP16 was stably transfected using the Superfect

transfection reagent (Qiagen), 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) for 10–14 days 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 according to the manufacturer's recommendations (Invitrogen). SiRNA oligonucleotides were chemically synthesized by Shanghai GeneChem Co., Ltd (Shanghai, China). The sequences of LRP16-siRNA374 and LRP16-siRNA668 were previously reported (Han *et al.* 2007). The siRNA sequence against ER α was referred as previously described (Cheng *et al.* 2007). The unrelated siRNA sequence (sense strand, 5'-TTCTCCGAACGTG-CACGT-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% (v/v) steroid-depleted 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 luciferase reporters were cotransfected with or without 0.5 µg ER α 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. Thirty hours 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^{II} Lumino-metry System (Promega). All experiments were performed in triplicate and repeated at least thrice.

Cell proliferation assay

A total of 1 × 10⁴ viable cells were seeded in 24-well plates. After cell attachment, the medium was replaced with 1 ml fresh RPMI 1640 supplemented with 1% (v/v) 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 isolation and northern analysis

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method using Triblue reagent (Biotec Co., Beijing, China). The procedure of the northern blot

analysis was previously described (Han *et al.* 2007). Briefly, 20 µg total RNA was electrophoresed through a 1% (w/v) agarose gel containing formaldehyde and was transferred to a Hybond N⁺ membrane (Amersham). The membranes were hybridized using the following probes labeled with [α -³²P]dCTP by random priming: 550 bp fragment of LRP16 (432–981 bp, NM_014067) and 267 bp of GAPDH (543–809 bp, NM_002046).

Western blot and antibodies

The expression of LRP16, ER α and GAPDH proteins were examined by western blot analysis as previously described (Han *et al.* 2007). Briefly, cell lysates were electrophoresed by SDS-PAGE using 12% (w/v) acrylamide gels and blotted onto PVDF membranes (Amersham). Blots were probed with the primary antibodies, washed and then incubated with HRP-labeled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and binding was detected using enhanced chemiluminescence. The rabbit anti-LRP16 antibody (1:3000) was used as previously described (Han *et al.* 2007). Rabbit anti-ER α (1:500) and c-Myc (1:200) were purchased from Santa Cruz. Mouse anti-GAPDH antibody (1:500) was purchased from Abcam (Cambridge, MA, USA).

Chromatin immunoprecipitation assays

SKOV3 cells (1 × 10⁶) were grown in 10 cm tissue culture plates in RPMI 1640 supplemented with 10% (v/v) steroid-depleted FBS. After 24 h, the cells were transfected with 10 µg plasmid DNA mixture (1:1 for pGL3-S₅ and pS5G-hER α) using Superfect reagent. Forty hours later, the transfected cells were treated with E₂ (10⁻⁸ M/l) for 3 h and were subjected to chromatin immunoprecipitation (ChIP) assays. In addition, SKOV3 cells (1 × 10⁷) treated with E₂ (10⁻⁸ M/l) were also used for ChIP assays, which were performed as previously described (Meng *et al.* 2007). Briefly, immunoprecipitation was carried out overnight at 4 °C with ER α (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'-GCGCCAGGCTCTCCAGCTCG-3', and reverse primer, 5'-CCCAGTGTCGCGGATGGAGC-3'.

Statistical analyses

Experiments were performed in triplicate and repeated at least thrice, and the results were expressed as the mean ± S.E.M. Statistical analysis was performed using Statview 5.0 software. Paired Student's *t*-tests or two-way ANOVA followed by the

Student-Newman-Keuls test were used where applicable to assess significant differences between groups. $P < 0.05$ was considered to be statistically significant.

Results

LRP16 expression is upregulated by E₂ in BG-1 cells, but is downregulated in SKOV3 cells

Previously published work from our laboratory demonstrated that E₂ upregulated the level of the human LRP16 gene through ER α 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 ER α -positive human ovarian cancer BG-1 and SKOV3 cells, we performed northern blot analysis. As seen in Fig. 1A, the mRNA expression level of LRP16 was upregulated 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, in SKOV3 cells, LRP16 expression was not upregulated by E₂ treatment; conversely, it was down-regulated by E₂ in a dose-dependent fashion (Fig. 1B). Compared with the LRP16 expression level in cells cultured under steroid-deprived culture conditions (Fig. 1B lane 1), a dramatic decrease was observed in cells under normal conditions (FBS without steroid deprivation) (Fig. 1B, lane 6), indicating that the endogenous estrogen in culture medium is enough to suppress LRP16 expression after a long-term exposure of cells to it. To confirm that the LRP16 mRNA

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 level of LRP16 mRNA was determined by northern blot analysis. Total RNA was used as the loading control. (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. LRP16 mRNA level was determined by northern blot analysis. Total RNA was used as the loading control. Western blots were probed for LRP16 and GAPDH. N, cells were cultured in medium containing FBS without steroid deprivation. (C and 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, ER α and GAPDH. (E) SKOV3 cells were cultured in routine culture medium and were treated with ICI 182 780 for the indicated times (left panel). SKOV3 cells were cultured in medium containing steroid-stripped FBS for at least 3 days, treated with E₂ and ICI 182 780, and then were cultured for the indicated time points (right panel). Immunoblots were probed for LRP16 and GAPDH. The experiments shown in A–E were repeated at least thrice.

levels were indicative of protein levels, the expression level of LRP16 protein was examined in E₂-treated SKOV3 cells using western blot analysis and the results showed the consistent change with that observed at the mRNA level (Fig. 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 in SKOV3 cells, over a 72 h time-period (Fig. 1C). A more than twofold decrease in LRP16 protein level was observed as early as 6 h after addition of E₂, and this was continued to 48 h. At 72 h after treatment of E₂, the expression of LRP16 protein decreased to a much lower level. To rule out the possibility that E₂-induced LRP16 decrease resulted from altered ER α expression, we measured the ER α protein levels in E₂-treated cells. As shown in Fig. 1D, E₂ treatment (10⁻⁸ M/l) did not significantly change the expression of ER α protein during the 72 h time course. These results demonstrated that *LRP16* is not an estrogen upregulated target gene in estrogen-insensitive SKOV3 ovarian carcinoma cells as it is in estrogen-responsive epithelial cancer cells, but is an estrogen downregulated gene which exhibits a sensitive and continuous response to E₂ treatment.

To assess the effect of antiestrogen on the expression level of LRP16 in SKOV3 cells, the selective estrogen antagonist, ICI 182 780 (100 nM) was used to treat proliferating cells. Total protein over a 72 h time course was extracted and western blot analysis was used to determine the LRP16 protein level. As shown in Fig. 1E (left panel), there was no significant increase of LRP16 protein level in cells after ICI 182 780 addition. The LRP16 protein level was downregulated by the addition of E₂ (10⁻⁸ M/l E₂) even in the presence of ICI 182 780 (Fig. 1E, right panel), indicating that antiestrogen treatment can not effectively antagonize the E₂ suppression of LRP16 expression in estrogen-resistant ovarian cancer cells.

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

Figure 2 ER α 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 ER α expression vector or empty vector. Thirty-six hours after transfection, cells were treated with or without E₂ (10⁻⁸ M/l) and cultured for an additional 12 h. Immunoblots were probed for ER α , LRP16 and GAPDH. (B) SKOV3 cells were cultured in steroid-deprived medium for at least 3 days, and then were transfected with the ER α -specific siRNA or the control-siRNA oligonucleotides. Forty-eight hours after transfection, total protein was extracted and subjected to immunoblotting analysis using the indicated antibodies. The experiments shown in A and B were repeated at least thrice.

To further address the regulatory roles of liganded and unliganded ER α 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 (Fig. 3). Cotransfection of ER α did not significantly change the luciferase activities for pGL3-S_{B1} and the control vector pGL3-Basic, but indeed resulted in a two- to fourfold increase of reporter gene activities for pGL3-S₀, pGL3-S₂, pGL3-S₄ and pGL3-S₅ constructs (Fig. 3). However, the unliganded ER α 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 ER α 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 ER α to LRP16 gene promoter

To analyze whether the increased promoter activity of LRP16 by unliganded ER α is the direct result of recruitment of ER α , we performed ChIP assays. The LRP16 promoter containing construct pGL3-S₅, which was authenticated to be activated by ER α in the absence of E₂ as illustrated in Fig. 3, together with ER α 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 ER α was analyzed by ChIP (Fig. 4A). In the absence of E₂ treatment, we repeatedly detected a high level of ER α binding at the promoter of LRP16. However, ER α 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 non-specific IgG group.

Figure 3 Unliganded and liganded ERα regulation of *LRP16* gene promoter activity in SKOV3 cells. (A) The graphic illustration of luciferase reporters driven by different LRP16 promoter fragments. (B) 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. Thirty hours after transfection, cells were treated with E₂ (10⁻⁸M) 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 thrice; results are expressed as mean ± S.E.M. Two-way ANOVA followed by the Student-Newman-Keuls test were performed for assessing significant differences between groups **P* < 0.05, ***P* < 0.01, comparison between ERα transduction-induced activity with respective basal activity. #*P* < 0.05, ##*P* < 0.01, comparison between ERα transduction and E₂ treatment-induced activity.

Next, we performed ChIP-analyses of the endogenous LRP16-promoter (-476 to -241 bp) and the results showed that binding of unliganded ERα occurs at the natural, endogenous promoter in the estrogen-resistant SKOV3 cells, and that this is lost after E₂ treatment (Fig. 4B). These results indicated that *LRP16* is a primary target gene of the unliganded ERα in SKOV3 cells.

LRP16 does not significantly modulate the growth responsiveness of SKOV3 cells to E₂

Previous reports from our laboratory demonstrated that overexpression of LRP16 promotes proliferation of estrogen 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 SKOV3 cells and performed cell proliferation assays. After 10 to 14-day G418 screening, the drug-resistant

Figure 4 E₂ inhibition of ERα recruitment to LRP16 gene promoter in SKOV3 cells. (A) SKOV3 cells were transiently cotransfected with ERα and pGL3-S₅. Forty hours 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 'Materials and Methods'. (B) SKOV3 cells were treated with E₂ (10⁻⁸ M/l) or DMSO for 3 h and were subjected to immunoprecipitation and PCR as described in A. The experiments shown in A and B were repeated thrice.

clones appeared and were mixed for amplified culture. All of the parental cells were killed by G418 within this period. The expression level of LRP16 was measured within 30 days after transfection by western blot analysis and the results showed that the ectopic transfection markedly increased the LRP16 expression (Fig. 5A). As shown in Fig. 5B, LRP16 overexpression did not significantly promote SKOV3 cell proliferation either in the absence or presence of E₂ treatment. 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 did not change the expression level of the GAPDH gene (Fig. 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. Significant difference of cell growth between LRP16-siRNA and control-siRNA transfected cell groups was not observed at any time point during the culture period when the cells were cultured without E₂ stimulation. Similarly, the growth rate of SKOV3 cells was not markedly altered by LRP16 knockdown when the cells were treated with E₂ (Fig. 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 (Fig. 6C). These findings suggested that LRP16 does not significantly 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 ERα target genes such as c-Myc and c-fos in E₂-sensitive breast cancer cells can still

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 GAPDH within 30 days 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 $\pm$ S.E.M. number of cells counted in triplicate dishes from at least three independent experiments. Student's *t*-test was performed between values. No significant difference was observed at each data point between the indicated two cell groups (*P* > 0.05).

be observed in SKOV3 cells (Hua *et al.* 1995). To determine whether LRP16 functions as an active ER α coactivator in SKOV3 cells as it does in estrogen-dependent ER α -positive epithelial cancer cells, we tested the effect of LRP16 expression on ER α -mediated transcription by using a 3 $\times$ ERE-TATA-Luc reporter construct. SKOV3 cells were cultured in steroid-stripped medium for at least 3 days, and then were cotransfected with 3 $\times$ ERE-TATA-Luc, ER α and LRP16-siRNA374, LRP16-siRNA668 or control-siRNA. As shown in Fig. 7A, E₂ (10⁻⁸ M/l) stimulation elicited a twofold increase of ER α -mediated reporter gene activity in control-siRNA expressing cells, which was in agreement with the previous report (Hua *et al.* 1995). However, only

Figure 6 Effect of LRP16 knock-down 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. Forty-eight hours after transfection, total protein was extracted and subjected to immunoblotting analysis using the indicated antibodies. (B) SKOV3 cells were cultured in medium supplemented with steroid-stripped FBS (5%, v/v) for 3 days and then transiently transfected with LRP16-siRNA374 or control-siRNA. Forty-eight hours 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 $\pm$ S.E.M. of at least three independent experiments. Student's *t*-test was performed between values. No significance difference was observed at each data point between the indicated two cell groups (*P* > 0.05). (C) SKOV3 cells transfected with the indicated siRNAs were cultured in steroid-deprived medium for 7 days. Immunoblots were probed for LRP16 and GAPDH.

Figure 7 Effect of LRP16 knock-down on ER α -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 3 $\times$ ERE-TATA-Luc reporter and the effector molecule ER α and LRP16-siRNAs or control-siRNA oligonucleotides. The relatively 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 $\pm$ S.E.M. Two-way ANOVA followed by the Student-Newman-Keuls test were used to assess significant differences between groups. * $P < 0.05$, comparison between E₂-induced activity with respective basal activity. # $P < 0.05$, comparison between E₂-induced activity with only ER α -transfected activity. (B) SKOV3 cells were cultured in steroid-stripped medium for at least 3 days, then transiently transfected with the indicated siRNA oligonucleotides. Forty-eight hours after transfection, cells were treated with E₂ or DMSO for an additional 3 h. Immunoblots were probed for c-Myc and GAPDH.

a 1.5-fold increase of the reporter gene activity by E₂ stimulation was observed in LRP16-siRNA668 transfected cells, and a 0.1-fold increase in LRP16-siRNA374 transfected cells. Next, we measured the E₂ induction of c-Myc protein in LRP16-inhibited SKOV3 cells. As illustrated in Fig. 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 ER α signaling transduction and inhibition of LRP16 expression can efficiently suppress ER α -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 ER α coactivator, decreased expression of LRP16 in SKOV3 cells is capable of attenuating 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' ER α -activated signaling transduction. The antagonism between these two parallel estrogen signaling pathways underscores a novel mechanism of estrogen unresponsiveness of ovarian cancer.

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 being the genomic or classical action of ER α (Cheski *et al.* 2007, Heldring *et al.* 2007), several lines of evidence demonstrate the existence of an estrogen-repressed signaling pathway in various estrogen target cells and it is possibly linked to the pathogenesis of some diseases (Zubairy & Oesterreich 2005, Cheski *et al.* 2007). 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 mechanism whereby estrogen prevents inflammatory diseases associated with menopause (Ammann *et al.* 1997, Pfeilschifter *et al.* 2002, Pai *et al.* 2004). Even in estrogen-sensitive MCF-7 breast cancer cells and PEO-1 ovarian cancer cells, the number of estrogen downregulated 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 crosstalk between estrogen-repressed and estrogen-activated pathways in estrogen-insensitive ovarian cancer cells. The crosstalk between different ER α -mediated signaling pathways was also observed in other cases. For instance, results from the analysis of a non-classical ER α knock-in mice model suggested crosstalk between ERE-dependent and independent ER α 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 been 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 contexts. For instance, *Cyr61* is upregulated by estrogen in PEO-1 ovarian cancer cells (O'Donnell *et al.* 2005), but is downregulated in MCF-7 cells (Sampath *et al.* 2001). *FN1* is downregulated in PEO-1 cells (O'Donnell *et al.* 2005) yet is upregulated in other cell types (Woodward *et al.* 2001, Mercier *et al.* 2002). In this study, the effects 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 ER α in SKOV3 cells revealed a 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 ER α and Sp1 transcription factor at a 1/2 ERE/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 (Fig. 3). The observation that ER α is able to bind to this region in the absence of estrogen stimulation by ChIP analysis revealed that LRP16 is a primary target of ER α . Similarly, estrogen-repressed cyclin G2, tumour necrosis factor α (TNF α) and E-cadherin genes are also ER α primary target genes (Oesterreich *et al.* 2003, Cvoro *et al.* 2006, Stossi *et al.* 2006). Similar to the binding of unliganded ER α to the promoter region of *LRP16* gene, the unliganded ER α can bind to the AP1/NF- κ B element of TNF α promoter region and enhance its transcription; however, it will be removed from TNF α promoter region as in the case of *LRP16* gene after estrogen treatment (Cvoro *et al.* 2006). Unliganded ER α can enhance cyclin G2 transcription by binding to a 1/2 ERE/Sp1 site within its promoter, or enhance E-cadherin transcription by binding to the most proximal region of its promoter, which does not contain any classical ERE but three E-boxes. Similarly, ER α will be removed from the binding sites of cyclin G2 and E-cadherin genes upon estrogen stimulation (Oesterreich *et al.* 2003, Stossi *et al.* 2006). By computer-based analysis, three sites including a 1/2 ERE/GC-rich Sp1 site, a NF- κ B 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 a self-feedback regulation loop in steroid nuclear receptor-mediated signaling pathway (Zwijnsen *et al.* 1997, Shi *et al.* 2001, Lauritsen *et al.* 2002, Hong *et al.* 2005). LRP16 is upregulated by estrogen in estrogen-sensitive epithelial cancer cells, and in turn, it enhances ER α -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 ER α -mediated transactivation and involved in proliferation of estrogen-responsive breast cancer cells (Han *et al.* 2007). This feed-forward mechanism for ER α activation may reflect the self-maintaining nature of ER α signaling in estrogen-sensitive target cells and may be involved in the progression of estrogen-dependent cancers. So, disruption of ER α feed-forward activation pathway such as by blocking estrogen-induced LRP16 upregulation may predispose estrogen-sensitive cells to insensitive cells. This opinion was supported by our previous observation that knock-down of LRP16 in estrogen-dependent MCF-7 cells impaired estrogen-stimulated growth (Han *et al.* 2007). However, as we demonstrated in this study (Figs 5 and 6), the ectopic modulation of LRP16 expression in estrogen-insensitive SKOV3 cells did not significantly change cell proliferation rate and the growth response to E₂ treatment. This observation suggested that the estrogen repression of LRP16 expression in SKOV3 cells may not sufficiently induce the resistance of cells to estrogen.

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 ER α -mediated transcription by functioning as an ER α coactivator in SKOV3 cells.

Declaration of interest

All authors declare that there is no conflict of interest.

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