Histol Histopathol (2017) 32: 1187-1196

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Effect of estradiol on the expression of angiogenic factors in epithelial ovarian cancer

Macarena Valladares[1,2*], Francisca Plaza-Parrochia[1*], Macarena Lépez[1], Daniela López[1], Fernando Gabler[3], Patricio Gayan[4], Alberto Selman[5], Margarita Vega[1,5] and Carmen Romero[1,5]

[1]Laboratory of Endocrinology and Reproduction Biology, Clinical Hospital, University of Chile, [2]Biotechnology Laboratory, Bernardo O'Higgins University, [3]Department of Pathology, School of Medicine, San Borja Arriarán Clinical Hospital, University of Chile, [4]National Institute of Cancer and [5]Department of Obstetrics and Gynecology, School of Medicine, Clinical Hospital, University of Chile, Santiago, Chile

*Equally first authors

Summary. Introduction: Ovarian cancer presents a high angiogenesis (formation of new blood vessels) regulated by pro-angiogenic factors, mainly vascular endothelial growth factor (VEGF) and nerve growth factor (NGF). An association between endogenous levels of estrogen and increased risk of developing ovarian cancer has been reported. Estrogen action is mediated by the binding to its specific receptors (ERα and ERβ), altered ERα/ERβ ratio may constitute a marker of ovarian carcinogenesis progression. Objective: To determine the effect of estradiol through ERα on the expression of NGF and VEGF in epithelial ovarian cancer (EOC). Methodology: Levels of phosphorylated estrogen receptor alpha (pERα) were evaluated in well, moderate and poorly differentiated EOC samples (EOC-I, EOC-II, EOC-III). Additionally, ovarian cancer explants were stimulated with NGF (0, 10 and 100 ng/ml) and ERα, ERβ and pERα levels were detected. Finally, human ovarian surface epithelial (HOSE) and epithelial ovarian cancer (A2780) cell lines were stimulated with estradiol, where NGF and VEGF protein levels were evaluated. Results: In tissues, ERs were detected being pERα levels significantly increased in EOC-III samples compared with EOC-I ($p<0.05$). Additionally, ovarian explants treated with NGF increased pERα levels meanwhile total ERα and ERβ levels did not change. Cell lines stimulated with estradiol revealed an increase of NGF and VEGF protein levels ($p<0.05$). Conclusions: Estradiol has a positive effect on pro-angiogenic factors such as NGF and VEGF expression in EOC, probably through the activation of ERα; generating a positive loop induced by NGF increasing pERα levels in epithelial ovarian cells.

Key words: Epithelial Ovarian cancer, NGF, VEGF, Estradiol, Estradiol Receptors

Offprint requests to: Carmen Romero, Laboratory of Endocrinology and Reproduction Biology, Clinical Hospital, University of Chile, Department of Obstetrics and Gynecology, School of Medicine, Clinical Hospital, University of Chile, Santos Dumont # 999, Santiago, Chile. e-mail: cromero@hcuch.cl

DOI: 10.14670/HH-11-874

Introduction

Ovarian cancer is the fourth leading cause of cancer death in western populations being the epithelial ovarian cancer (EOC) the most common among them (80% - 90%). Also, it constitutes a gynecological condition that presents high lethality mainly due to few symptoms associated, resulting in late detection of the disease (Ali et al., 2012; Stasenko et al., 2015). Different factors have been associated with a high risk of developing ovarian cancer, such as hormonal replacement therapy, family history and infertility (Leitzmann et al., 2009), where estrogens may contribute to ovarian epithelium alterations that could condition to cell malignancy (Risch, 1998; Wright et al., 2011; Mungenast and Thalhammer, 2014).

Among the main features associated with the development of ovarian cancer is high angiogenesis (formation of new blood vessels), regulated mainly by

vascular endothelial growth factor (VEGF) (the most important angiogenic factor), by nerve growth factor (NGF) and its specific receptor tyrosine/kinase TRKA (Lukanova and Kaaks, 2005; Tapia et al., 2011). In EOC, the process of angiogenesis is reflected by high protein and transcript levels of VEGF, partly stimulated by NGF, respect to normal ovaries (Julio-Pieper et al., 2006, 2009; Campos et al., 2007). This contributes to the high growth and aggressiveness (metastasis) of ovarian cancer that result in a low rate of survival.

On the other hand, there is evidence of an association between circulating estrogen levels and increased risk of developing female neoplasias such as breast, endometrial and ovarian cancer (Brown and Hankinson, 2015); nevertheless, controversies exist regarding that hormonal replacement therapy might increase the risk of ovarian cancer (Anderson et al., 2003). As known, canonical estrogen action is initiated by its binding to specific receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ) that belong to the superfamily of nuclear receptors expressed in normal and malignant ovarian cells (Enmark and Gustafsson, 1999). This mechanism of action involves the binding of estrogen-receptor complex to DNA specific sites known as estrogen-response elements (ERE) and the subsequent activation of ER specific genes like proliferation-related genes. It is known that VEGF and NGF genes have estrogen-response elements (Toran-Allerand, 1996a; Scharfman and MacLusky, 2008). This action is through the formation of homodimers (ERα/ERα, ERβ/ERβ) and/or heterodimers (ERα/ERβ) with the concomitant stimulation of ovarian epithelial cell proliferation (Cunat et al., 2004). The ERβ isoform is highly expressed in normal ovarian surface epithelium (OSE) and benign tumors, whereas ERα is highly expressed in malignant ovarian tumors. Therefore, the transcript of ERβ decreases with tumor progression (Rutherford et al., 2000). This is in agreement with the differential expression of ERα or β during ovarian carcinogenesis, being ERα over-expressed in cancer compared to ERβ, which suggests that estrogen-induced proteins may act as ovarian tumor-promoting agents (Cunat et al., 2004). Moreover, altered ERα/ERβ ratio may be considered as a marker of ovarian carcinogenesis (Cunat et al., 2004).

In addition to estradiol binding, the activity of ER is modulated by phosphorylation at several key amino-acidic residues. In fact, the activation of ER could involve the phosphorylation of serine residues (Ser), which appears to influence the recruitment of coactivators, resulting in the potentiation of the transcriptional process mediated by this receptor. The sites of phosphorylation of ERα could be Ser104, Ser106 and Ser118; the latter being the major site of phosphorylation in response to estrogen or activation by MAPK (le Goff et al., 1994; Chen et al., 2002).

Based on the above evidences and in consideration that VEGF and NGF genes have estrogen-response elements besides the high levels of these pro-angiogenic factors and high angiogenesis observed in EOC, the main objective of this study was to determine whether estradiol through its specific receptors modifies the expression of pro-angiogenic factors such as NGF and VEGF in EOC and whether NGF induces the activation of pERα in epithelial ovarian cells.

Materials and methods

Subjects

Ovarian tissue samples were obtained from the Pathology Department of the University of Chile Clinical Hospital and from the National Cancer Institute. The women who participated in the study gave written informed consent, approved by the ethics committee of the different institutions that participated.

Ex-vivo studies

Paraffin-embedded ovarian tissues of the four groups of patients were obtained. The samples were classified into four study groups by an experienced pathologist: Inactive Normal Ovary (I-Ov) (n=9), Serous Epithelial Ovarian Carcinoma with well differentiated grade I (EOC I) (n=12), moderate differentiated grade II (EOC II) (n=12) and poorly differentiated grade III (EOC III) (n=12). These samples were subjected to immunohistochemistry for the detection of ERα, ERβ and pERα.

In vitro studies

Explant studies: Forty five fresh samples (100 mg approximately) from epithelial ovarian cancer were cultured for 2 h in 24 well plates. Each well contained 1 mL of DMEM/Ham-F12 (Sigma Chemicals, St Louis, MO, USA) supplemented with bicarbonate (600mg/L), penicillin (50 mg/L), gentamicin (80 mg/L), streptomycin (50 mg/L) and ketoconazole (5 mg/L) in the presence of increasing concentrations of NGF (10 and 100 ng/ml) for 2 h (Sigma, St. Louis, MO, USA). Further, the explants were fixed in Bouin and embedded in paraffin for immunohistochemical studies to evaluate different isoforms of ERs.

Cell lines studies

The cell lines HOSE and A2780 were used. HOSE cells were obtained from normal human ovarian surface epithelial of a postmenopausal patient, immortalized with SV40-Tag (Maines-Bandiera et al., 1992). A2780 is a drug-sensitive human ovarian cancer cell line with epithelial morphology that was established using EOC tissue from an untreated patient (Hamilton et al., 1984). Both cell lines are representative models of control epithelial ovarian tissue and ovarian cancer tissue, respectively, with respect to morphology and expression of NGF and TRKA (Tapia et al., 2011; Vera et al., 2012).

The cells were propagated in DMEM/Ham-F12 medium without phenol red (Sigma- Aldrich Co. Saint

Louis, MO, USA) supplemented with 10% FBS in the presence of 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate and 25 g/ml Amphoterecin B (Hyclone™ Thermo Fisher Scientific, Rochester, NY, USA) and cultured at 37°C with 5% CO_2.

The cells were cultured for 24 h at a ratio of 200,000 cells/plate treated with $3.7x10^{-11}$, $3.7x10^{-9}$ and $3.7x10^{-7}$ mM of estradiol in DMEM HAM F12 without serum. An immunocytochemistry in 4-well Lab-Tek® II Chamber Slides™ was done to evaluate NGF and VEGF content.

Immunohistochemistry

This technique was used to assess the localization and semi-quantitation of total ERα, ERβ and pERα Ser118 proteins in tissues from the four studied groups and EOC explants treated with NGF.

Immunostaining was performed on 5-µm sections of formalin fixed paraffin-embedded ovarian biopsies. Briefly, tissue sections were deparaffinized in xylene and hydrated in a series of graded alcohols. The sections were incubated in an antigen retrieval solution (10 mM sodium citrate buffer, pH 6) at 95°C for 20 min. Endogenous peroxidase activity was prevented by incubating the samples in 3% hydrogen peroxide for 5 min. Nonspecific antibody binding was blocked with kit Histostain SP (Zymed Laboratories Inc, San Francisco, CA, USA). The ovarian explants were incubated for 18 h at 4°C with specific antibodies anti-ERα (sc-8005, Santa Cruz, CA, USA) (1:50), ERβ (sc-6820, Santa Cruz, CA, USA) (1:50) and pERα Ser 118 (#2511, Cell Signaling Technology®, Danvers, MA, USA). Negative controls were analyzed on adjacent sections incubated without the primary antibody and using non-immune species specific antisera. The slides were incubated for 20 min with the biotinylated anti-mouse or anti-rabbit secondary antibody (1:300). The reaction was developed by the streptavidin-peroxidase system, and DAB (3-3' diaminobenzidine) was used as the chromogen; counterstaining was carried out with hematoxylin. The slides were evaluated in a Nikon optical microscope (Nikon Inc., Melville, NY, USA). Each sample was evaluated by percentage (%) of positive stained cells obtained from counting 1000 cells and by H-Score; intensity of brown staining was evaluated as 1 (mild), 2 (moderate) and 3 (severe), considering the % of positive cells as the sum of H-Score 2 and 3 intensities.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature and permeabilized with 0.1% Triton X 100 in PBS at room temperature for 10 min. Endogenous peroxidase blocking was performed with hydrogen peroxide at 3% for 15 min. Non-specific binding was blocked using 5% milk in PBS for 10 min. After overnight incubation with anti-ERα (#sc-8005, Santa Cruz Biotechnology, CA, USA) (1:50), anti-ERβ (#sc-6820, Santa Cruz Biotechnology, CA, USA) (1:50), anti-VEGF, which detect VEGF121 (#05-443, Upstate, Lake Placid, NY, USA) (1:1000) or anti-NGFβ (#ab64136, Abcam, Cambridge, UK) (1:1000), the anti-rabbit and anti-mouse secondary antibodies (1:300) were applied for 30 min at 37°C. DAB staining was performed and counterstained with Harris Hematoxylin (1:5). Finally, the material was dehydrated with graded alcohols (70-95-100-100%) and cleared in xylene, coverslipped and examined under an optical microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan). Images were acquired with a MicroPublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada) (Tapia et al., 2011). The evaluation of brown staining was done by Image Pro Plus 6.1 software, measuring IOD (Integrated optical density) expressed as arbitrary units (AU).

Statistical analysis

To calculate the number of tissues and *in vitro* experiments per group was assuming α=0.05 and β=0.2, a difference between means of 0.25 and standard deviation according to our previous studies (Tapia et al., 2011). Comparisons between groups were performed by Kruskal-Wallis and Dunns post- test. p-values <0.05 were considered significant. Statistical tests were performed using Graph Pad Prism 6.0.

All procedures performed in these studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its subsequent amendments or comparable ethical standards.

Results

Phosphorylation of estrogen receptor α (pERα) in epithelial ovarian cancer samples

Phosphorylation rate of steroid receptors can be considered as an approach of its transcriptional activity. A semi quantitative analysis of phosphorylated ERα (pERα) levels were evaluated in well, moderate and poorly differentiated epithelial ovarian cancer samples (EOC-I; EOC-II and EOC-III) and compared with I-Ov by the immunohistochemical technique. The pERα was localized principally in the epithelium of the four study groups, as shown in Fig. 1A. The protein levels of the phosphorylated receptor were similar in groups I-Ov, EOC I and EOC II ($p>0.05$). However, in EOC III, levels of pERα significantly increased compared with EOC I ($p<0.05$) (Fig. 1B).

Correlation between the expression of TRKA receptor and pERα in EOC

Furthermore, the activation of pERα can be enhanced by the action of growth factors such as NGF through its TRKA receptor; the data of TRKA was

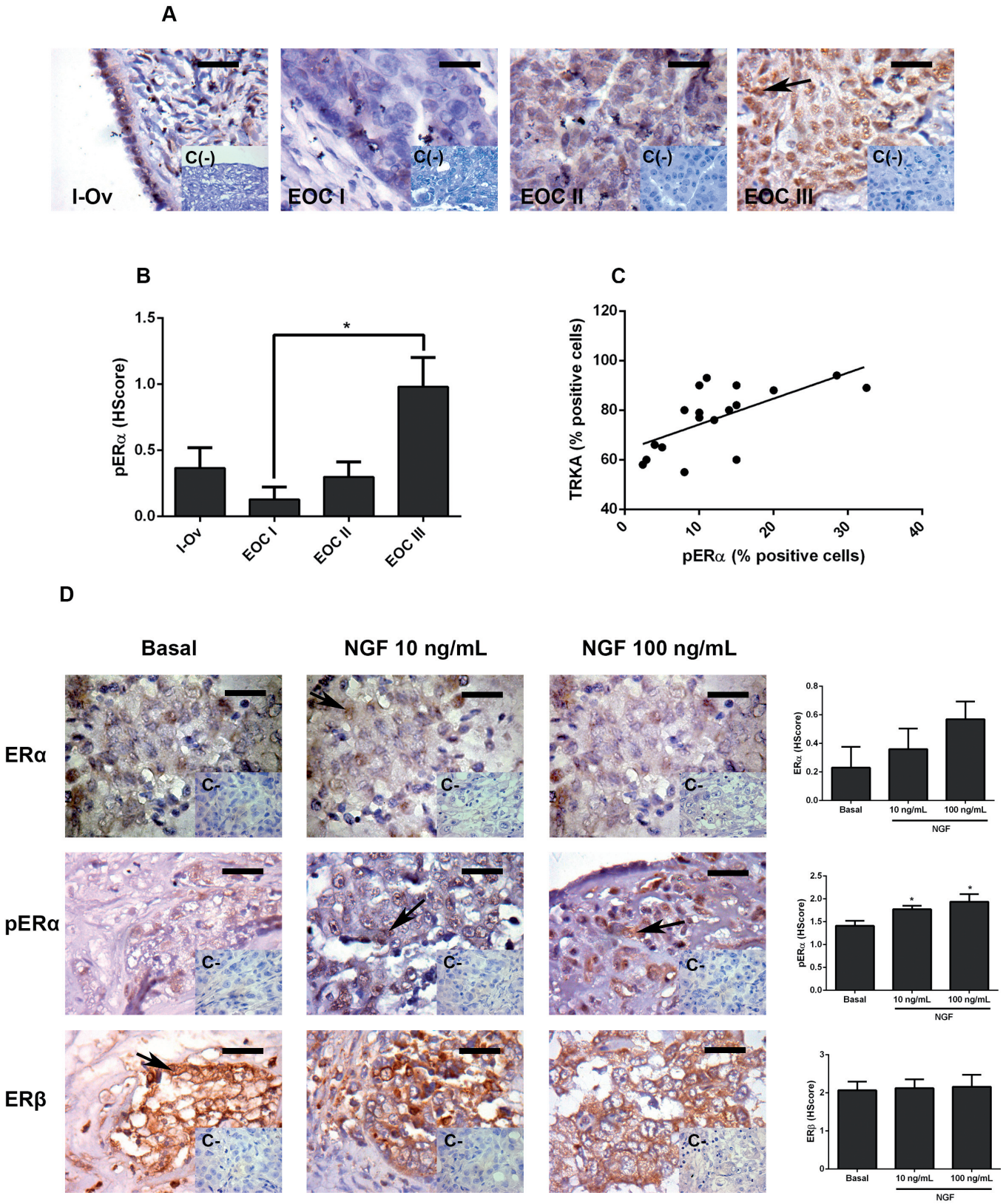

Fig. 1. A. Immunohistochemistry of phosphorylated estrogen receptor α (pERα) in ovarian tissues from inactive ovaries (I-Ov) and epithelial ovarian cancer I, II or III (EOC I, EOC II or EOC III). **B.** The semi-quantitative analysis was performed by HScore. **C.** Graph of correlation between percentages of tyrosine receptor kinase A (TRKA) positive cells and estrogen receptor α phosphorylated (pERα) positive cells in epithelial ovarian cancer tissues in different grade of differentiation. R^2=0.42 and p=0.004. **D.** Immunohistochemistry of estrogen receptor α (ERα), estrogen receptor α phosphorylated (pERα) and estrogen receptor β (ERβ) of explants of ovarian tissue stimulated with nerve growth factor (NGF) at 10 and 100 ng/mL for 2 h. The semi-quantitative analysis were performed by HScore, * p<0.05 compared to basal condition. Arrows: positive staining. Insert: C (-): negative control. The results were expressed as mean + standard error of the mean (SEM). * p<0.05. Scale bars: 50 μm.

obtained in previous studies of our group, being 12.4±1.9 % positive cells (Tapia et al., 2011). The analysis of the correlation assay showed that pERα protein levels correlated positively and significantly with TRKA positive cells in EOC samples in different stages of differentiation (p=0.004 and R^2=0.42) (Fig. 1C).

Effect of NGF on Isoforms of ER (ERα and ERβ) and pERα protein levels in epithelial ovarian cancer explants

Growth factors as NGF can activate pathways that allow phosphorylation of various molecules, including steroid receptors. To determine if NGF modifies protein levels of estrogen receptors, ERα, its phosphorylated form and ERβ were evaluated in ovarian tissues. The sub-cellular immunodetection of ERα and pERα isoforms were principally at the nuclear level, whereas, the location of ERβ was mainly in the cytoplasm (Fig. 1D), suggesting that the main action of estradiol is through ERα.

The evaluation of total protein levels for ERα and ERβ revealed no changes when explants were treated with NGF (10 ng/mL and 100 ng/mL) (HScore ERα=Basal: 0.23±0.15; NGF 10 ng/mL: 0.36±0.14; NGF 100 ng/mL: 0.57±0.12 AU) (HScore ERβ=Basal: 2.07±0.23; NGF 10 ng/mL: 2.12±0.23; NGF 100 ng/mL: 2.16±0.31 AU). However, in these same samples, a significant increase of pERα protein levels was found with stimuli of NGF 10 and 100 ng/ml compared to the basal condition (p=0.03) (HScore pERα=Basal: 1.41±0.11; NGF 10 ng/mL: 1.77±0.08; NGF 100 ng/mL: 1.94±0.17 AU).

Detection of ERα and ERβ in ovary cell lines HOSE and A2780

To evaluate the relevance of estrogen action, two ovarian cell lines were used: HOSE and A2780 to

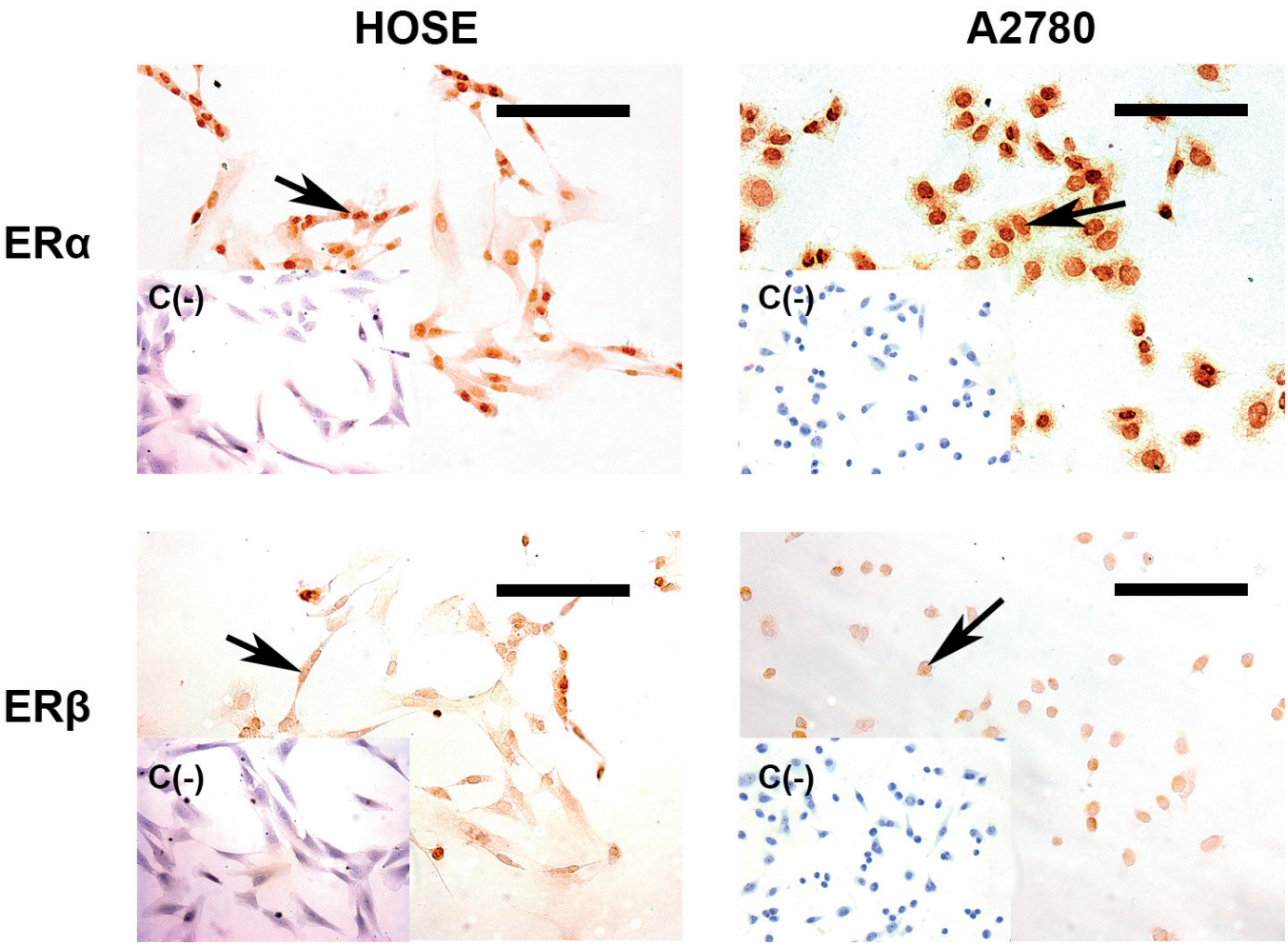

Fig. 2. Microphotography of immunohystochemistry of estrogen receptor α (ERα) and estrogen receptor β (ERβ) in HOSE and A2780 ovary cell lines. Arrows: positive staining. Inset: C (-): negative control. Scale bars: 50 μm.

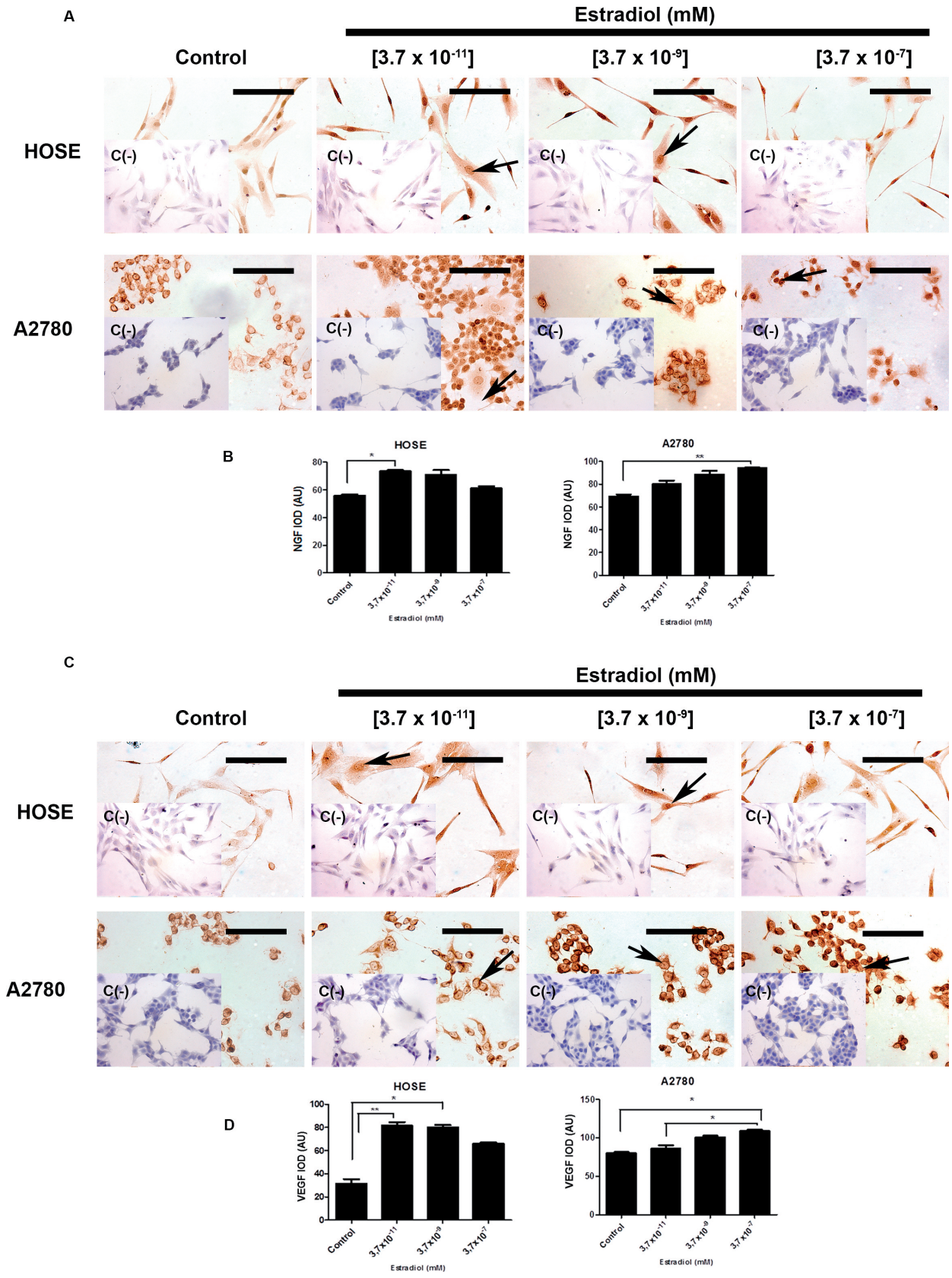

Fig. 3. A. Immunodetection of nerve growth factor (NGF) in HOSE and A2780 ovarian cell lines with $3.7x10^{-11}$, $3.7x10^{-9}$ and $3.7x10^{-7}$ mM of estradiol. **B.** Graphics of semi-quantitive analysis of measurement of NGF. **C.** Immunodetection of vascular endothelial growth factor (VEGF) in HOSE and A2780 ovarian cell lines with $3.7x10^{-11}$, $3.7x10^{-9}$ and $3.7x10^{-7}$ mM of estradiol. Arrows: positive staining. Inserts: C (-): negative control. **D.** Graphics of measurement of VEGF protein levels evaluated by immunocytochemistry. Scale bars: 50 μm.

determine ERα and ERβ expression (Fig. 2). In both cell lines the immunodetection of ERα and ERβ were found mainly in the nucleus. Interestingly, in A2780 cells was found high levels of immunodetection of ERα compared with ERβ. These cells represent an appropriate model of EOC, based on the similar results found in EOC samples that have been reported (Tapia et al., 2011; Vera et al., 2012).

Protein levels of NGF in ovary cell lines stimulated with estradiol

Given that NGF promoter has a response element to estrogen besides the high degree of phosphorylation of ERα in ovarian cancer, it was necessary to determine whether estradiol treatment generates changes in NGF protein levels in ovarian cells. In HOSE cells, the stimulus of $3.7x10^{-11}$ mM of estradiol exerted an increase in NGF protein levels compared to controls (32%; $p<0.05$); whereas, in A2780 cell cultures estradiol treatment provoked an increase of 90% in NGF protein content versus the controls ($p<0.01$). Even though estradiol exerts a stimulatory effect in both cell lines, the increase in NGF protein levels in A2780 cells was greater than in HOSE cell lines (Fig. 3A,B). These results suggest that estradiol induces NGF expression in EOC and, in turn, NGF may induce the activation of ERα.

VEGF protein levels in ovary cell lines HOSE and A2780 stimulated with estradiol

The same as for NGF, VEGF promoter has an estrogen response element and as mentioned before, NGF and VEGF are increased in ovarian cancer. Thus, it is of relevance to determine whether estradiol favors the increment of VEGF protein levels in these cell lines (Fig. 3C,D). Thus, the semi-quantitative analysis of VEGF in HOSE cells showed a significantly increase in protein levels under estradiol treatment, increasing 142% with $3.7x10^{-11}$ mM ($p<0.01$) and 136% with $3.7x10^{-9}$ mM stimulus ($p<0.05$) compared to control condition. Meanwhile, differences were evident in A2780 cells between $3.7x10^{-7}$ mM estradiol stimuli versus control condition, detecting an increase of 37.5% ($p<0.05$). Besides, $3.7x10^{-11}$ mM of estradiol exerts an increase of 25% compared to $3.7x10^{-7}$ mM of the steroid ($p<0.05$). These data show that the response of HOSE cells to estradiol treatment is higher than in A2780 cell line, probably due to the lower VEGF protein levels found in HOSE cells in basal condition than in A2780.

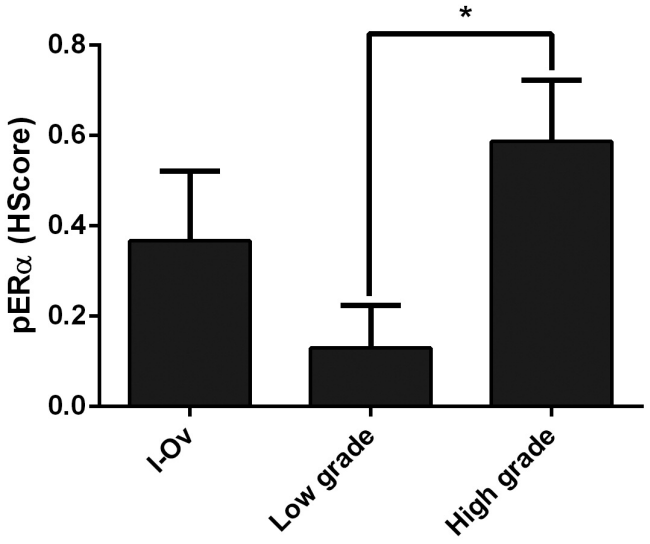

Fig. 4. Graph of semi-quantitative analysis performed by HScore of Immunohistochemistry of phosphorylated estrogen receptor α (pERα) in ovarian tissues from inactive ovaries (I-Ov) and epithelial ovarian cancer of Low grade (EOC I) and High grade (EOC II plus EOC III). The results were expressed as mean ± standard error of the mean (SEM). * $p<0.05$.

Discussion

This study addresses the importance of estradiol action through its receptors in the progression of EOC. In fact, the data of the present study show changes in estrogen receptor levels during progression of EOC and the modulation by estradiol of NGF and VEGF protein levels in epithelial ovarian cells, gaining knowledge on the mechanism by which estrogens could be associated with malignancy of ovarian cancer. Previous studies showed a role of estrogen-regulated pathways in the etiology and progression of ovarian cancer, common to other hormone-dependent cancers, such as breast and endometrial cancer (Modugno et al., 2012; Schuler et al., 2013; Labrie, 2014). Apparently in EOC, the progression of cancer is mostly related with ER α/β ratio and not to the individual receptors; therefore, it is broadly discussed the prognostic value of receptors levels (Pearce and Jordan, 2004; Cunat et al., 2004). Several reports indicate that beta isoform of ER is a molecule that protects against the mitogenic activity of ERα, being diminished in undifferentiated stages (Cunat et al., 2004; Lazennec, 2006). In the present study, protein levels of the phosphorylated form of ERα increased in the state of greater undifferentiation, as EOC III, compared with EOC I. This can be partially explained by the effect of NGF through TRKA receptor, that positively correlates with pERα levels, based on reports indicating that NGF/TRKA are highly expressed in EOC, especially in poorly differentiated ovarian cancer (Tapia et al., 2011). Usually, the EOC is grouped into low and high-grade (Fig 4); however, we consider interesting to show the progressive differentiation changes observed in tissues.

The developing of EOC occurs principally in advanced aged women, especially during post menopause. As known, after menopause serum estradiol levels decrease due to the cessation of the ovarian function. However, when a tumor is present, the intra-tumor estrogen levels increase, participating in the

development of this cancer (Cunat et al., 2004; Mungenast and Thalhammer, 2014). Likewise, elevated levels of serum estrogens may play a role in the development of other cancers. An example of this is the use of estrogen replacement therapy in the absence of progestagens that favors the development of endometrial and ovarian cancer (Persson, 2000). Nevertheless, the clinical implications of estrogen exogenously administered as hormone replacement therapy generating a risk factor of EOC is still under controversy. Some authors determined that estrogen hormone replacement therapy may increase the risk of ovarian cancer (Rodriguez et al., 1995; Lacey et al., 2002), this has been evident even in combination therapy of estrogen and progestin (Anderson et al., 2003). However, other group indicate that hormone replacement therapy might even improve survival in EOC (Eeles et al., 2015), additionally that there is no conclusive evidence that antiestrogens or aromatase inhibitors have a therapeutic effect on EOC (Cunat et al., 2004). The methodological discrepancy in the studies does not yet conclusive evidence to define the actual clinical importance of estrogen in the pathogenesis of ovarian cancer, however the present study contributes to elucidate the molecular mechanisms involved in this pathology.

The estradiol effect in the angiogenesis process has been described in several cancers, among them the ovarian cancer (Auersperg, 2013; Jiang et al., 2016; Ciucci et al., 2016). In *in vivo* ovarian cancer study, estrogen increase microvessel density (Ciucci et al., 2016). This could be explained by the increase levels of macrophages stimulated by estrogens, favoring the initiation of vasculogenesis and angiogenesis, due to the increment of type 2 cytokines secretion, which promote angiogenesis and remodeling (Ausperperg, 2013; Ciucci et al., 2016). However, in the present paper, an alternative mechanism has been described, we found a link of estrogen to growth factors, which would in turn increase angiogenesis processes in EOC. Considering, the increase of angiogenesis favored by growth factors previously described in *in vivo and in vitro* cancer models (Romon et al., 2010; Tapia et al., 2011).

In addition, several reports indicate that estradiol stimulates NGF production in different tissues, such as in MCF-7 breast cancer cells, where estradiol increases NGF levels and favors cellular proliferation through its TRKA receptor (Chiarenza et al., 2001); moreover, in targets of sympathetic innervations, estrogen increases NGF levels (Bjorling et al., 2002). In agreement with these investigations, the data of the present work indicates that estradiol stimulation exerts an increase in NGF protein levels in ovarian cell lines (HOSE and A2780). Consistent with this, it has been described response elements for estrogen receptor in TRKA gene (Sohrabji et al., 1994; Toran-Allerand, 1996a,b); also, estrogen has been shown to affect TRKA expression in sensory neurons (Sohrabji et al., 1994; Lanlua et al., 2001). In this same context, our results showed that estradiol increased VEGF levels in ovarian cancer and normal cell lines, which are in agreement with the results found in breast cancer cells, where it was found that VEGF is a target gene for ERα and ERβ (Applanat et al., 2008). It is noteworthy that we measured NGF and VEGF levels under estradiol stimuli, the growth factors receptors weren't evaluated, thus we could not conclude that the activity of the ligand is increased.

The estradiol stimuli generate increased levels of NGF and VEGF, being the cellular response more evident in HOSE cell line compared to A2780. This could be due to high levels of these growth factors present in carcinogenic origin A2780 cells; these cells stimulated with estradiol generate a non-remarkable increase, since the transcription and translation of these factors have a greater basal level than in non-cancerous origin HOSE cells. Additionally, this difference in the response of cells could be give to high activity of estrogen receptors α of HOSE cell line, considering the high amount of this protein expressed in the nucleus.

As already mentioned, different studies show that estrogens through its receptors may be associated with an increased risk of developing EOC (Rosenblatt and Thomas, 1993; Prior, 2005). One of the mechanisms that regulate ERs expression is the hypermethylation of CpG islands in the promoter, leading to a significant decrease in ER mRNA expression. The CpG islands are varying amounts of cytosine-phosphate-guanine dinucleotides, the presence of those nucleotides in the promoter of a gene (in this case, ERs) regulate the transcription activity, since the bound of methyl to 5 position of cytosine prevents gene transcription (Lennen et al., 2016). This regulatory mechanism has been studied in the promoters of ERβ in ovarian cancer tissues, where promoter hypermethylation is observed when compared with ovarian superficial epithelium cells, leading to a decrease in the expression of ERβ mRNA in ovarian cancer. This finding could partially explain the results observed in the present work, where a decrease of ERβ in the neoplastic progression of EOC and no changes in ERα expression were observed. Moreover, the differential expressions of estrogen receptors isoforms genes are related to genesis of EOC (Bardin et al., 2004; Cheng et al., 2004; Treeck et al., 2007).

In various cancer models, growth factors and steroids cross talked, favoring the development and growth tumor (Ignar-Trowbridge et al., 1995; Pietras et al., 2005; Bacallao et al., 2016). Particularly high levels of growth factors present in EOC could activate MAPK pathway, a transduction signaling path that stimulate several genes related with cellular process as proliferation, differentiation, metabolism among other (Julio-Pepier et al., 2009). It is known that MAPK increase phosphorylation of ERα (le Goff et al., 1994, Chen et al., 2002). Therefore, growth factors could increase the phosphorylation of this steroid receptor. Meanwhile, ERα activated (phosphorylated) increase growth factors levels acting on its response elements present in NGF and VEGF promoter regions (Scharfman and MacLusky, 2008; Toran-Allerand, 1996a). This

cross-talk between growth factors and estradiol could favor an activation loop which could be related to carcinogenesis or development of EOC.

Consequently, the present data indicate the importance of estradiol effect on NGF and VEGF expression, both important growth factors that have been involved in proliferation and angiogenesis processes in epithelial ovarian cancer. Additional, NGF induces the activation of ERα (pERα) producing a loop of estradiol action in EOC.

Acknowledgements. This work was funded with a grant from the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) #1110372, #1160139.

Declaration of interest. The authors declare that there are no conflicts of interest.

References

Ali A.Y., Farrand L., Kim J.Y., Byun S., Suh J.Y., Lee H.J. and Tsang B.K. (2012). Molecular determinants of ovarian cancer chemoresistance: new insights into an old conundrum. Ann. NY Acad. Sci. 1271, 58-67.

Anderson G.L., Judd H.L., Kaunitz A.M., Barad D.H., Beresford S.A., Pettinger M., Liu J., McNeeley S.G. and Lopez A.M. (2003). Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. JAMA 290,1739-1748.

Applanat M.P., Buteau-Lozano H., Herve M.A. and Corpet A. (2008). Vascular endothelial growth factor is a target gene for estrogen receptor and contributes to breast cancer progression. Adv. Exp. Med. Biol. 617, 437-444.

Auersperg N. (2013). The origin of ovarian cancers--hypotheses and controversies. Front. Biosci. (Schol. Ed.). 1, 709-19.

Bacallao K., Plaza-Parrochia F., Cerda A., Gabler F., Romero C., Vantman D. and Vega M. (2016). Levels of regulatory proteins associated with cell proliferation in endometria from untreated patients having polycystic ovarian syndrome with and without endometrial hyperplasia. Reprod. Sci. 23, 211-218.

Bardin A., Boulle N., Lazennec G., Vignon F. and Pujol P. (2004). Loss of ERβ expression as a common step in estrogen-dependent tumor progression. Endocr. Relat. Cancer 11, 537-551.

Bjorling D.E., Beckman M., Clayton M.K. and Wang Z.Y. (2002). Modulation of nerve growth factor in peripheral organs by estrogen and progesterone. Neuroscience 110, 155-167.

Brown S.B. and Hankinson S.E. (2015). Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers. Steroids 99, 8-10.

Campos X., Muñoz Y., Selman A., Yazigi R., Moyano L., Weinstein-Oppenheimer C., Lara H.E. and Romero C. (2007). Nerve growth factor and its high-affinity receptor TrkA participate in the control of vascular endothelial growth factor expression in epithelial ovarian cancer. Gynecol. Oncol. 104, 168-175.

Chen D., Washbrook E., Sarwar N., Bates G.J., Pace P.E., Thirunuvakkarasu V., Taylor J., Epstein R.J., Fuller-Pace F.V., Egly J.M., Coombes R.C. and Ali S. (2002). Phosphorylation of human estrogen receptor alpha at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. Oncogene 21, 4921-4931.

Cheng J., Lee E.J., Madison L.D. and Lazennec G. (2004). Expression of estrogen receptor β in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis. FEBS Lett. 566, 169-172.

Chiarenza A., Lazarovici P., Lempereur L., Cantarella G., Bianchi A. and Bernardini R. (2001). Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-71. Cancer Res. 61, 3002-3008.

Ciucci A., Zannoni G.F., Buttarelli M., Lisi L., Travaglia D., Martinelli E., Scambia G. and Gallo D. (2016). Multiple direct and indirect mechanisms drive estrogen-induced tumor growth in high grade serous ovarian cancers. Oncotarget. 7, 8155-8171.

Cunat S., Hoffmann P. and Pujol P. (2004). Estrogens and epithelial ovarian cancer. Gynecol. Oncol. 94, 25-32.

Eeles R.A., Morden J.P., Gore M., Mansi J., Glees J., Wenczl M., Williams C., Kitchener H., Osborne R., Guthrie D., Harper P. and Bliss J.M. (2015). Adjuvant hormone therapy may improve survival in epithelial ovarian cancer: Results of the aht randomized trial. J. Clin. Oncol. 33, 4138-4144.

Enmark E. and Gustafsson J.A. (1999). Oestrogen receptors: an overview. J. Int. Med. 246, 133-138.

Hamilton T.C., Young R.C. and Ozols R.F. (1984). Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches. Semin. Oncol. 11, 285-98.

Ignar-Trowbridge D.M., Pimentel M., Teng C.T., Korach K.S. and McLachlan J.A. (1995). Cross talk between peptide growth factor and estrogen receptor signaling systems. Environ. Health Perspect. 103, 35-38.

Jiang C.F., Li D.M., Shi Z.M., Wang L., Liu M.M., Ge X., Liu X., Qian Y.C., Wen Y.Y., Zhen L.L., Lin J., Liu L.Z. and Jiang B.H. (2016). Estrogen regulates miRNA expression: implication of estrogen receptor and miR-124/AKT2 in tumor growth and angiogenesis. Oncotarget 7, 36940-36955.

Julio-Pieper M., Lara H., Bravo J. and Romero C. (2006). Effects of nerve growth factor (NGF) on blood vessels area and expression of the angiogenic factors VEGF and TGFbeta I in the rat ovary. Reprod. Biol. Endocrinol. 4, 57-67.

Julio-Pieper M., Lozada P., Tapia V., Vega M., Miranda C., Vantman D., Ojeda S.R. and Romero C. (2009). Nerve growth factor induces vascular endotelial growth factor expression in granulose cells via a trkA receptor/mitogen-activated protein kinaseextracellularly regulated kinase 2-dependent pathway. J. Clin. Endocrinol. Metab. 94, 3065-3071.

Labrie F. (2014). All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause. J. Steroid Biochem. Mol. Biol. 145, 133-138.

Lacey J.V. Jr., Mink P.J., Lubin J.H., Sherman M.E., Troisu R., Hartge P., Schatzkin A. and Schairer C. (2002). Menopausal hormone replacement therapy and risk of ovarian cancer. JAMA 288, 334-341.

Lanlua P., Gangula P.R., Taglialatela G. and Yallampalli C. (2001). Gestational changes in calcitonin gene-related peptide, nerve growth factor, and its receptors in rat dorsal root ganglia. Biol. Reprod. 65, 1601-1605.

Lazennec G. (2006). Estrogen receptor beta, a possible tumor suppressor involved in ovarian carcinogenesis. Cancer Lett. 231, 151-157.

Leitzmann M.F., Koebnick C., Danforth K.N., Brinton L.A., Moore S.C., Hollenbeck A.R., Schatzkin A. and Lacey J.V. Jr. (2009). Body mass index and risk of ovarian cancer. Cancer 115, 812-822.

Leenen F.A.D., Muller C.P. and Turner J.D. (2016). DNA methylation: conducting the orchestra from exposure to phenotype? Clin. Epigenet. 8, 92-107.

le Goff P., Montano M.M., Schodin D.J. and Katzenellenbogen B.S. (1994). Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J. Biol. Chem. 269, 4458-4466.

Lukanova A. and Kaaks R. (2005). Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol. Biomarkers Prev. 14, 98-107.

Maines-Bandiera S.L., Kruk P.A. and Auersperg N. (1992). Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix. Am. J. Obstet. Gynecol. 167, 729-735.

Modugno F., Laskey R., Smith A.L., Andersen C.L., Haluska P. and Oesterreich S. (2012). Hormone response in ovarian cancer: time to reconsider as a clinical target? Endocr. Relat. Cancer 19, 255-279.

Mungenast F. and Thalhammer T. (2014). Estrogen biosynthesis and action in ovarian cancer. Front. Endocrinol. (Lausanne) 12, 192-203.

Pearce S.T. and Jordan V.C. (2004). The biological role of estrogen receptors α and β in cancer. Crit. Rev. Oncol. Hematol. 50, 3-22.

Persson I. (2000). Estrogens in the causation of breast, endometrial and ovarian cancers-evidence and hypotheses from epidemiological findings. J. Steroid Biochem. Mol. Biol. 74, 357-364.

Pietras R.J., Márquez D.C., Chen H.-W., Tsai E., Weinberg O. and Fishbein M. (2005). Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids 70, 372-381.

Prior J.C. (2005). Ovarian aging and the perimenopausal transition: the paradox of endogenous ovarian hyperstimulation. Endocrine 26, 297-300.

Risch H.A. (1998). Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J. Natl. Cancer Inst. 90, 1774-1786.

Rodriguez C., Calle E.E., Coates R.J., Miracle-McMahill H.L., Thun M.J. and Health C.W. Jr (1995) Estrogen replacement therapy and fatal ovarian cancer. Am. J. Epidemiol.141, 828-835.

Romon R., Adriaenssens E., Lagadec C., Germain E., Hondermarck H. and Le Bourhis X. (2010). Research nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways. Mol. Cancer 9, 157.

Rosenblatt K.A. and Thomas D.B. (1993). Lactation and the risk of epithelial ovarian cancer. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. I. J. Epidemiol. 22, 192-197.

Rutherford T., Brown W.D., Sapi E., Aschkenazi S., Munoz A. and Mor G. (2000). Absence of estrogen receptor-beta expression in metastatic ovarian cancer. Obstet. Gynecol. 96, 417-421.

Scharfman H.E. and MacLusky N.J. (2008). Estrogen–growth factor interactions and their contributions to neurological disorders. Headache 48, S77-S89.

Schuler S., Ponnath M., Engel J. and Ortmann O. (2013). Ovarian epithelial tumors and reproductive factors: a systematic review. Arch. Gynecol. Obstet. 287, 1187-1204.

Sohrabji F., Miranda R.C. and Toran-Allerand C.D. (1994). Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. J. Neurosci. 14, 459-471.

Stasenko M., Plegue M., Sciallis A.P. and McLean K. (2015). Clinical response to antiestrogen therapy in platinum-resistant ovarian cancer patients and the role of tumor estrogen receptor expression status. Int. J. Gynecol. Cancer 25, 222-228.

Tapia V., Gabler F., Muñoz M., Yazigi R., Paredes A., Selman A., Vega M. and Romero C. (2011). Tyrosine kinase A receptor (trkA): a potential marker in epithelial ovarian cancer. Gynecol. Oncol. 12, 13-23.

Toran-Allerand C.D. (1996a). Mechanisms of estrogen action during neural development: mediation by interactions with the neurotrophins and their receptors? J. Steroid Biochem. Mol. Biol. 56, 169-178.

Toran-Allerand C.D. (1996b). The estrogen/neurotrophin con- nection during neural development: is co-localization of estrogen receptors with the neurotrophins and their receptors biologically relevant? Dev. Neurosci. 18, 36-48.

Treeck O., Pfeiler G., Mitter D., Lattrich C., Piendl G. and Ortmann O. (2007). Estrogen receptor β1 exerts antitumoral effects on SK-OV-3 ovarian cancer cells. J. Endocrinol. 193, 421-433.

Vera C., Tapia V., Kohan K., Gabler F., Ferreira A., Selman A., Vega M. and Romero C. (2012). Nerve Growth Factor Induces the Expression of Chaperone Protein Calreticulin in Human Epithelial Ovarian Cells. Horm. Metab. Res. 44, 1-5.

Wright J.W., Jurevic L. and Stouffer R.L. (2011). Dynamics of the primate ovarian surface epithelium during the ovulatory menstrual cycle. Hum. Reprod. 26, 1408-1421.

Accepted January 24, 2017