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Estrogen-independent role of ERα in ovarian cancer progression induced by leptin/Ob-Rb axis

Ahmad Ghasemi[1,2] · Jafar Saeidi[3] · Mahnaz Mohtashami[4] · Seyed Isaac Hashemy[5,6]

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Abstract

Leptin induces ovarian cancer cell invasion via overexpression of MMP7, MMP9, and upA. In addition, the key role of ERα in leptin-increased cell growth was indicated. However, the influence of ER on leptin-mediated cell invasion remains still unknown. The present study was designed to evaluate the E2-independent effect of ERα/β on leptin-mediated cell invasion and cell proliferation in ovarian cancer. We utilized SKOV3 cancer (expressing OB-Rb and ERα/β, insensitive to estrogen) and OVCAR3 (expressing OB-Rb) cell lines to show the involvement of ER in leptin-mediated effects in an E2-independent manner. MTT, BrdU, and BD matrigel invasion assays were applied to analyze cell growth, proliferation, and invasion. The siRNA approach was used to confirm the role of ERα/β in leptin effects. Moreover, western blotting and Real-time PCR were employed to detect the OB-Rb, ER, MMP9/7, and upA proteins and mRNAs. Leptin, in the absence of E2, increased ERα expression in SKOV3 cells, which was attenuated using knockdown of OB-Rb gene by siRNA. The effect of leptin on the cell growth was promoted in the presence of PPT, but not in the presence of DNP and E2, which was lost when OB-Rb siRNA was transfected. Furthermore, ERα gene silencing and/or pre-incubation with ER antagonist (ICI 182,780, 10 nM) significantly reduced cell invasion and MMP9 expression stimulated by leptin. In conclusion, our findings demonstrated that ERα, but not ERβ, is involved in leptin-induced ovarian cancer in an E2-independent manner, providing new evidence for cancer progression in obesity-associated ovarian cancer.

Keywords Leptin · Cell invasion · Estrogen receptor · Estradiol · Ovarian cancer

Introduction

Obesity has been assumed as one of the most common health challenges in both developed and developing countries [1]. According to the world health organization (WHO) report in 2016, more than 1.9 billion adults aged 15 years and above were overweight and of these over 650 million were obese [2]. Obesity is a well-established risk factor for a number of cancers particularly those known as hormone-dependent such as breast [3], endometrial [4], and ovarian [5] cancers. Obesity can lead to changes in the secretion of adipocyte-derived hormones such as leptin and it was revealed that serum leptin level is significantly higher in obese individuals compared to healthy subjects [6, 7]. Leptin is an important adipokine secreted by adipocytes that plays a key role in energy homeostasis balance and in the regulation of food intake through binding to its transmembrane receptors (OB-Rb) [8, 9]. Previous studies have shown mitogenic and anti-apoptotic effects of leptin in different cancers [7, 10]. Moreover, leptin influences cell migration and invasion

✉ Ahmad Ghasemi
Ghasemia2@nums.ac.ir

✉ Seyed Isaac Hashemy
HashemyI@mums.ac.ir

1 Non-communicable Disease Research Center, Neyshabur University of Medical Sciences, Neyshabur, Iran

2 Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

3 Department of Physiology, School of Basic Science, Neyshabur Branch, Islamic Azad University, Neyshabur, Iran

4 Department of Biology, School of Basic Science, Neyshabur Branch, Islamic Azad University, Neyshabur, Iran

5 Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

6 Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

which are critical steps in tumor metastasis and cancer progression [11]. Ovarian cancer is the fifth leading cause of cancer-related deaths among women [12, 13]. The majority of ovarian cancer patients have already reached in advanced stage (III or IV) accompanied by widely metastatic disease largely due to the lack of early detection methods [14, 15]. Previous investigations have indicated a positive association between serum leptin levels and ovarian cancer [16, 17]. Our previous studies and the others have demonstrated that leptin induces cell migration and invasion of ovarian cancer cells [17–20].

Estrogen (E2) enhances different steps of cancer progression in epithelial ovarian cancer [21]. The biological effects of E2 are mediated via binding to the two forms of estrogen receptors (ERs), including alpha (ERα) and beta (ERβ) [22]. Ovaries express both ER-α and ER-β [23], which have tumorigenic and anti-tumorigenic influences, respectively [24–26]. The studies have shown higher expression levels of ERα in ovarian malignancies compared with benign tumors and normal tissues [27–29]. In addition, it was reported that ERβ is expressed in ovarian normal and non-metastatic tissues [29–31]. Leptin induces ERα activation through the MAPK signaling pathway, even in the absence of E2 [32]. Leptin also induces the activation of aromatase enzyme, which converts androgens into estrogens [33]. It was demonstrated that leptin can crosstalk with E2 and its receptors in gynecological cancers [33–38]. Moreover, leptin-induced cell growth was attenuated by ER antagonist, ICI, in MCF7 breast cancer cell line [32, 39].

In ovarian cancer, the involvement of ERα in leptin-mediated cell growth was observed [29], however, its contribution in the stimulation of cell migration and invasion by leptin remains unclear. In the present study, it was investigated whether ER plays a crucial role in leptin-induced cell invasion in ovarian cancer cells. In addition, the E2-independent roles of ER in leptin-induced activation in SKOV3 which is ER-positive and E2-insensitive [27, 40] and also OVCAR3 which is not express ER are evaluated [29].

Materials and methods

Materials

RPMI, FBS, and other growth supplements were purchased from Gibco (Germany). Recombinant human leptin was obtained from R&D Systems (Minneapolis, MN, USA). ICI 182,780, an antagonist of ERα/β, as well as 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), a specific ERα agonist, and 2,3-bis(4-hydroxyphenyl)-propionitrile (DNP), a highly potent ERβ agonist, were purchased from Tocris Bioscience (Bristol, United Kingdom). Goat anti-mouse IgG-HRP: sc-2005, monoclonal antibodies against ERα: sc-73479, ERβ: sc-53494, Matrix metalloproteinase-9 (MMP9): sc-393859, and B-actin (C4): sc-47778 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Human OB-R Antibody: MAB867 was purchased from R&D Systems. Also, small interfering RNA (siRNA) (sc-36115) and siRNA transfection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments

Ovarian cancer cell lines were purchased from National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). SKOV3 and OVCAR3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin in a humidified incubator with 95% air and 5% CO_2 at 37 °C. The confluent cells were harvested by trypsinization, collected by centrifugation, and were treated with leptin (100 ng/ml). To evaluate whether ERs are contributed in leptin-mediated cell proliferation and invasion in ovarian cancer cells in E2-independent manner, cells were pretreated with ER antagonist (ICI 182,780, 10 nM), DNP (1 µM), PPT (1 µM), E2 (10^{-7} M), or transfected by siRNA molecule target to ER, and their effects were determined in the presence of leptin.

MTT assay

The effect of leptin on cell growth was analyzed using the MTT assay as described previously [18]. The OVCAR3 and SKOV3 cells were cultured with 5×10^3 cells/well density in 96-well plates containing RPMI medium with 10% FBS and incubated overnight. After incubation with determined treatments for 48 h, MTT tetrazolium salt (50 µl, of 2 mg/ml) was added to each well and the cells were incubated at 37 °C for 4 h. Subsequently, the mediums were removed, the produced Formosan crystals in the viable cells were dissolved in DMSO, and the optical density of samples measured at 570 nm. The cell viability percentage was calculated using the following formula: (mean OD of treated group/mean OD of non-treated group × 100).

BrdU assay

Cell proliferation was evaluated by colorimetric immunoassay based on bromodeoxyuridine (BrdU) according to the manufacturer's instructions (Roche, Mannheim, Germany). Briefly, 5×10^3 cells per well were cultured in 96-well plates in complete medium for 24 h. SKOV3 and OVCAR3 cells were incubated with leptin in the presence or absence of DNP, PPT, ICI 182,780, and E2 for 48 h. The growth medium then was supplemented with BrdU-labeling solution (20 µl) and cells were incubated for an additional 4 h. Subsequently, the culture medium was replaced with Fixodent

solution and samples were incubated with peroxidase-conjugated anti-BrdU antibody for 90 min. After removing the unbound anti-BrdU-POD, substrate solution was added to medium and color reaction was stopped with sulfuric acid addition after 3–5 min. Sample absorbance was detected at 450 nm using a spectrophotometric plate reader.

Matrigel invasion assay

Ovarian cancer cell invasion was measured by an invasion assay kit with Matrigel-coated inserts (8 μm, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. In Brief, SKOV3 or OVCAR3 cells (2×10^5 cells per insert) were placed in upper chambers in serum-free medium and the RPMI medium containing 10% FBS was used as the chemoattractant in the lower chambers. The cells were pretreated with ICI 182,780 or MMP9 inhibitor for 60 min before leptin treatment. In another set of experiment, cells were transfected with ERα/β siRNA or incubated with E2, and then cell invasion was measured in the presence of leptin. After 24-h incubation with treatments, non-invading cells remaining on the outer side of filters were gently removed with a cotton swab. Invading cells on the bottom of filters were fixed in methanol, washed three times with PBS, stained with 1% crystal violent, and counted on six different random fields under a light microscope at ×400 magnification.

Real-time RT-PCR experiments

The mRNA expression levels of OB-Rb, ERα, ERβ, MMP9, MMP7, and urokinase-type plasminogen activator (upA) were determined using Quantitative Real-Time RT-PCR. In brief, total RNA was extracted from SKOV3 and OVCAR3 cells using with the Trizol reagent (Invitrogen, USA). A first-strand cDNA synthesis kit (Takara Shuzo, Otsu, Japan) was used to synthesize cDNA from total RNA (2 μg). The amplified cDNA was used as the template for Quantitative RT-PCR using the SYBR Green kit (Amplicon). Reactions were performed in 96-well plates on an ABI 7500 sequence detection system (Applied Bio-systems) for 40 cycles using the following conditions: denaturation at 95 °C for 15 min, annealing at 57 °C for 5 s, and elongation at 72 °C for 5 s. Amplification specificity of PCR reactions was confirmed by melting point curve generated at the end of each run. Finally, ΔCt was calculated for target genes, and GAPDH as the endogenous housekeeping gene and $2^{-\Delta\Delta Ct}$ analysis was used to quantify relative gene expression. The primers used for Real-Time RT-PCR were indicated in Table 1.

SiRNA-mediated knockdown of ER

OB-Rb, ERα, ERβ siRNA, and a scrambled siRNA containing a random sequence were transfected into OVCAR-3 and SKOV3 cells using a siRNA transfection reagent (Santa Cruz Biotechnology) as explained previously [18]. Cells were cultured in six-well plates and transfected with a pool of siRNA at a final concentration of 40 nM. Finally, the transfected cells were incubated with determined treatments for 24 h and used for MTT, BrdU, Matrigel invasion assay, Real-time PCR, and western blotting analysis.

Western blotting

Protein contents of ERα, ERβ, and MMP9 were measured using western blot analysis. To quantify protein expression levels of ERα, ERβ, and MMP9, western blot analysis was performed. After incubation with determined treatments, total protein from each experiment was extracted using ice-cold RIPA buffer (20 mM Tris–HCl pH 7.5, 0.5%, Nonidet P-40, 0.5 mM PMSF, 100 mM β-glycerol 3-phosphate, and 0.5% protease inhibitor cocktail). The samples were incubated on ice for 2 h with gentle vortexing every 15 min, centrifuged at 10,000×*g* for 10 min at 4 °C, and then the supernatant was served as the cytosolic extracts. Total protein content in the cytosolic extract was obtained with Bradford reagent assay, and protein concentration was analyzed using the linear equation generated from the protein standard curve. Same amounts of lysate were loaded into 10% SDS-PAGE gels, and separated proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech.). Blocking of membranes was performed in PBS containing 5% non-fat dry milk and 0.1% Tween 20. PVDF membranes were incubated with primary antibodies against ERα, ERβ,

Table 1 Sequences of the primers used for the analysis of mRNA gene expression

Gene name	Forward primer	Revers primer
OB-Rb	5′-TGAGGTATCATAGGAGCAGCC-3′	5′-TGTTGGTGGAGAGTCAAGTGA-3′
MMP9	5′-CCGGACCAAGGATACAGTT-3′	5′-CGGCACTGAGGAATGATCTA-3′
MMP7	5′-GTCTCTGGACGGCAGCTATG-3′	5′-GATAGTCCTGAGCCTGTTCCC-3′
uPA	5′- GTCCTCCGGATTCCATCCAC -3′	5′-GATAGTCCTGAGCCTGTTCCC-3′
ERα	5′-AGCACCCTGAAGTCTCTGGA-3′	5′-GATGTGGGAGAGGATGAGGA-3′
ERβ	5′-CAGTTATCACATCTGTATGCGG-3′	5′-ACTCCATAGTGATATCCCGA-3′
GAPDH	5′-CTCCCGCTTCGCTCTCTG -3′	5′-TCCGTTGACTCCGACCTTC -3

or MMP9 overnight at 4 °C. Following washing with 10 ml PBST (three times, every 10 min), blots were treated with diluted horseradish peroxidase-conjugated secondary antibody (1:2000) for 60 min. The reactive signals of the target proteins were detected with ECL detection kit (Amersham Corp, Arlington Heights, IL, USA). The band intensity was analyzed using Image J software (http://imagej.nih.gov/ij/). The expressions of ERα, ERβ, and MMP9 were normalized to endogenous controls before the differences relative to non-treated samples were calculated.

Statistical analysis

All experiments were carried out in triplicate, and values are reported as the mean ± SD of three separate experiments. Data were analyzed using a Non-parametric one-way analysis of variance (ANOVA) followed by Dunnett's test with the statistical program SPSS 18.0. A p value < 0.05 was considered statistically significant.

Results

Leptin promoted ERα expression in SKOV3 cells

Both mRNA and protein levels of ERα and ERβ were measured in SKOV3 and OVCAR3 ovarian cancer cell lines. The results of western blot demonstrated that SKOV3 express a higher level of ERα than ERβ, while OVCAR3 cells express the slight level of both ERα and ERβ (Fig. 1a). We next evaluated the effect of leptin on the ERα and ERβ expressions of ovarian cancer cells after 24-h treatment using Real-time PCR. As shown in Fig. 1b, the effect of 50 and 100 ng/ml leptin on the mRNA expression of ERα, but not ERβ, was markedly higher in SKOV3 cells than untreated cells (2.8 ± 0.9 fold for 50 ng/ml, and 4.6 ± 1.01 fold for 100 ng/ml vs. control, respectively; $p < 0.01$). The exposure to leptin showed no significant inductive effects on ERα and ERβ mRNA expressions in OVCAR3 cells.

In another set of experiment, we performed western blotting for ERα and β in SKOV3 and OVCAR3 cells in the presence or absence of leptin (100 ng/ml). In consistent with Real-time PCR results, the findings of western blotting (Fig. 1c, d) also showed that treatment with leptin increased the protein expression of ERα in SKOV3 cell line (4.03 ± 0.45 fold vs. control, $p < 0.01$). These results indicated that leptin enhances mRNA and protein expression of ERα in SKOV3 cells.

OB-Rb gene silencing inhibited leptin-induced ERα expression in SKOV3 cells

Both SKOV3 and OVCAR3 cells were transfected with OB-Rb siRNA (40 nM) or scrambled control siRNA, and expressions of ERα and ERβ were then measured in the presence or in the absence of 100 ng/ml leptin. As shown

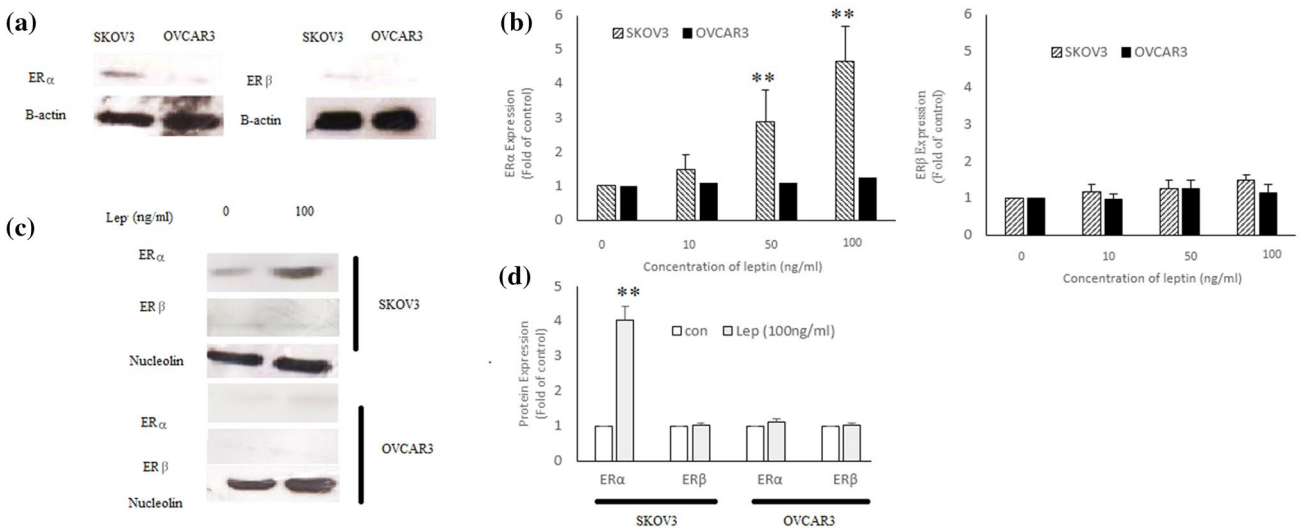

Fig. 1 Leptin promoted the ER expression in ovarian cancer cell lines: **a** total protein was extracted from SKOV3 and OVCAR3 cells, and equal amounts of proteins were analyzed with western blotting using specific antibodies for ERα and ERβ. **b** Cells were incubated with various concentrations of leptin (0, 10, 50, 100 ng/ml) for 24 h, total RNA was collected, and mRNA gene expression was measured by Real-time PCR. GAPDH was applied as an endogenous control and $2^{-\Delta\Delta Ct}$ method was used for quantitation of data. **c** SKOV3 cells were treated with 100 ng/ml of leptin for 24 h, and subsequently the expression of ERα/β was evaluated using western blotting. **d** The histogram represents the quantitative analysis of ER protein expression, which was normalized for Nucleolin expression. The values represent the mean ± SD ($n = 3$). Statistical analysis was performed by ANOVA (**$p < 0.01$ vs. control)

in Fig. 2a, the OB-Rb gene silencing markedly attenuated stimulative influence of leptin on mRNA expression of ERα (leptin: 4.6 ± 1.01 fold; leptin + ERα siRNA: 2.8 ± 0.4 fold; vs. control; $p < 0.01$) in SKOV3, but not in OVCAR3, cells. No significant change was observed in expression of ERβ in response to leptin in both cell lines. The effect of OB-Rb gene silencing on ERα expression was confirmed by western blotting (leptin: 4.1 ± 5.8 fold; leptin + ERα siRNA: 2.38. ± 0.2 fold; vs. control; $p < 0.01$) in SKOV3 (Fig. 2b). These results demonstrated that OB-Rb is directly involved in leptin-induced ERα expression of SKOV3 cells.

ERα is involved in leptin-mediated cell growth of SKOV3 cells

To investigate whether ER has an essential role in stimulative effects of leptin on ovarian cancer cell growth, the cells were pretreated with a specific inhibitor of ER (ICI 182,780) for 60 min, and subsequently stimulated with leptin for 48 h. MTT results (Fig. 3a, b) demonstrated that pretreatment with ICI 182,780 markedly blocked the leptin-induced cell growth in SKOV3 (leptin: 173 ± 6.6%; leptin + ICI 182,780:145 ± 9%; vs. control; $p < 0.05$ and $p < 0.01$, respectively), but not in OVCAR3 cells, suggesting the key role of ER in leptin-induced effects in ER expressing cell line.

In the separate experiment, cells were pretreated with PPT and DNP for 1 h before leptin treatment. As depicted in Fig. 3a, b, although a significant increase in leptin-mediated cell growth was observed in the presence of PPT in SKOV3 cells (leptin + PPT: 195.3 ± 3.5%; alone leptin: 173 ± 6.6; vs. control; $p < 0.05$), no significant enhance was observed in the presence of DNP. Cell transfection with OB-Rb siRNA significantly reduced leptin-induced cell growth in SKOV3 cells (leptin: 173 ± 6.6%; leptin + OB-Rb siRNA: 123.3 ± 5.5; vs. control; $p < 0.05$) and in OVCAR3 (leptin: 170 ± 10; leptin + OB-Rb siRNA: 130.5 ± 6.5; vs control; $p < 0.05$), while cell transfection with scrambled siRNA showed no change in leptin-enhanced growth of ovarian cancer cells (% cell growth in leptin + scrambled siRNA experiment, in SKOV3: 170.9 ± 7.4; in OVCAR3: 171 ± 9; vs. control). The inductive effect of PPT on leptin influence was attenuated by gene silencing of both OB-Rb (leptin + PPT: 195.3 ± 3.5%; leptin + PPT + OB-Rb siRNA: 136 ± 12% vs. control; $p < 0.01$) and ERα (leptin + PPT: 195.3 ± 3.5%; leptin + PPT + ERα siRNA: 149 ± 8% vs. control; $p < 0.01$). Pretreatment with either ERα or ER β agonists did not show any significant effects on cell growth in OVCAR3 cells.

To show the E2-independent contribution of ERα on leptin-mediated cell proliferation and growth, the cells were treated with leptin (100 ng/ml), E2 (10^{-7} M) or both for 48 h and the cell growth and proliferation were detected. As depicted in Fig. 3, no inductive effect was observed on leptin-induced cell growth of SKOV3 and OVCAR3 in the presence of E2. Moreover, the involvement of ERα in leptin influences was confirmed using BrdU assay (Fig. 3c, d). Taken together, given that treatment with E2 did not exhibit additional effects on leptin-induced cell growth, while using antagonists, agonist, and siRNA against ERα significantly changed these processes induced by leptin, it can be concluded that ERα, independent of E2, is contributed in leptin-induced effects.

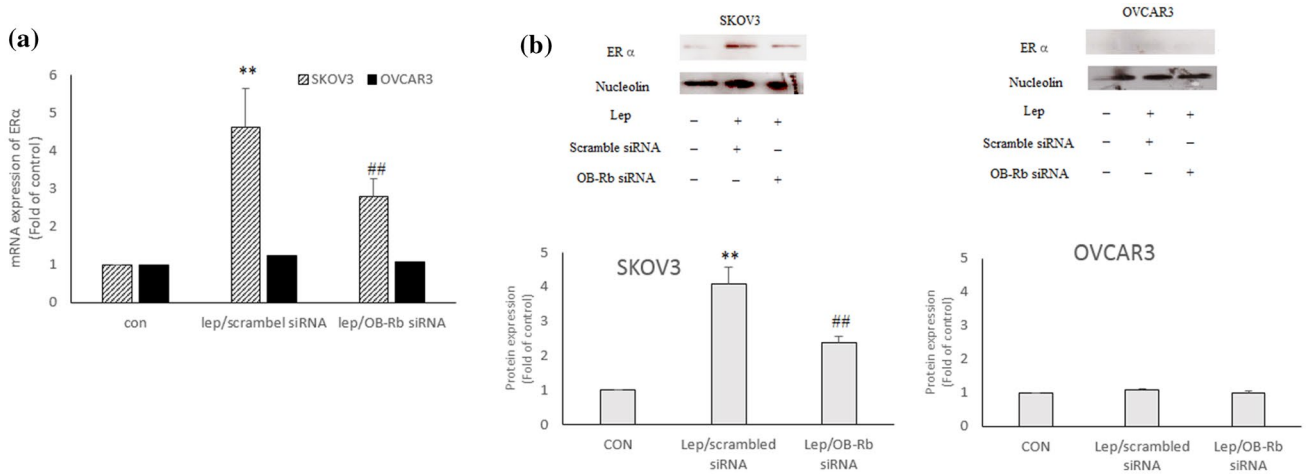

Fig. 2 Knockdown of OB-Rb by siRNA decreased mRNA and protein expressions of ERα induced by leptin treatment: ovarian cells were transfected with a scrambles sequence and OB-Rb siRNA (40 nM) for 72 h and incubated with leptin (100 ng/ml) for 24 h. mRNA and protein were extracted from transfected cells and subjected to Real-time PCR and western blotting. **a** The histogram represents the quantitative analysis of mRNA expression of ERα in the presence and/or absence of leptin. Values were normalized by GAPDH. **b** The protein levels of ERα in cell lysates were detected using specific antibodies. The data represent the mean ± SD ($n = 3$). Statistical analysis was performed by ANOVA (**$p < 0.01$ vs. non-treated control; $^{\#\#}p < 0.01$ vs. leptin/scramble treatment)

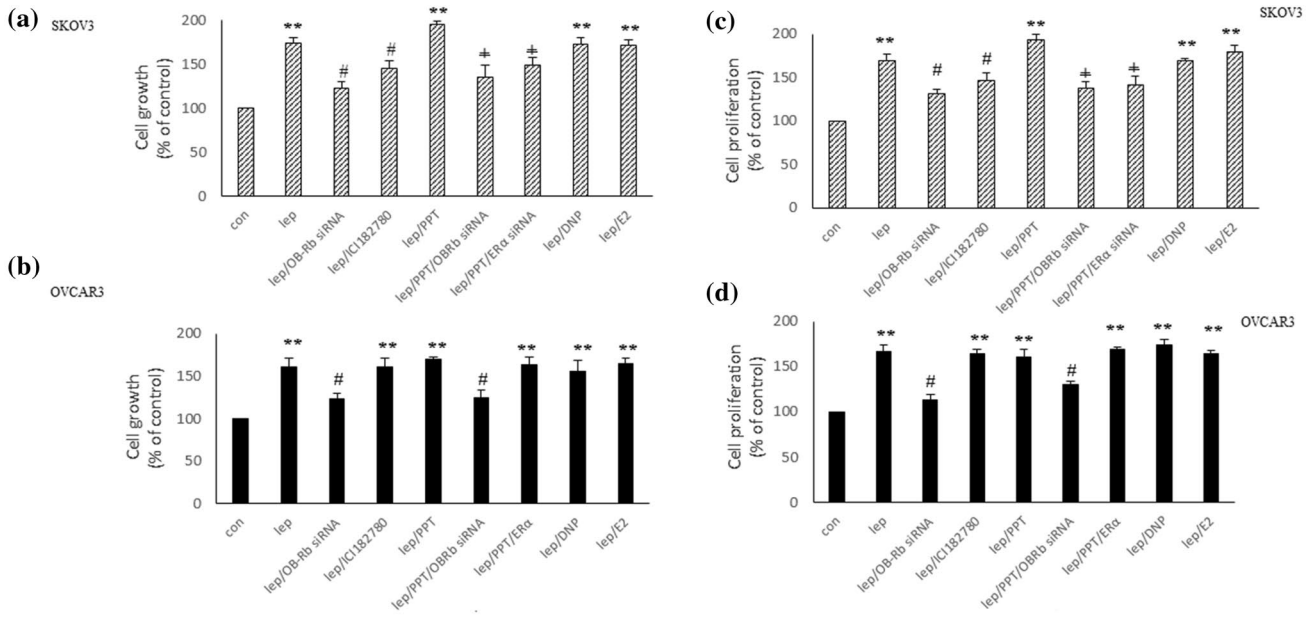

Fig. 3 ERα is involved in leptin-induced cell growth of SKOV3 cells in an E2-independent manner: SKOV3 and OVCAR3 cells were incubated with indicated treatments for 48 h, and cell growth was analyzed by MTT assay **(a**, **b)** and Brdu assay **(c**, **d)**. Results (mean ± SD) were calculated as the percentage of corresponding control values ($n = 3$). Statistical analysis was performed by ANOVA (**$p < 0.01$ vs. non-treated control; $^{\#\#}p < 0.01$ vs. leptin treatment; $^{+}p < 0.05$ vs. leptin/PPT treatment)

ERα is involved in leptin-induced cell invasion in SKOV3 cells

To analyze whether ER plays a pivotal role in leptin-mediated cell invasion in ovarian cancer, the cells were pre-incubated with ICI 182,780, and cell invasion was then analyzed in the presence of leptin using Matrigel invasion assay. Cell treatment with leptin enhanced cell invasion in SKOV3 cells (leptin: 4.9 ± 0.3 fold vs. control; $p < 0.01$), whereas 60-min pre-incubation with ICI 182,780 decreased the stimulative effect of leptin in ovarian cancer cell invasion (leptin: 4.9 ± 0.3 fold; leptin + ICI 182,780: 2.4 ± 0.6 fold; $p < 0.01$). In the OVCAR3 cells, although leptin promoted cell invasion, no significant decrease was observed in leptin-induced cell invasion in the presence of ICI 182,780 (Fig. 4a, b).

For further investigation of the effects of ERα or ERβ on leptin-mediated cell invasion, we evaluated the effect of ERα or ERβ gene silencing on the invasive effect of leptin in SKOV3 cells. As shown in Fig. 5a, b, cell transfection with siRNA targeting ERα leads to a significant reduction in cell invasion mediated by leptin (leptin: 4.9 ± 0.3 fold; leptin + ERα siRNA: 2.5 ± 0.6 fold; $p < 0.01$), whereas this influence was not detected in ERβ-siRNA or scrambled siRNA-transfected cells, demonstrating the crucial role of ERα, but not ERβ, in leptin-dependent ovarian cancer cell invasion. As observed in Fig. 5a, b, co-treatment with E2 (10^{-7} M) did not impact on leptin-mediated cell invasion in SKOV3 cells, suggesting the key role of ER in leptin impact in an E2-independent manner. These results indicated that ER is involved in leptin-promoted cell invasion in E2-insensitive ovarian cancer cell line.

ERα gene knockdown inhibited the stimulative effects of leptin on MMP9 expressions in SKOV3 cells

Previously, we demonstrated that upA, MMP7, and MMP9 overexpressions are contributed in leptin-mediated cell progression of ovarian cancer cells. To examine whether ERα is involved in overexpression of these proteins by leptin, we analyzed the effect of ERα gene silencing on the mRNA expression of them after stimulation by leptin. Real-time PCR results demonstrated that the transfection with siRNA targeting ERα gene, but not scrambled gene, had an inhibitory effect on leptin-induced expression of MMP9 (leptin: 8.8 ± 1 fold; leptin + ERα siRNA: 4.7 ± 0.4 fold; $p < 0.01$), but not on MMP7 and upA (Fig. 6a–c). In a separate experiment, the inhibitory effect of ERα gene silencing on leptin-mediated MMP9 overexpression was confirmed by western blotting (leptin: 6.6 ± 0.35 fold; leptin + ERα siRNA: 3.43 ± 0.37 fold; $p < 0.01$) (Fig. 6d). In addition, we confirmed MMP9 is contributed in leptin-mediated cell invasion by using Matrigel invasion assay. As shown in Fig. 6e, pretreatment with MMP9 inhibitor (Abcam, ab142180) significantly reduced the effect of leptin on cell invasion. These results suggest that ERα is involved in leptin-induced cell invasion by increasing MMP9 expression.

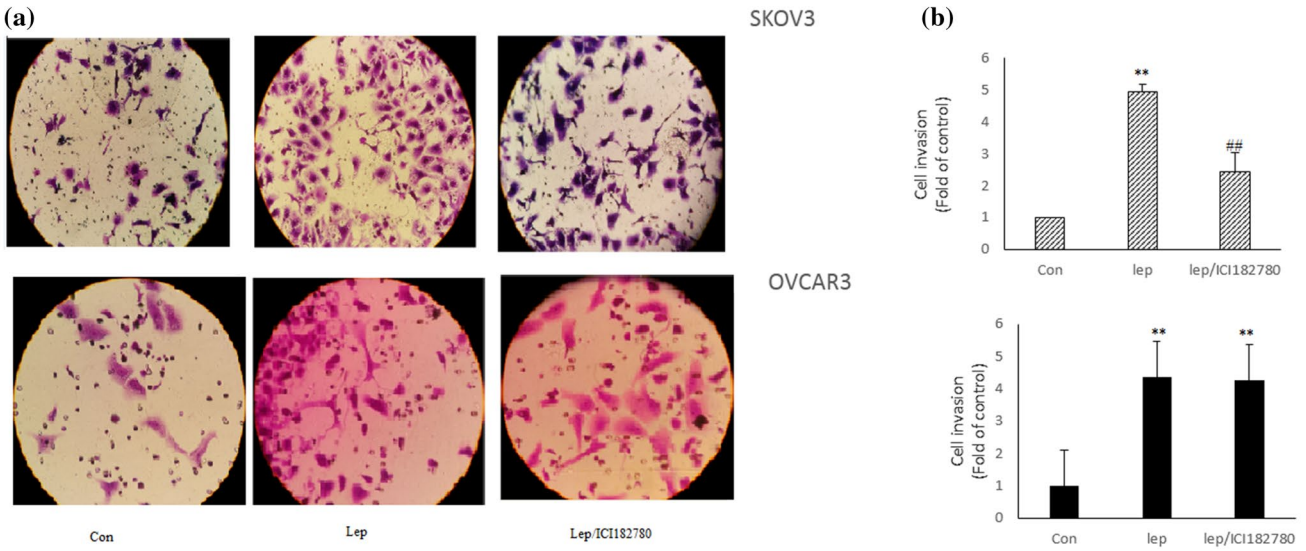

Fig. 4 Pretreatment with ICI 182,780 attenuated the effect of leptin on cell invasion of SKOV3 cells. **a** 1×10^5 cells were cultured in the upper chamber of Transwell inserts. Before 24-h incubation with leptin (100 ng/ml), SKOV3 and OVCAR3 cells were pre-incubated with ICI 182,780 (ER antagonist) for 60 min. **b** The histogram represents the quantitative analysis of the invaded cells. Cell invasion was calculated by counting the invaded cells in six randomly selected fields within each membrane; the values were averaged and presented as fold of control. All data shown were expressed as mean ± SD ($n=3$). Statistical analysis was performed by ANOVA (**$p<0.01$ vs. non-treated control; $^{\#\#}p<0.01$ vs. leptin treatment)

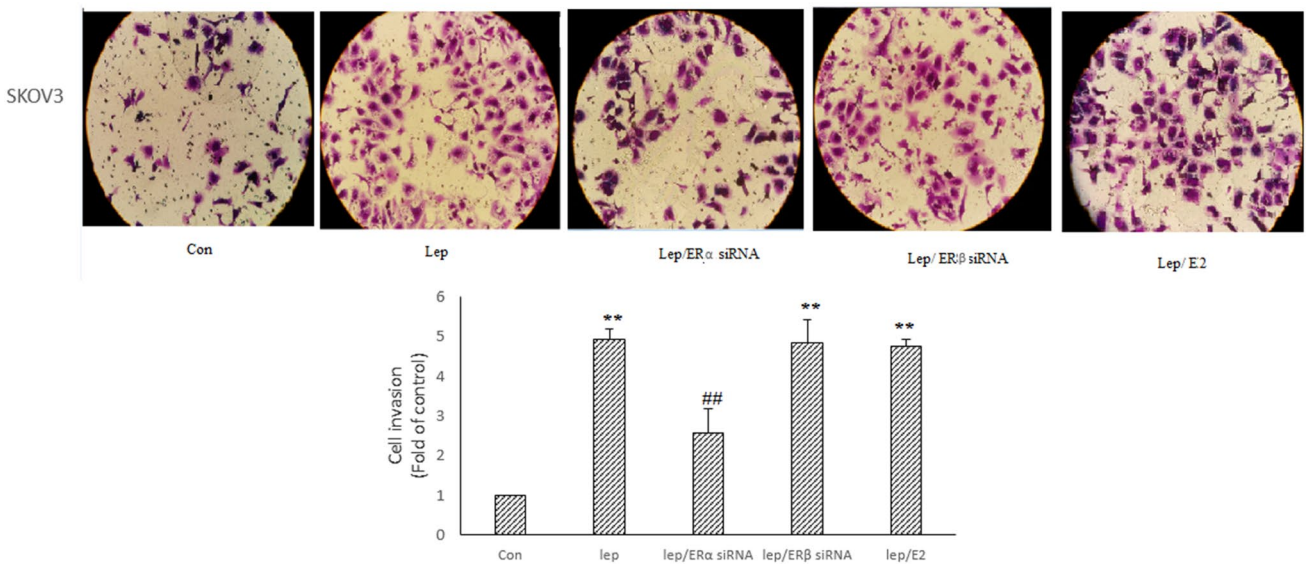

Fig. 5 ERα gene silencing blocked leptin-mediated cell invasion in SKOV3 cells. **a** 1×10^5 of siRNA-transfected cells and non-transfected cells were seeded in Transwell inserts, incubated with indicated treatment for 24 h and cell invasion was measured using Matrigel invasion assay as indicated. **b** The values represent an average of invaded cells in six randomly selected fields, which presented as fold of control. All data shown were expressed as mean ± SD ($n=3$). Statistical analysis was performed by ANOVA (**$p<0.01$ vs. non-treated control; $^{\#\#}p<0.01$ vs. leptin treatment)

Discussion

Obesity drives cancer progression by several mechanisms including alterations in leptin regulation [6, 7]. In this regard, several studies reported that leptin induces cell growth, proliferation, migration, and invasion in different cancer cell types [7, 10, 11]. In ovarian cancer, it was demonstrated that increased serum leptin levels promote cancer

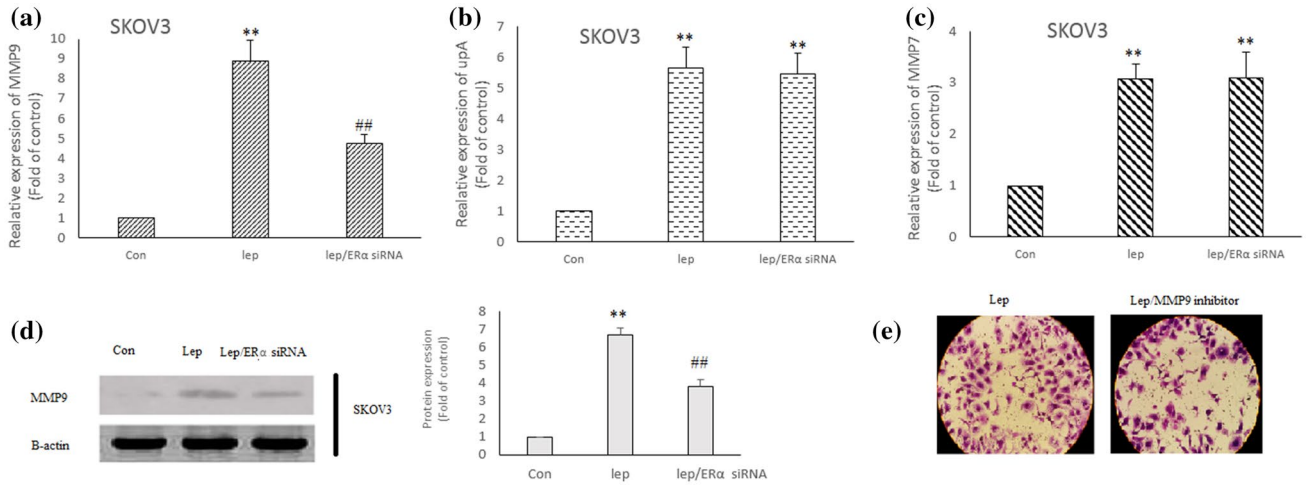

Fig. 6 The ERα is contributed in a leptin-mediated increase of MMP9 expression in SKOV3 cells. **a** Cells were transfected with ERα siRNA (40 nM), transfected cells were treated with leptin (100 ng/ml) for 24 h, total RNA was extracted, and then the mRNA expressions of the MMP7, MMP9, and upA were measured using Real-time PCR. **b** After transfection with siRNA, the cells were treated with leptin (100 ng/ml), total protein was extracted, and protein expression of MMP9 was detected by western blotting. The histogram represents the quantitative analysis of MMP9 protein expression, which was normalized for B-actin expression. **c** SKOV3 cells were pretreated with MMP9 inhibitor for 60 min before 24-h treatment with leptin and then cell invasion was analyzed using Matrigel invasion assay. All data shown were expressed as mean ± SD ($n = 3$). Statistical analysis was performed by ANOVA (**$p < 0.01$ vs. control. ##$p < 0.05$ vs. leptin)

development and progression through OB-Rb signaling pathways [17]. However, the exact molecular mechanisms underlying the response to leptin are still poorly known. In this investigation, we focused on the contribution of ERα/β in leptin-induced cell invasion in ovarian cancer cell lines. Previously, we reported that leptin induces cell invasion and migration in SKOV3 and OVCAR3 cells, but these effects were not observed in CaoV-3 cells which do not express OB-Rb [18, 19]. Previous investigations have also demonstrated that a leptin/OB-Rb axis may crosstalk with the ER and promote tumor development and cancer progression [32, 34, 36]. Therefore, we hypothesized that ERα/β are involved in leptin-mediated effect in ovarian cancer. The data presented here show that ERα, but not ERβ, as well as MMP9 overexpression have an essential role in the leptin-mediated cell proliferation and invasion in a ligand-independent manner in ovarian cancer.

Apart from E2, it was indicated that some factors, including growth factors [41] and leptin [32, 33] may also induce ER activation. In addition, leptin overexpressed ERα in the absence of E2 in BG-1, an E2-sensitive ovarian cancer cell line. In our experiment, we observed an increased expression of ERα in response to different concentrations of leptin in SKOV3, an E2-insensitive ovarian cancer cell line. Furthermore, we observed blocking of OB-Rb expression using siRNA attenuated leptin-induced ERα expression in SKOV3 cells, confirming a directional crosstalk between leptin/OB-Rb axis and ERα in ovarian cancer. In this line, the inductive influence of leptin and the inhibitory effect of OB-Rb blocking on ERα expression were reported in ER-positive breast cancer cell lines [34, 42–45]. In addition, increased level of ERα expression was detected following treatment with leptin in ovarian cancer [29], prostate cancer [46], and ATDC5 cells [47, 48]. In vivo studies on endometrial carcinoma [38] and breast cancer [49] also demonstrated a positive correlation between OB-Rb expression and ER status. In contrast with these results, Shen et al. reported that leptin has no significant effect on ERα expression, whereas ERβ expression is markedly increased in leptin-incubated HepG2 cells compared to non-treated control cells [50].

Previously, Chen et al. reported an increase in cell growth following treatment with ERα agonist (PPT) in SKOV3 and OV2008 ovarian cancer cells [51]. On the other hand, Choi et al. revealed the contribution of ERα pathway in leptin-mediated cell growth in ovarian cancer cells [29]. In the present study, we found that blocking of ERα or OB-Rb significantly decreased leptin-promoted cell growth and proliferation in SKOV3 cells. In addition, an increase in leptin-mediated cell growth and proliferation was observed after treatment with PPT, but not DNP, which was decreased after OB-Rb gene silencing by siRNA. These findings confirmed that the crosstalk between ERα and OB-Rb is involved in cell growth and proliferation that was increased following treatment with leptin. In this line, Fusco et al. demonstrated a crosstalk between OB-Rb and ERα, which plays a key role in leptin-mediated cell proliferation in breast cancer cells [34]. In another study, it was indicated that ERα is involved

in cell proliferation induced by leptin through autophagy indication [43]. Moreover, leptin-promoted proliferative influence in prostate cancer was attributed to up-regulation of ERα, aromatase, and cytochrome P450 1B1 (CYP1B1) enzymes [46].

The clinical significance of ER subtypes and variants in ovarian cancer cell invasion was revealed [52, 53]. Recently, Haque et al. reported that leptin induces cancer cell invasion in ERα-positive cell lines, but not ERα-negative breast cancer cells [44]. In another study, it was also revealed that leptin produced by obese adipose stromal/stem cells (obASCs) markedly increases the expression of EMT and metastasis genes in ER+ breast cancer cells [45].

Using ER antagonist and knockdown of ERα by siRNA, for the first time, we revealed a pivotal role of ERα in the leptin-induced cell invasion in ovarian cancer cells. Our findings also demonstrated that cell treatment with E2 showed no significant influence on both cell proliferation and cell invasion promoted by leptin in SKOV3 cells, indicating the E2-independent role of ERα in leptin effect. In our previous studies, we demonstrated that MMP7, MMP9, and upA play key roles in leptin-promoted cancer progression in SKOV3 ovarian cancer cells [18, 19]. Accordingly, we examined the role of ERα in overexpression of these proteins by leptin in SKOV3 cells. No significant reduction was detected in the leptin-mediated expressions of MMP7 and upA after knockdown of ERα by siRNA. However, ERα gene silencing markedly reduced the expression of MMP9 induced by leptin. In addition, our results showed that inhibition of MMP9 significantly reduced leptin-mediated cell invasion of SKOV3 cells, suggesting that ERα has an important role in leptin-promoted cell invasion in ovarian cancer cells by affecting MMP9.

Conclusion

Briefly, in the present study, we revealed a crosstalk between leptin/OB-Rb axis and ERα for cancer growth, and confirmed the E2-independent effect of ERα in leptin-promoted cell growth in ovarian cancer cells. We also demonstrated that ERα is involved in leptin-mediated cell invasion and MMP9 expression of ovarian cancer cell line. Taken together, these results provide a new mechanism for leptin-induced cancer cell progression in ovarian cancer.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

References

1. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 384:766–781
2. Organization WH (2016) World Health Organization obesity and overweight fact sheet
3. Picon-Ruiz M, Morata-Tarifa C, Valle-Goffin JJ, Friedman ER, Slingerland JM (2017) Obesity and adverse breast cancer risk and outcome: mechanistic insights and strategies for intervention. CA: Cancer J Clin 67:378–397
4. Shaw E, Farris M, McNeil J, Friedenreich C (2016) Obesity and endometrial cancer. Obesity and Cancer. Springer, Cham, pp 107–136
5. Tworoger SS, Huang T (2016) Obesity and ovarian cancer. Obesity and Cancer. Springer, Cham, pp 155–176
6. Roberts DL, Dive C, Renehan AG (2010) Biological mechanisms linking obesity and cancer risk: new perspectives. Annu Rev Med 61:301–316
7. Louie SM, Roberts LS, Nomura DK (2013) Mechanisms linking obesity and cancer. Biochim Biophys Acta (BBA)-Mol Cell Biol Lipids 1831:1499–1508
8. Trayhurn P, Bing C (2006) Appetite and energy balance signals from adipocytes. Philos Trans R Soc Lond B 361:1237–1249
9. Margetic S, Gazzola C, Pegg G, Hill R (2002) Leptin: a review of its peripheral actions and interactions. Int J Obes 26:1407
10. VanSaun MN (2013) Molecular pathways: adiponectin and leptin signaling in cancer. Clin Cancer Res. https://doi.org/10.1158/1078-0432.CCR-12-0930
11. Ray A, Cleary MP (2017) The potential role of leptin in tumor invasion and metastasis. Cytokine Growth Factor Rev. https://doi.org/10.1016/j.cytogfr.2017.11.002
12. Vargas AN (2014) Natural history of ovarian cancer. Ecancermedicalscience 8:465. https://doi.org/10.3332/ecancer.2014.465
13. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA: Cancer J Clin 61:69–90
14. Romero I, Bast RC Jr (2012) Minireview: human ovarian cancer: biology, current management, and paths to personalizing therapy. Endocrinology 153:1593–1602
15. Lengyel E (2010) Ovarian cancer development and metastasis. Am J Pathol 177:1053–1064
16. Leitzmann MF, Koebnick C, Danforth KN, Brinton LA, Moore SC, Hollenbeck AR, Schatzkin A, Lacey JV Jr (2009) Body mass index and risk of ovarian cancer. Cancer: Interdiscip Int J Am Cancer Soc 115:812–822
17. Uddin S, Bu R, Ahmed M, Abubaker J, Al-Dayel F, Bavi P, Al-Kuraya KS (2009) Overexpression of leptin receptor predicts an unfavorable outcome in Middle Eastern ovarian cancer. Mol Cancer 8:74
18. Ghasemi A, Hashemy SI, Aghaei M, Panjehpour M (2017) RhoA/ROCK pathway mediates leptin-induced uPA expression to promote cell invasion in ovarian cancer cells. Cell Signal 32:104–114
19. Ghasemi A, Hashemy SI, Aghaei M, Panjehpour M (2018) Leptin induces matrix metalloproteinase 7 expression to promote ovarian cancer cell invasion by activating ERK and JNK pathways. J Cell Biochem 119:2333–2344

20. Kato S, Abarzua-Catalan L, Trigo C, Delpiano A, Sanhueza C, García K, Ibañez C, Hormazábal K, Diaz D, Brañes J (2015) Leptin stimulates migration and invasion and maintains cancer stem-like properties in ovarian cancer cells: an explanation for poor outcomes in obese women. Oncotarget 6:21100
21. Cunat S, Hoffmann P, Pujol P (2004) Estrogens and epithelial ovarian cancer. Gynecol Oncol 94:25–32
22. Matthews J, Gustafsson J-Å (2003) Estrogen signaling: a subtle balance between ERα and ERβ. Mol Interv 3:281
23. Brandenberger AW, Tee MK, Jaffe RB (1998) Estrogen receptor alpha (ER-α) and beta (ER-β) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-β in neoplastic tissues. J Clin Endocrinol Metab 83:1025–1028
24. Liao XH, Lu DL, Wang N, Liu LY, Wang Y, Li YQ, Yan TB, Sun XG, Hu P, Zhang TC (2014) Estrogen receptor α mediates proliferation of breast cancer MCF-7 cells via a p21/PCNA/E2F1-dependent pathway. FEBS J 281:927–942
25. Lazennec G (2006) Estrogen receptor beta, a possible tumor suppressor involved in ovarian carcinogenesis. Cancer Lett 231:151–157
26. Andersen CL, Sikora MJ, Boisen MM, Ma T, Christie A, Tseng G, Park YS, Luthra S, Chandran U, Haluska P (2017) Active estrogen receptor-alpha signaling in ovarian cancer models and clinical specimens. Clin Cancer Res. https://doi.org/10.1158/1078-0432.CCR-16-1501
27. Lau K-M, Mok SC, Ho S-M (1999) Expression of human estrogen receptor-α and -β, progesterone receptor, and androgen receptor mRNA in normal and malignant ovarian epithelial cells. Proc Natl Acad Sci 96:5722–5727
28. Vierikko P, Kauppila A, Vihko R (1983) Cytosol and nuclear estrogen and progestin receptors and 17 beta-hydroxysteroid dehydrogenase activity in non-diseased tissue and in benign and malignant tumors of the human ovary. Int J Cancer 32:413–422
29. Choi J-H, Lee K-T, Leung PC (2010) Estrogen receptor alpha pathway is involved in leptin-induced ovarian cancer cell growth. Carcinogenesis 32:589–596
30. Rutherford T, Brown WD, Sapi E, Aschkenazi S, Muñoz A, Mor G (2000) Absence of estrogen receptor-β expression in metastatic ovarian cancer. Obstet Gynecol 96:417–421
31. Pujol P, Rey J-M, Nirde P, Roger P, Gastaldi M, Laffargue F, Rochefort H, Maudelonde T (1998) Differential expression of estrogen receptor-α and-β messenger RNAs as a potential marker of ovarian carcinogenesis. Cancer Res 58:5367–5373
32. Catalano S, Mauro L, Marsico S, Giordano C, Rizza P, Rago V, Montanaro D, Maggiolini M, Panno ML, Andó S (2004) Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor α in MCF-7 cells. J Biol Chem 279:19908–19915
33. Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Andò S (2003) Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. J Biol Chem 278:28668–28676
34. Fusco R, Galgani M, Procaccini C, Franco R, Pirozzi G, Fucci L, Laccetti P, Matarese G (2010) Cellular and molecular crosstalk between leptin receptor and estrogen receptor-α in breast cancer: molecular basis for a novel therapeutic setting. Endocr Relat Cancer 17:373–382
35. Binai NA, Damert A, Carra G, Steckelbroeck S, Löwer J, Löwer R, Wessler S (2010) Expression of estrogen receptor alpha increases leptin-induced STAT3 activity in breast cancer cells. Int J Cancer 127:55–66
36. Ray A, Nkhata KJ, Cleary MP (2007) Effects of leptin on human breast cancer cell lines in relationship to estrogen receptor and HER2 status. Int J Oncol 30:1499–1509
37. Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H (1999) Leptin directly stimulates aromatase activity in human luteinized granulosa cells. Mol Hum Reprod 5:708–713
38. Méndez-López LF, Zavala-Pompa A, Cortés-Gutiérrez EI, Cerda-Flores RM, Davila-Rodriguez MI (2017) Leptin receptor expression during the progression of endometrial carcinoma is correlated with estrogen and progesterone receptors. Arch Med Sci: AMS 13:228
39. Garofalo C, Sisci D, Surmacz E (2004) Leptin interferes with the effects of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells. Clin Cancer Res 10:6466–6475
40. Hua W, Christianson T, Rougeot C, Rochefort H, Clinton GM (1995) SKOV3 ovarian carcinoma cells have functional estrogen receptor but are growth-resistant to estrogen and antiestrogens. J Steroid Biochem Mol Biol 55:279–289
41. Hall JM, Couse JF, Korach KS (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276:36869–36872
42. Chen X, Zha X, Chen W, Zhu T, Qiu J, Røe OD, Li J, Wang Z, Yin Y (2013) Leptin attenuates the anti-estrogen effect of tamoxifen in breast cancer. Biomed Pharmacother 67:22–30
43. Raut PK, Choi DY, Kim SH, Hong JT, Kwon TK, Jeong JH, Park P-H (2017) Estrogen receptor signaling mediates leptin-induced growth of breast cancer cells via autophagy induction. Oncotarget 8:109417
44. Haque I, Ghosh A, Acup S, Banerjee S, Dhar K, Ray A, Sarkar S, Kambhampati S, Banerjee SK (2018) Leptin-induced ER-α-positive breast cancer cell viability and migration is mediated by suppressing CCN5-signaling via activating JAK/AKT/STAT-pathway. BMC Cancer 18:99
45. Strong AL, Ohlstein JF, Biagas BA, Rhodes LV, Pei DT, Tucker HA, Llamas C, Bowles AC, Dutreil MF, Zhang S (2015) Leptin produced by obese adipose stromal/stem cells enhances proliferation and metastasis of estrogen receptor positive breast cancers. Breast Cancer Res 17:112
46. Habib CN, Al-Abd AM, Tolba MF, Khalifa AE, Khedr A, Mosli HA, Abdel-Naim AB (2015) Leptin influences estrogen metabolism and accelerates prostate cell proliferation. Life Sci 121:10–15
47. Li X-F, Wang S-J, Jiang L-S, Dai L-Y (2013) Stage specific effect of leptin on the expressions of estrogen receptor and extracellular matrix in a model of chondrocyte differentiation. Cytokine 61:876–884
48. Wang SJ, Li XF, Jiang LS, Dai LY (2012) Leptin regulates estrogen receptor gene expression in ATDC5 cells through the extracellular signal regulated kinase signaling pathway. J Cell Biochem 113:1323–1332
49. Koda M, Kanczuga-Koda L, Sulkowska M, Surmacz E, Sulkowski S (2010) Relationships between hypoxia markers and the leptin system, estrogen receptors in human primary and metastatic breast cancer: effects of preoperative chemotherapy. BMC Cancer 10:320
50. Shen M, Shi H (2016) Estradiol and estrogen receptor agonists oppose oncogenic actions of leptin in HepG2 cells. PLoS ONE 11:e0151455
51. Chan KK-L, Leung TH-Y, Chan DW, Wei N, Lau GT-Y, Liu SS, Siu MK, Ngan HY-S (2014) Targeting estrogen receptor subtypes (ERα and ERβ) with selective ER modulators in ovarian cancer. J Endocrinol 221:325–336
52. Chan KK, Siu MK, Jiang Y-X, Wang J-J, Wang Y, Leung TH, Liu SS, Cheung AN, Ngan HY (2017) Differential expression of estrogen receptor subtypes and variants in ovarian cancer: effects on cell invasion, proliferation and prognosis. BMC Cancer 17:606
53. Lu Z, Zhang Y, Yan X, Chen Y, Tao X, Wang J, Jia N, Lyu T, Wang J, Ding J (2014) Estrogen stimulates the invasion of ovarian cancer cells via activation of the PI3K/AKT pathway and

regulation of its downstream targets E-cadherin and α-actinin-4. Mol Med Rep 10:2433–2440