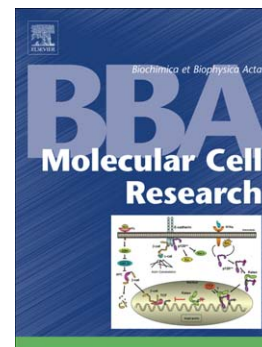# Accepted Manuscript

Regulation of leptin expression by 17beta-estradiol in human placental cells involves membrane associated estrogen receptor alpha

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Regulation of leptin expression by 17beta-estradiol in human placental cells involves membrane associated estrogen receptor alpha

Short title: Leptin expression in placental cells is regulated by estradiol.

Summary sentence: 17β-estradiol, a master hormone in reproductive processes, regulates leptin expression in trophoblastic BeWo cells and placental explants through nongenomic actions involving membrane associated ERalpha.

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Abstract

The placenta produces a wide number of molecules that play essential roles in the establishment and maintenance of pregnancy. In this context, leptin has emerged as an important player in reproduction. The synthesis of leptin in normal trophoblastic cells is regulated by different endogenous biochemical agents, but the regulation of placental leptin expression is still poorly understood. We have previously reported that 17β-estradiol (E_2) up-regulates placental leptin expression. To improve the understanding of estrogen receptor mechanisms in regulating leptin gene expression, in the current study we examined the effect of membrane-constrained E_2 conjugate, E-BSA, on leptin expression in human placental cells. We have found that leptin expression was induced by E-BSA both in BeWo cells and human placental explants, suggesting that E_2 also exerts its effects through membrane receptors. Moreover E-BSA rapidly activated different MAPKs and AKT pathways, and these pathways were involved in E_2 induced placental leptin expression. On the other hand we demonstrated the presence of ERα associated to the plasma membrane of BeWo cells. We showed that E_2 genomic and nongenomic actions could be mediated by ERα. Supporting this idea, the downregulation of ERα level through a specific siRNA, decreased E-BSA effects on leptin expression. Taken together, these results provide new evidence of the mechanisms whereby E_2 regulates leptin expression in placenta and support the importance of leptin in placental physiology.

Abbreviations list

E_2: 17β-estradiol; E-BSA: β-Estradiol 6-(O-carboxymethyl)oxime:BSA; ER: estrogen receptor; GPR: G protein-coupled membrane receptor; ERE: estrogen responsive element; LEP: leptin; Luc: luciferase; βgal: beta galactosidase; qRT-PCR: quantitative real-time RT-PCR; shRNA: short

hairpin RNA; siRNA: small interfering RNA; MAPK/ERK: mitogen-activated kinase; MEK: MAPK kinase; AKT: protein kinase B; PI3K: phosphotidylinositol 3-kinase; SAPK/JNK: stress-activated protein kinase/c-Jun NH_2-terminal kinase; JAK/STAT: Janus kinase/signal transducers and activators of transcription; PKA: protein kinase A; PLC: phospholipase C; PKC: protein kinase C; hCG: human chorionic gonadotropin; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; HDAC1: histone deacetylase 1; Sp-1: Stimulating protein 1; a.u.: arbitrary units.

1. Introduction

Steroids play an important role in the growth, differentiation, metabolism, reproduction, and morphogenesis of higher organisms. Particularly, its actions are required for the development and maintenance of reproductive tissues [1]. In addition to their physiological function, steroid hormones also play a crucial role in many pathological processes, including endocrine, cancerous, and metabolic diseases.

The most potent and dominant estrogen in humans is 17β-estradiol (E_2) [2]. Estradiol exerts its actions through two members of the nuclear receptor superfamily, estrogen receptor (ER)α and ERβ, and a G protein-coupled membrane receptor, GPR30 [3]. Mechanisms by which ERα and ERβ bind ligand, dimerize, associate with coactivators or corepressors, and regulate gene transcription through binding to target genes, are well-known and are typically referred to as "genomic" actions [4]. ER-dependent activation of gene expression also involves DNA-bound ER that subsequently interacts with other DNA-bound transcriptions factors and direct ER-transcription factor (protein–protein) interactions where ER does not bind promoter DNA [5].

Recent evidence suggests that, along with gene regulation, E_2 also mediates rapid cellular effects (non-genomic or extranuclear pathways) [5]. Estradiol rapidly activates several protein kinases [*e.g.*, MAPKs, phosphotidylinositol 3-kinase (PI3K), and protein kinase C (PKC)] and phosphatases, as well as the release of several cyclic amines (cAMP, cGMP) and calcium, in a variety of cell types [6]. It is now firmly established that estrogen and all sex steroid receptors exist in discrete cellular pools outside the nucleus. Estrogen receptors have been localized to the plasma membrane where both ERα and ERβ function in a wide variety of cells and organs. In ligand-dependent fashion, each ER pool contributes to the overall, integrated effects of estrogens producing biological outcomes. Both ERα and ERβ, the two ER isoforms, localize in many cells to the plasma membrane and to cytoplasmic organelles including mitochondria and the endoplasmic reticulum [7].

The blastocyst development and implantation is governed by an extremely complicated but cooperative interplay of various cellular factors and biomolecules, which are regulated in a defined spatio temporal fashion. One of the important aspects of blastocyst implantation is the involvement of regulatory molecules, primarily embryotrophic factors, which encompass second messengers, transcription factors, proteases, growth factors and cytokines [8].

In particular, leptin has been proposed to play a relevant role in implantation and in placenta growth and survival during pregnancy [9-11]. Leptin hormone, the product of the *LEP* gene, is a 16000 MW non-glycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang *et al* [12].

In humans, the placenta is a significant source of maternal circulating leptin, with levels in the second and third trimesters being up to 200% of those in the first trimester or in the non-gravid situation [13]. Many physiological roles have been suggested for leptin in human pregnancy. Both leptin and leptin receptors have been identified in the placental syncytiotrophoblast, which suggests the potential for autocrine and paracrine mechanisms. Leptin also activated the release of proinflammatory cytokines and prostaglandins from human placental explants, further implicating leptin as a modulator of placental endocrine function. On the other hand, deregulation of leptin metabolism and/or leptin function in the placenta may be implicated in the pathogenesis of various disorders during pregnancy, such as recurrent miscarriage, gestational diabetes, intrauterine growth retardation, and preeclampsia [14, 15].

The complete regulation of leptin production in the placenta is still poorly understood. Previous work demonstrated that hCG and cAMP enhances leptin expression through a crosstalk between PKA and MAPK signaling pathways [16, 17].

Human placenta is known to synthesize estrogens during pregnancy [18], in association with the cytotrophoblast invasion [19, 20]. Moreover, there is evidence to suggest that placentally derived estrogen may play an autocrine role in trophoblast differentiation [21, 22].

Estrogens have been reported to regulate leptin expression by acting on a portion of the estrogen response element in the leptin promoter [23]. The presence of estrogen receptor in primate

trophoblast [22] suggests that, this is an estrogen receptor-mediated phenomenon. Estrogen receptor ER α and β proteins have also been localized in nuclei of cultured human syncytiotrophoblast cells using immunohistochemistry [22].

Although these findings suggest the importance of estrogens in leptin biosynthesis, the mechanisms that mediate leptin expression by estrogen in human placenta remain undefined. In our previous work [24, 25], we showed that E_2 indeed upregulates leptin expression in human placental cells probably involving genomic and nongenomic action by a crosstalk between ERα and the MAPK and PI3K signal transduction pathways. To improve the understanding of estrogen receptor mechanisms in regulating placental leptin expression, we here examined the effect of membrane-constrained E_2 conjugate, E-BSA, on leptin expression in human placental cells. Our present results suggest that E_2, a key regulator of leptin expression in placenta, involves ERα membrane associated via nonclassical estrogen receptor pathway.

2. Materials and methods

2.1. Cell culture and treatments

The human choriocarcinoma cell line BeWo was grown in 45% Dulbecco Modified Eagle Medium (DMEM) and 45% HAM F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma Chemical Company, St. Louis, MO) at 37ºC in 5% CO_2.

The effect of 17β-estradiol (E_2; Sigma) was previously tested at different doses and 10 or 100 nM E_2 was used in subsequent experiments [24]. The antiestrogen ICI 182,780 (AstraZeneca, UK, London) was used to assay the specificity of estrogen effects. Membrane estrogen actions were evidenced incubating with estradiol covalently linked to BSA (E-BSA, β-Estradiol 6-(O-carboxymethyl)oxime:BSA; Sigma). E-BSA was filtered prior to use in order to eliminate free E_2 according with a previously described method [26]. Briefly, 900 μl of E-BSA (400 μM in estradiol

dissolved in 50 mM Tris–HCl pH 8.5) was added to a centrifugal filter unit with a MW cut-off of 3,000 (Millipore, Billerica, MA) and subjected to centrifugation at $13,000 \times g$ for 30 min. The retained fraction was washed 3 times with buffer and recovered. Volume was adjusted to 900 μl. The efficacy of filtration to remove free E_2 was verified by Stevis *et al*. [26].

The specific PI3K inhibitors Wortmannin (0.1 μM) and LY-294,002 (1 μM), the MEK inhibitor PD98059 (50 μM), the p38-MAPK inhibitor SB 203580 (5 μM) and the JNK inhibitor SP600125 (5 μM) (Sigma) were also used. Treatments were performed in DMEM-F12 media without phenol red and supplemented with 1% charcoal stripped FCS unless otherwise stated. Serum present in incubation media was reduced from 10% to 1% to lower non-specific effects.

2.2. Placental explants collection and processing

Human placentas (n = 15) were obtained after cesarean section or vaginal delivery following normal term pregnancies and immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory, where they were washed 2–3 times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and thoroughly rinsed with cold DMEM-F12 medium. None of the donor patients suffered from anomalous pregnancy. Samples were obtained under informed consent, and the study was approved by the Hospital Universitario Virgen Macarena (Seville, Spain) ethics committee.

2.3. Treatments of placental explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium without FCS (n = 1 explant/tube, 3 replicates per treatment). Placental explants were maintained in a shaking water bath at 37°C during 5 min to equilibrate temperature and incubated in the same

medium supplemented or not with E_2 or E-BSA. For leptin analysis by western blot or qRT-PCR, placental explants were incubated during 4 h and for signaling experiments they were incubated during 10 min as indicated in each Legend. When indicated pretreatment with ICI 182,780 or pharmacological inhibitors of signaling pathways were performed during 20 min. Explants were removed from the bath, centrifuged for 2 min at 2000 g at 4°C and resuspended in 500 μl of lysis buffer (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 10 mg/ml phenylmethanesulfonyl fluoride (PMSF)) during 30 min at 4°C on an orbital shaker and later centrifuged at $10000 \times g$ for 20 min. Supernatants were analyzed by Western blot. For real-time PCR, after thoroughly washing with PBS, the explants were immediately frozen at -80 ºC and stored until extraction of total RNA.

2.4. Membrane isolation

Membrane fractions were isolated by sucrose centrifugation according with a previously described method [27]. Briefly, BeWo cells were grown on 100 mm dishes in DMEM-F12 plus 1% FCS for 48 h, were washed with icecold PBS and scraped into 0.75 mL sucrose buffer (50 mM Tris, 1 nM EDTA, 200 mM sucrose, 4 mM $NaHPO_4$, 1 μM Na_3VO_4, 1 μM NaF). Cells were homogenized by passing the sample forty times through a syringe needle. Homogenates were spun at $1000 \times g$ at 4 ºC. The pellet was resuspended in solubilization buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5% Triton X-100 plus protease and phosphatase inhibitors) and labeled "nuclear." Supernatant was spun at $20000 \times g$ for 75 min at 4 °C. The pellet was resuspended in solubilization buffer and labeled "membrane" and supernatant was labeled "cytoplasm." All the fractions were analyzed by Western blot.

2.5. Western blot analysis

Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at $10000 \times g$ for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by

Bradford [28], using bovine serum albumin (BSA) as standard. Lysates were mixed with Laemmli´s sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, separated by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia, Buckinghamshire, UK). Membranes were equilibrated in 1x PBS and non-specific binding sites were blocked by 5% non-fat milk in PBS at room temperature for 1 h. Membranes were then immunoblotted with polyclonal rabbit anti-human leptin Y20 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), with polyclonal rabbit anti-phospho-mitogen-activated protein kinase (MAPK) kinase 1 and 2 (MEK1/2) (Ser217/Ser221; 1:3000; Sigma), with polyclonal rabbit anti-phospho-MAPK 1 and 2 (ERK1/2) (Thr202/Tyr204; 1:3000; Sigma), with polyclonal mouse anti-phospho-AKT (Ser 472/473) antibody (1:3000; BD Biosciences Pharmingen, NJ), with monoclonal mouse anti-phospho-p38MAPK (Thr180/Tyr182; 28B10; 1:3000; Cell Signaling Technology, Danvers, MA), with monoclonal mouse anti-phospho-pSAPK/JNK (Thr183/Tyr185; G9; 1:3000; Cell Signaling Technology), with polyclonal rabbit anti ERα (1:500; Santa Cruz), with polyclonal rabbit anti ERβ (1:500; Santa Cruz), with polyclonal rabbit anti HDAC1 (1:2000; Santa Cruz), or with polyclonal rabbit anti G-protein β subunit (1:1000; Santa Cruz), as indicated in each figure.

Loading controls were performed by immunoblotting the same membranes with polyclonal rabbit anti-actin (1:1000; Sigma), with monoclonal mouse anti-α tubulin (1:1000; Sigma), with polyclonal rabbit anti-total-MAPK kinase 1 and 2 (MEK 1/2; 1:3000; Sigma), with polyclonal rabbit anti-total-MAPK 1 and 2 (ERK 1/2; 1:3000; Sigma) or with polyclonal rabbit anti-total-AKT antibody (1:3000; Sigma).

Bound antibodies were detected using horseradish peroxidase-linked donkey anti-rabbit IgG (1:10000; Amersham Pharmacia) or peroxidase-linked sheep anti-mouse IgG (1:10000; Amersham Pharmacia) and visualized by the Amersham Pharmacia ECL Chemiluminescence signaling system

and a Bio-Imaging Analyzer Fujifilm LAS-1000. Quantification of protein bands was performed by densitometry using Scion Image software (Scion Corporation, Washington, DC).

2.6. Plasmids

The luciferase (Luc) reporter construct pL1951 is based on pGL-3 basic vector. They were kindly provided by Oksana Gavrilova (Bethesda, MD) [29]. pRSV-βgal contains the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) promoter and was used to normalize the efficiency of individual transfections.

The plasmid ERE-*Luc* is an ERE (estrogen responsive element) promoter driven luciferase reporter plasmid and was kindly provided by Dr. Adalí Pecci (FCEN-UBA, Buenos Aires) [30].

The Sh2 vector produces an intracellular short hairpin (sh) RNA to mediate ERα mRNA degradation through formation of the 'RNA-induced silencing complex'. It was kindly provided by Dr. Yunus Luqmani (Faculty of Pharmacy, Kuwait University) [31]. It is based on pSingle-tTS-shRNA plasmid (Clontech, Mountain View, CA), and contains the sequence 5' GATTGGCCAGTACCAATGATTCAAGAGATCATTGGTACTGGCCAATC 3' cloned into Xho I and HindIII sites, under the control of the tetracycline-inducible pTRE-U6 promoter.

To perform transient transfection assays, plasmids were purified using the Maxipreps Wizard kit (Promega Co., Madison, WI), and the concentration of DNA was estimated spectrophotometrically.

2.7. Transient transfection experiments

For transient transfection experiments, BeWo cells were plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 ml of DMEM-F12 plus 10% FCS. Cells were incubated for 24 h. Medium was replaced and transfection of cells was performed according to the standard liposome-mediated method.

When reporter plasmids were used, the sensitivity of the method was determined in this cell type, performing a standard dose of reporter plasmid vs. light emission (data not shown). Typically 5 μg

of the Luc reporter, 5 μg of pRSV-βgal internal control construct and/or 5 μg of each expression plasmid were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc., Grand Island, NY). The medium was replaced after 5 h with DMEM-F12 1% charcoal stripped FCS as indicated in each figure, plus the addition of the different effectors. Transfection analysis was performed in duplicate in at least three independent experiments.

Transfection of cells with Sh2 plasmid was performed according to the method previously described, using 5 μg of Sh2/well. Two days after transfection, the medium was replaced with DMEM-F12 10% containing 500 μg/ml G418 (Sigma). A standard dose-response curve was performed to evaluate cell sensitivity to G418 (data not shown).

Cell derived clones were serially expanded and passed at least 10 times in G418 containing medium before using for analysis. Expression of ERα shRNA was induced incubating cells with DMEM-F12 1% charcoal stripped FCS containing 5 μg/ml doxycycline (Pfizer, New York, NY) during 48 h.

2.8. Assays for Luc and β-galactosidase activities

Luc activity in cell lysates was measured using the Luciferase Assay System (Promega). Cells were washed with PBS and harvested 48 h after the transfection procedure using 50 μl of lysis buffer. Cell extracts were centrifuged and 30 μl of the supernatant were mixed with 50 μl of Luc assay buffer. Luc activity was measured with a junior luminometer (Hidex, Turku, Finland). β-galactosidase activity was assayed using 1 mg of o-nitrophenyl β-D-galactopyranoside (AmResco, Solon, OH) as the substrate in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.07% β-mercaptoethanol) and incubated at 37 ºC until yellow staining. The product was determined by absorption at 420 nm. This value was used to correct variations in transfection efficiency. Luc results were calculated as the ratio of Luc activity per unit of β-galactosidase activity. Duplicate samples were analyzed for each data point.

2.9. Quantitative real-time RT-PCR (qRT-PCR) assay

Abundance of leptin mRNA was determined by qRT-PCR. Total RNA was extracted from placental explants using TRISURE reagent, according to the manufacture instructions (Bioline Co., Essex, UK). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse transcribed at 55 ºC during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Indianapolis, IN). Quantitative real-Time PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: leptin: forward, 5' GAACCCTGTGCG GATTCTT 3'; reverse, 5' CCAGGTCGTTGGATATTTGG 3'; and cyclophilin: forward, 5' CTTCCCCGATGAGAACTTCA 3'; reverse, 5' TCTTGGTGCTCTCCACCTTC 3'. Quantitative RT-PCR Master Mix Reagent kit was obtained from Qiagen (SensiMix^{TM} *Plus* SYBR Kit), and PCRs were performed on a MJ Mini BioRAD gradient thermal cycler (Bio-Rad, Hercules, CA). A typical reaction contained 10 μM of forward and reverse primer, 3 μl of cDNA, and the final reaction volume was 25 μl. The reaction was initiated by preheating at 95°C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 sec at 95°C, annealing 30 sec at 58°C min and extension 30 sec at 72°C. The threshold cycle (CT) from each well was determined by the BioRAD CFX Manager Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophylin), and relative to the untreated control.

2.10. Immunofluorescence

BeWo cells were grown on coverslips overnight in DMEM-F12 plus 10% FCS. Then were washed three times in PBS and fixed 5 min at RT with 2.0% paraformaldehyde, 0.5% Nonidet P-40 (NP-40)

and 0.15 M sucrose in PBS pH 7.4; freshly prepared, according a previous described method [32]. At the end of the fixation period, the cells were washed three times with PBS, and blocked 1-h at RT with 10% BSA in PBS. The primary antibodies were diluted in 0.5% BSA in PBS and were added to the cells ON at 4 ºC; next, the cells were washed six times PBS. A Cy3-labeled antirabbit IgG (Invitrogen) was added to the cells in the dark for 6 h at 4 ºC. Washed cells were stained with Hoechst 33342, mounted with Mowiol 4-88 (Sigma), and stored in the dark at 4 ºC until examined for fluorescence. Photomicrographs were taken using a Nikon Eclipse E600W microscope (Nikon, Tokyo, Japan) equipped with a Y-FL epifluorescence attachment and a digital camera (Nikon Coolpix 5000) or using an Eclipse E800 Nikon C1 laser confocal microscope.

2.11. Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as mean ± standard error (S.E.M) in arbitrary units (a.u.). Arbitrary units were calculated as normalized band intensity in Western blot analysis or normalized Luc activity in reporter assays, where control values were taken as 1. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad Software, San Diego, CA). Statistical significance was assessed by ANOVA followed by different post hoc tests, as indicated in each figure. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Estradiol-BSA enhances endogenous leptin expression in placental cells

We have previously observed that E_2 was able to induce leptin expression not only in BeWo cells but also in human placental explants. Our results strongly suggest that E_2 action probably involves both genomic and nongenomic actions [24]. In order to evidence the involvement of membrane receptors in E_2 signaling, we aimed to study the effect of membrane-constrained E_2 conjugate, E-BSA, on leptin expression in human placental cells.

As seen in Figure 1A, E-BSA enhanced leptin expression in BeWo cells as determined by Western blot analysis. This effect was dose dependent, reaching a 2.2-fold maximal increase at 100 nM. We next performed transient transfection assays with a vector containing the regulatory region of leptin gene from -1951 bp fused to the Luc reporter gene (pL1951). As shown in Figure 1B, in transfected BeWo cells, treatment with 10 nM E-BSA for 48h induced a 5.6-fold maximal increase in Luc activity. A control treatment with 100 nM E_2 was included; it significantly enhanced leptin expression as previously reported [24]. In order to analyze whether E-BSA stimulates leptin at the transcriptional level in a more physiological model, additional experiments were performed in placental explants. Figure 1C shows that E-BSA significantly enhanced leptin mRNA expression, measured by qRT-PCR in human placental explants. Maximal effect was achieved at 10 nM with a 3.9-fold increase. Control treatment with 0.1 nM E_2 significantly enhanced leptin expression.

To confirm that E-BSA is not a membrane-permeable compound, BeWo cells were transfected with a luciferase reporter construct driven by a minimal promoter containing tandem repeats of a canonical ERE, and then stimulated with E_2 or E-BSA as indicated for 48 h (Figure 1D). Compared with control, E_2 triggered over a 3.7-fold increase in ERE-driven Luc activity, whereas E-BSA had no effect.

Taken together, these results suggest that E-BSA increases placental leptin expression through estrogen membrane-initiated actions.

3.2. Estradiol-BSA activates multiple signal transduction pathways

Estrogen action mediated by membrane receptors are frequently associated with the activation of many protein-kinase cascades [33]. So, our next aim was to investigate whether E-BSA could activate different signaling transduction pathways in placental cells.

As shown in Figure 2, ERK1/2, AKT, p38-MAPK and JNK phosphorylation were increased with E-BSA treatment in n BeWo cells, and these effects were dose dependent. On the other hand, E-

BSA increased MEK, ERK1/2 and AKT phosphorylation in placental explants, but did not affect p38-MAPK and JNK phosphorylation (Figure 3).

These results suggest that E-BSA is able to activate MAPKs and PI3K signal transduction pathways in placental cells.

3.3. Estradiol-BSA involves multiple signaling pathways in the induction of leptin expression

Recent evidence suggest that estrogen membrane initiated actions may indirectly influence gene expression, through the activation of signal transduction pathways that eventually act on target transcription factors [2]. E-BSA enhanced leptin expression and also activated different signaling pathways in placental cells, therefore we decided to analyze if those signaling pathways are involved in E-BSA induction of leptin expression.

Results obtained in transfected BeWo cells (Figure 4A), showed that leptin promoter activity enhanced by E-BSA was significantly reduced when cells were pretreated with pharmacological inhibitors of MEK (50 μM PD98059), p38-MAPK (5 μM SB 20358), JNK (5 μM SP600125) and PI3K (0.1 μM Wortmannin and 1 μM LY-294,002) pathways. It was observed that basal leptin expression seemed to be dependent on JNK activation as the treatment with SP600125 alone significantly decreased promoter activity.

Similar experiments were performed in human placental explants, a more physiological model. In these experiments explants were pretreated with the same pharmacological inhibitors and then incubated with 10 nM E-BSA for 4 h. Treatments with all the inhibitors tested significantly blocked the effect of E-BSA on leptin expression measured by qRT-PCR (Figure 4B), suggesting that MAPKs and PI3K pathways are involved in E-BSA leptin induction.

3.4. ERα is localized both in nucleus and plasma membranes of BeWo cells

Estrogen receptors have been localized to the plasma membrane where both ERα and ERβ function in a wide variety of cells and organs [7]. The physiologic role of these plasma membrane binding sites for estrogens is currently under intense investigation. We decided to investigate if ERα and ERβ estrogen receptors are present in plasma membrane of BeWo cells by two different experimental approaches: Western blot of subcellular fractions and immunofluorescence.

In total sample the 66 KDa band corresponding to ERα was observed. Analyzing subcellular fractions obtained by sucrose gradients of BeWo cells, we detected ERα in nuclear and also in membrane fractions (Figure 5A). Three detectable bands were observed between 66 and 50 KDa, probably due to partial degradation or to fractionation artifacts.

By the other side, ERβ appears to be mainly nuclear, although it has been slightly detected in cytosol of BeWo cells (Figure 5A).

In order to evidence whether the treatment with E_2 or E-BSA alters the distribution of ERα, similar cellular fractionation experiments were performed in the presence of the hormone. As it can be seen in Figure 5B (Western blot results) and in 5C (relative ERα expression), ERα localization was not modified after 48 h of incubation in the presence of 100 nM of E_2 or E-BSA. The time of the treatment was choose according previous results, where it was demonstrated that the presence of E_2 or E-BSA produced a maximal increase in leptin expression at 48 h of treatment [24].

HDAC1 and G-protein β subunit detection were used as control of quality of nucleus and membrane fractions respectively. As it can be seen in Figures 5A and 5B both proteins were only revealed in their specific compartments.

By a different experimental approach as immunofluorescence, similar results were obtained. ERα was detected mainly in nucleus and in plasma membrane of BeWo cells (Figure 6A) meanwhile ERβ is predominantly nuclear (Figure 6B). Immunofluorescence results were confirmed by confocal microscopy (Figure 6D and 6E).

Negative controls in the absence of primary antibodies were also performed (Figure 6C and 6F).

These results strongly suggest that ERα is localized both in nucleus and plasma membrane of trophoblastic cells, and could be involved in estrogen regulation of placental leptin expression.

3.5. Estradiol action probably involves ERα membrane receptors

Finally, we analyzed if ERα membrane receptor could mediate membrane-initiated estrogen actions involved in placental leptin expression.

The antiestrogen ICI 182,780 was used to assay if E-BSA effects depends on the presence of functional estrogen receptors. When placental explants were pretreated with 100 nM ICI 182,780, the stimulatory effect of E-BSA on leptin expression was prevented (Figure 7A). Moreover, in the presence of 100 nM ICI 182,780, E-BSA failed to induce ERK 1/2 phosphorylation (Figure 7B). By the other side 100 nM ICI 182,780 increased AKT phosphorylation, probably due to some agonist effect of this antiestrogen (Figure 7C). These results suggest that ICI 182,780 can block some of the effects of E-BSA in placental explants.

In a different experimental approach siRNA silencing against ERα was carried out. BeWo cells were stable transfected with Sh2 construct to decrease ERα protein. Expression of ERα siRNA was induced incubating cells with 5 μg/ml doxycycline, which showed no effect on ERα or ERβ expression in non-transfected BeWo cells (Figure 8A).

In Figure 8B it is shown that ERα siRNA expression produced a marked reduction in the amount of ERα protein in BeWo cells measured by Western blot. The ERα silencing was specific, as ERβ level was not modified in Sh2 transfected cells treated with doxycycline. In Sh2 transfected cells, not induced with doxycycline, the incubation with 100 nM estradiol or 1-100 nM E-BSA increased leptin promoter activity (Figure 8D). Besides, ERα protein was not totally downregulated when ERα siRNA expression was induced with doxycycline. In this scenario the treatment with E_2 or E-BSA failed to induce leptin expression (Figure 8C) and leptin promoter activity (Figure 8D). On the other hand, it was previously demonstrated that E-BSA treatment probably does not exert its effect

on leptin expression through an intracellular ER (Figure 1D). All together these results strongly suggest that in BeWo cells, the membrane receptor/s involved in E-BSA induced leptin expression could be membrane associated ERα.

4. Discussion

The steroid hormone 17β-estradiol (E$_2$) is an estrogen that regulates growth, differentiation, and function in a wide array of target tissues, including the male and female reproductive tracts, mammary gland, and skeletal and cardiovascular systems [34]. It exerts some effects through the action of classical estrogen receptors ERα and ERβ, which act as ligand-activated transcription factors and regulate gene expression [33]. In addition, E$_2$ can elicit rapid responses from membrane-associated receptors, like activation of protein kinase pathways, which can result in the modification of transcriptional activity of conventional transcription factors. Thus the cellular effects of E$_2$ will depend on the specific receptors expressed and the integration of their stimulatory and inhibitory signaling events [35].

During human pregnancy, the production of E$_2$ raises eighty fold, from 0.75 nM preovulatory peak to 60 nM at term. Estradiol influences various aspects of placental function and fetal development in humans and primates [36], and plays a role in the regulation of fetal growth, onset of parturition, placental steroideogenesis, release of neuropeptides, release of glycoproteins and leptin secretion [37]. Leptin is a key hormone in trophoblast proliferation and survival [25], that regulates cell proliferation by inhibiting apoptosis [10, 11], stimulates protein synthesis [38], and regulates fetal growth and development [39].

The regulation and physiology of leptin in placenta is largely unknown, and in particular little is known about the mechanisms involved in regulation of leptin expression by E$_2$ in placental cells. It was previously reported that E$_2$ increased leptin production in cultured human cytotrophoblastic cells from first trimester placenta [37]. Moreover, it was demonstrated that E$_2$ enhanced leptin

promoter activity in JEG-3 cells, suggesting that E_2 regulates *LEP* gene expression via promoter activation [23]. Those investigations suggest that E_2 could be an important regulator of leptin expression in placental cells. Our previous results indicated that E_2 induces leptin expression not only in BeWo cells but also in human placental explants probably involving both soluble and membrane estrogen receptors [24].

In this regard, we aimed to study the regulation of leptin expression by E-BSA, an estrogen analogue that evidence membrane-iniciated estrogen actions [40]. We used two experimental models: BeWo choriocarcinoma cells and human placental explants. BeWo cells maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling [41-43]. Normal placental explants from healthy donors were also studied to confirm the physiological relevance of the results.

When the effect of E-BSA on leptin expression was analyzed in BeWo cells and human placental explants, a significant upregulation was observed. Moreover, we observed that E-BSA was able to increase leptin promoter activity and leptin mRNA transcription. These genomics effects could not be attributed to classic nuclear ERs, as E-BSA, in contrast to E_2, failed to stimulate the reporter activity of ERE-Luc plasmid. These results confirmed the membrane-constraint property of E-BSA and suggested that its actions are probably triggered by plasma membrane receptors.

We observed that high concentration of E-BSA was less efficient on leptin expression induction than low concentration in both experimental models. Similar results were obtained with E_2 effect on leptin expression in placental cells [24], and are also in agreement with the bifasic modulation of human cytotrophoblastic leptin secretion by E_2 [37]. The underlying molecular mechanism is not completely understood. It has been proposed that high doses of E_2 or E-BSA could downregulate the expression of estrogen receptors, as observed in other reproductive tissues, but this hypothesis remains to be studied in placental cells.

Membrane-initiated actions by E_2 could regulate gene expression through the activation of different signaling pathways such us PLC/PKC; p38/MAPK; JAK/STAT; Pak1; casein kinase I-g2; and sphingosine kinase [44].

Our results suggest that E-BSA activates MEK-ERK and PI3K signaling pathways both in placental explants and BeWo cells. The activation of p-38 MAPK and JNK signaling pathways by E-BSA were only evidenced in BeWo cells. Further experiments will be needed to clarify the differences between these experimental models. Despite these differences, all the signaling pathways analyzed are probably involved in E-BSA regulation of placental leptin expression as evidenced in the pharmacological inhibition experiments.

Our results are in agreement with previous reports, where it has been shown that E_2 can exert rapid non-genomic effects in human placental explants, inducing ERK 1/2 and AKT phosphorylation. The blockage of these pathways in BeWo cells prevented the effect of E_2 on leptin expression both at protein and transcriptional levels [24].

Various regulatory elements have been identified within the leptin promoter, e.g., cAMP, estrogen, androgen, insulin and glucocorticoid response elements, CCATT/enhancer and SP1 binding sites, suggesting a direct regulation of leptin expression through different transcriptional pathways [25]. Several transcription factors that recognize those regulatory elements could be interacting with nuclear estrogen receptors or could be modulated by protein kinases activated by membrane-iniciated estrogen actions, as it has been widely reported [2]. Further experiments will be needed to understand the possible mechanisms of integration of membrane signaling and nuclear effects of E_2 involved in human placental leptin expression.

It is known that E_2 exerts its actions through two members of the nuclear receptor superfamily, estrogen receptor ERα and ERβ, and also through a G protein coupled membrane receptor, GPR30 [45]. The presence of estrogen receptor in primate trophoblast suggests that, as in adipose tissue, the effect of estrogen on leptin expression is an estrogen receptor-mediated phenomenon [46].

Although term placenta has been found to specifically bind estrogens, cellular localization of ERα in trophoblasts remains unclear [36]. Immunohistochemistry in term placenta sections showed that the ERα was strongly expressed in cytotrophoblast nuclei, lying beneath trophoblastic syncytium, which showed no staining of the nuclei. ERα immunoreactivity was also detected in villous vessels and sinusoids [36]. By the other side, reverse transcription-polymerase chain reaction and Western analyses showed the presence of ERα transcripts and protein in BeWo cells. The BeWo cell ER binds to an ERE consensus sequence, suggesting that these cells express a functional ER [47].

It has also been reported that human term placental tissues express ERβ and it was localized in the syncytiotrophoblast layer and in only few cells of the cytotrophoblast; no staining was observed in the stromal region. The presence of ERβ mRNA was also detected in BeWo choriocarcinoma cell line, and its expression –but not the ERα expression- is down-regulated by E_2 [48]. Our results suggest that BeWo cells express ERβ and it appears to be mainly nuclear, although cytosolic ERβ was also detected by immunofluorescence.

Previous results support the involvement of ERα in placental leptin expression, as the over-expression of ERα in BeWo cells increased the stimulatory effect of E_2 on leptin promoter activity [24]. Moreover, it has been proposed that the expression of ERα, and not of ERβ, evidenced the stimulatory effect of E_2 on leptin expression in JEG-3 cells [23].

In the present work we showed that BeWo cells express ERα mainly detected in nucleus and plasma membrane, but also cytosolic protein was detected by immunofluorescence.

These results are consistent with previous reports of Bukovsky *et al*. [36]. They observed that trophoblast cultures derived from normal placenta exhibited surface ERα expression. Nondividing trophoblast cells showed cytoplasmic/perinuclear or perinuclear/nuclear ERα immunoreactivity, which was accompanied by diminution of cell surface staining [36].

The blockage of E-BSA effects on ERK phosphorylation and leptin mRNA expression with the antiestrogen ICI 182,780 that ERα could be the receptor involved in membrane-initiated estrogen

actions that regulate leptin expression in placental cells. Moreover, decreased expression of ERα through a specific siRNA significantly dimished the effect of E-BSA on leptin expression.

Membrane initiated estradiol signaling have been reported in the regulation of rapid cell signaling in the control of sexual receptivity and in the estrogen positive feedback regulating the LH surge [49]. Our results suggest that leptin physiology may also depend of membrane-associated estrogen effects.

In the late 1990s was revealed the existence of the receptor GPR30, a member of the G protein– coupled receptor superfamily that mediates estrogen-dependent kinase activation as well as transcriptional responses [50]. GPR30 transcripts were reported to be widely distributed in normal and malignant human tissues, although there are discrepancies in the reported expression levels in placenta [50]. Besides, ICI 182,780 is also a GPR30 agonist [50], and can rapidly induce a significant increase in ERK2 and AKT phosphorylation, failing to block some E_2 effects [51, 52]. Further experiments will be needed to analyze if GPR30 is expressed in trophoblastic cells and participates in E_2 up-regulation of placental leptin expression.

5. Conclusions

In summary, in this study, we provide evidence that E-BSA induces leptin expression through the activation of multiple signaling pathways that could regulate leptin gene expression. The possible mechanism of regulation of placental leptin expression by E_2 is a multifactorial process, and may depend on the presence, concentration and localization of ERs, transcription factors, co-regulatory proteins and signal transducers, among others, as illustrated in the model based in current data shown in Figure 9. Our results provide new evidence of the regulatory mechanisms of placental leptin expression and support the importance of leptin in reproduction.

6. References

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Legends

Figure 1

E-BSA enhances leptin expression in placental cells

A, BeWo cells were plated in complete DMEM-F12 media supplemented with 1% charcoal stripped FCS and incubated during 48 h with different doses of E-BSA. Cell extracts were prepared as indicated in Materials and Methods. Proteins were separated on SDS-PAGE gels, and leptin expression was determined by Western blot analysis. Molecular weights were estimated using standard protein markers. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. Bands densitometry is shown in lower panels. Molecular mass (kDa) is indicated at the right of the blot.

B, BeWo cells were transiently transfected with pL1951 plasmid construction and treated with E_2 or E-BSA as indicated during 48 h in DMEM-F12 media supplemented with 1% charcoal stripped FCS. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity.

C, Placental explants were processed as previously described and treated with E_2 or E-BSA during 4 h. Total RNA was extracted as described in Materials and Methods. Leptin mRNA was quantified with real-time RT-PCR. Cyclophilin was used as internal standard.

D, BeWo cells were transiently transfected with ERE-Luc plasmid construction and treated with E_2 or E-BSA as indicated during 48 h in DMEM-F12 media supplemented with 1% charcoal stripped FCS. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity.

Results are expressed as mean ± SEM for three independent experiments performed in duplicates. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Dunnett's multiple comparison post hoc test. * $p < 0.05$ vs. control. a.u.: arbitrary units.

Figure 2

E-BSA activates multiple signaling pathways in BeWo cells

A-D, BeWo cells were preincubated 18 h in DMEM-F12 media supplemented with charcoal stripped 1% FCS, and then treated with E-BSA for 15 min. Cell extracts were prepared as indicated in Materials and Methods. ERK1/2 (A), AKT (B), p38-MAPK (C) and JNK (D) phosphorylation were determined by Western blot as indicated in Materials and Methods. The same membranes were analyzed by immunoblot using anti-total ERK 1/2 or AKT. Loading controls were performed by immunoblotting the same membranes with anti-α-tubulin as indicated.

Band densitometry is shown in lower panel in arbitrary units. Results from a representative experiment are shown. They are expressed as mean ± SEM of two independent experiments.

Asterisks indicate significant differences from the control according to Dunn's multiple comparison post hoc test. * p<0.05 vs. control.

Figure 3

E-BSA activates multiple signaling pathways in placental explants

A-E, Placental explants were processed as previously described and treated with E-BSA for 10 min. MEK (A), ERK 1/2 (B), AKT (C), p38-MAPK (D) and JNK (E) phosphorylation were determined by Western blot as indicated in Materials and Methods. The same membranes were analyzed by immunoblot using anti-total MEK, ERK 1/2 or AKT. Loading controls were performed by immunoblotting the same membranes with anti-α-tubulin as indicated.

In Western blot analysis, samples were loaded in a 12% SDS-PAGE. Band densitometry is shown in lower panel in arbitrary units. Results from a representative experiment are shown. They are expressed as mean ± SEM of two independent experiments.

Band densitometry is shown in lower panel in arbitrary units. Results from a representative experiment are shown. They are expressed as mean ± SEM of two independent experiments. Asterisks indicate significant differences from the control according to Dunn's multiple comparison post hoc test. * p<0.05 vs. control.

Figure 4

Different signaling pathways are involved in leptin expression induced by E-BSA

A, BeWo cells were transfected with pL1951 and were pretreated for 20 min with different pharmacological inhibitors: 50 μM PD98059 (PD), MEK inhibitor, 5 μM SB 203580 (SB), p38-MAPK inhibitor, 5 μM SP600125 (SP), JNK inhibitor, 0.1 μM Wortmannin (W) and 1 μM LY-294,002 (LY), PI3K inhibitors. Cells were incubated for 48 h with (black bars) or without 1 nM of E-BSA. Cell extracts were prepared as indicated in Materials and Methods and Luc activity was normalized to β-galactosidase activity.

B, Placental explants were pretreated for 20 min with different pharmacological inhibitors: 50 µM PD98059 (PD), MEK inhibitor, 5 µM SB 203580 (SB), p38-MAPK inhibitor, 5 µM SP600125 (SP), JNK inhibitor, 0.1 µM Wortmannin (W) and 1 µM LY-294,002 (LY), PI3K inhibitors. Cells were incubated for 4 h with (black bars) or without 10 nM of E-BSA. Total RNA was extracted as described in Materials and Methods. Leptin mRNA was quantified with real-time RT-PCR. Cyclophilin was used as internal standard.

Results are expressed as mean ± SEM for three independent experiments performed in triplicates. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test. * $p<0.05$ vs. control, # $p<0.05$ vs cells treated with E-BSA. a.u.: arbitrary units.

Figure 5

ERα was detected in plasma membrane fractions of BeWo cells

A, Subcellular fractions were isolated from BeWo cells by sucrose centrifugation as described in Materials and Methods and ERα, ERβ, HDAC1, G-protein β subunit and α-tubulin expression were analyzed by Western blot. N: nuclear, M: membrane and C: cytosolic fractions. T: total cell lysate.

B, BeWo cells were plated in complete DMEM-F12 media supplemented with charcoal stripped 1% FCS and incubated during 48 h with 100 nM E_2 or 100 nM E-BSA. Subcellular fractions were isolated by sucrose centrifugation as described in Materials and Methods and ERα, HDAC1, G-protein β subunit and α-tubulin expression were analyzed by Western-blot.

C, ERα band densitometry is shown, relative to α-tubulin expression. Results are expressed as mean ± SEM. Results from a representative experiment are shown. Three independent repetitions were performed. a.u.: arbitrary units.

Figure 6

Immunolocalization of ERs in BeWo cells

A-F, BeWo cells were fixed, permeabilized and blocked as described in Materials and Methods. Cells were incubated ON with anti ERα (A and D), anti ERβ (B and E) or with no primary antibody as negative control (C and F). A Cy3-labeled anti-rabbit was used to detect bounded primary antibodies. Cells were stained with Hoechst 33342, mounted with Mowiol 4-88, and examined in a conventional fluorescence microscope (A-C) or in a confocal fluorescence microscope (D-F). Representative images of four independent experiments are shown. Bar size: 50μm. Neg: Negative control.

Figure 7

The antiestrogen ICI 182,780 antagonized E-BSA effects

A, Placental explants were processed as previously described, pretreated during 20 min with 100 nM ICI 182,780 and then further incubated with E-BSA as indicated during 4 h. Total RNA was extracted as described in Materials and Methods. Leptin mRNA was quantified with real-time RT-PCR. Cyclophilin was used as internal standard.

B-C, Placental explants were processed as previously described, pretreated during 20 min with 100 nM ICI 182,780 and then further incubated with E-BSA as indicated for 10 min. ERK 1/2 (B) or AKT (C) phosphorylation were determined by Western blot as indicated in Materials and Methods. Total ERK 1/2 or AKT protein levels in cell extracts were determined.

Results are expressed as mean ± SEM for three independent experiments performed in triplicates. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Dunnett's multiple comparison post hoc test. * $p < 0.05$ vs. control. a.u.: arbitrary units.

Figure 8

ERα silencing diminished E-BSA effect on leptin expression

A-B, BeWo cells (A) or stably ERα silenced cells (B) were plated in complete DMEM-F12 media supplemented with charcoal stripped 1% FCS and incubated during 48 h with (black bars) or without (gray bars) 5μg/ml doxycycline. Cell extracts were prepared as indicated in Materials and Methods. Proteins were separated on SDS-PAGE gels, and ERα and ERβ expression were determined by Western blot analysis.

C. Stably ERα silenced cells were plated in complete DMEM-F12 media supplemented with 1% charcoal stripped FCS and pretreated 5 h with (black bars) or without (gray bars) 5μg/ml doxycycline, and then treated with E_2 or E-BSA as indicated for 48 h. Leptin expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membrane with anti-β-actin. Bands densitometry is shown in lower panels.

D. Stably ERα silenced cells were transiently transfected with pL1951 plasmid construction and pretreated with (black bars) or without (gray bars) 5μg/ml doxycycline. Cells were incubated with E_2 or E-BSA as indicated during 48 h in DMEM-F12 media supplemented with1% charcoal stripped FCS. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity.

Results are expressed as mean ± SEM for three independent experiments performed in duplicates. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test. * $p < 0.05$ vs. control, # $p < 0.05$ vs cells not treated with doxycycline. a.u.: arbitrary units.

Figure 9

Proposed model of E_2 mechanisms involved in induced leptin expression in trophoblastic cells

E_2 action involves nuclear and membrane-associated estrogen receptor alpha (ERα). The activation of several signaling pathways in response to E_2 regulates leptin expression. Another transcription factors (TF) could also be involved.

Figure 1

Figure 2
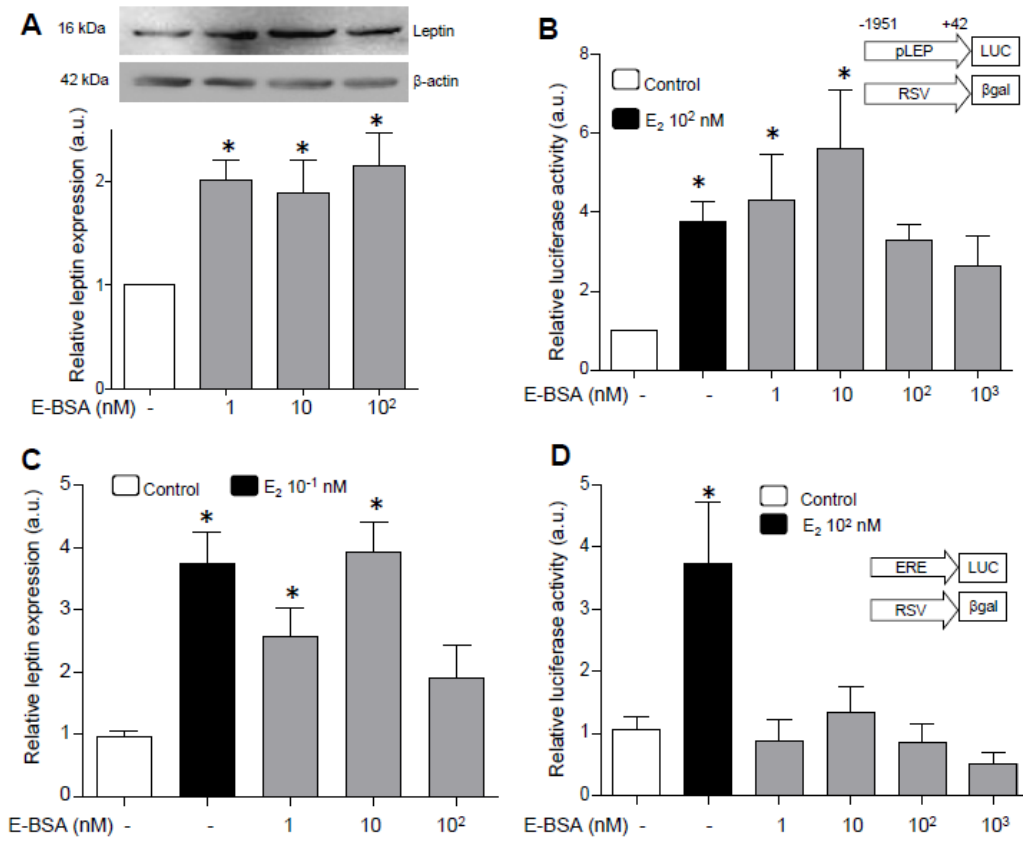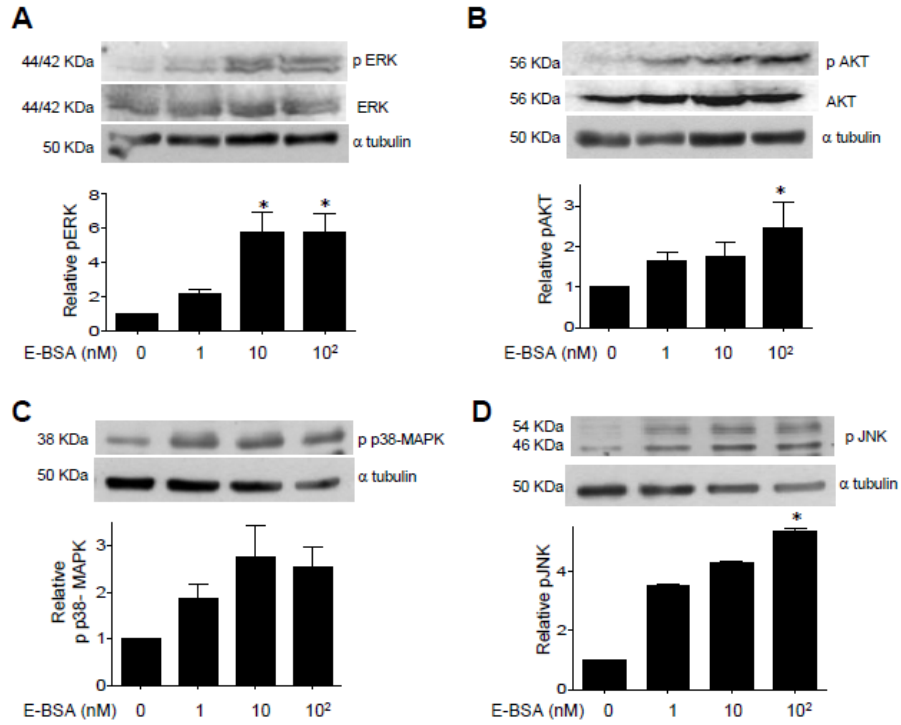
Figure 3

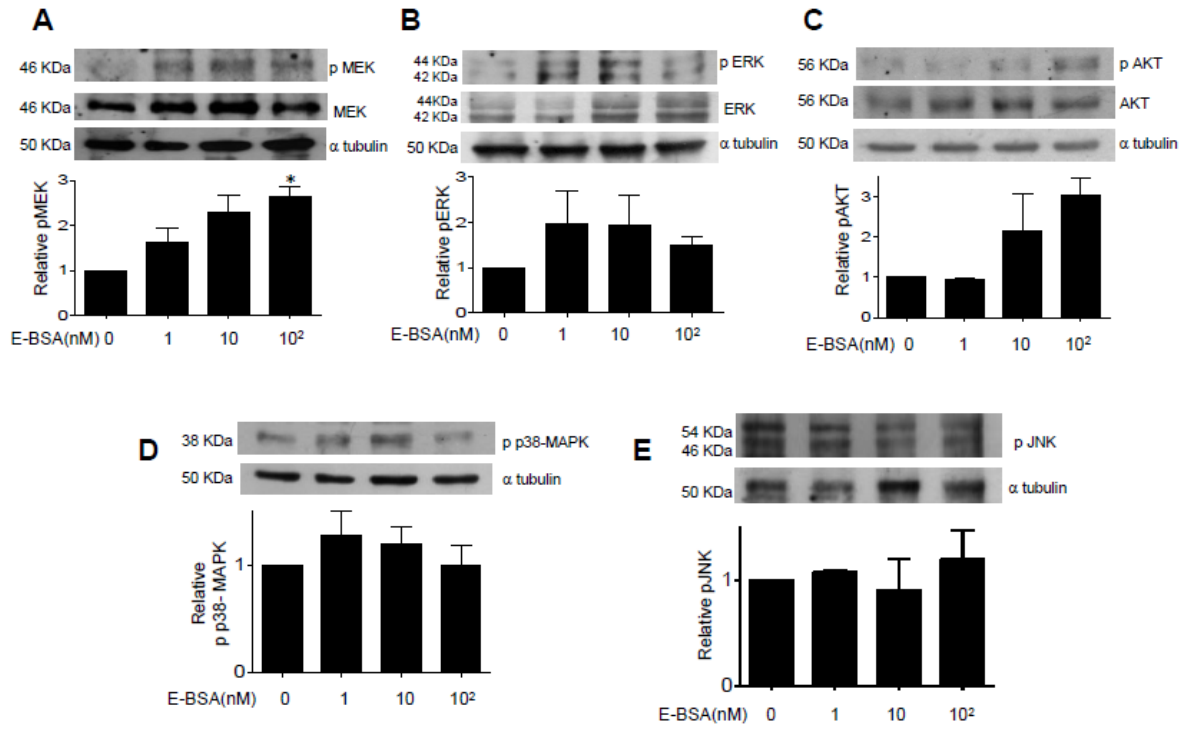**Figure 4**

Figure 5

Figure 6

| Hoechst | Immunofluorescence | | Hoechst | Immunofluorescence |

A ... ERα
B ... ERβ
C ... Neg
D ... ERα
E ... ERβ
F ... Neg

Figure 7

A

Relative leptin expression (a.u.)

| E-BSA (nM) | 0 | 1 | 10 | 0 | 1 | 10 |
| ICI (nM) | - | - | - | 10^2 | 10^2 | 10^2 |

B

44/42 KDa — pERK
44/42 KDa — ERK

Relative pERK (a.u.)

| E-BSA (nM) | 0 | 10 | 0 | 10 |
| ICI (nM) | 0 | 0 | 10^2 | 10^2 |

C

56 KDa — pAKT
56 KDa — AKT

Relative pAKT (a.u.)

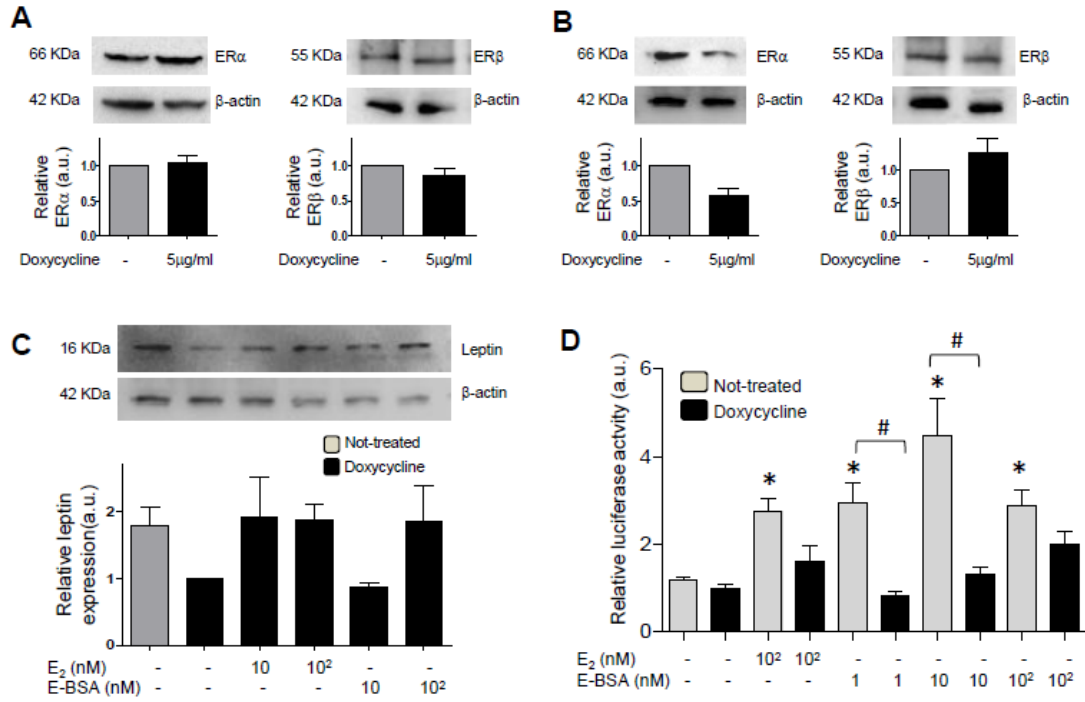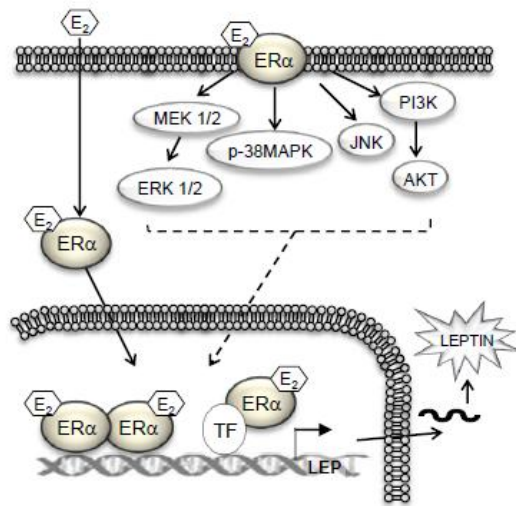| E-BSA (nM) | 0 | 10 | 0 | 10 |
| ICI (nM) | 0 | 0 | 10^2 | 10^2 |
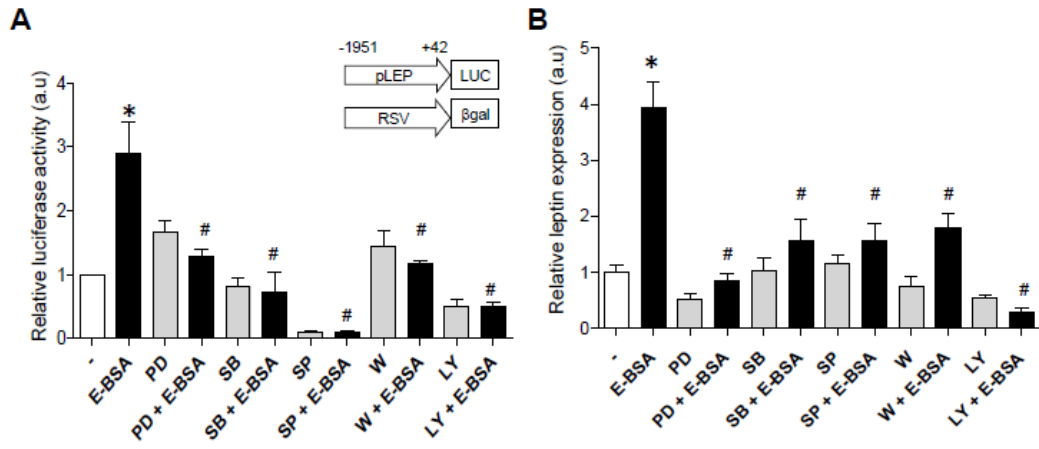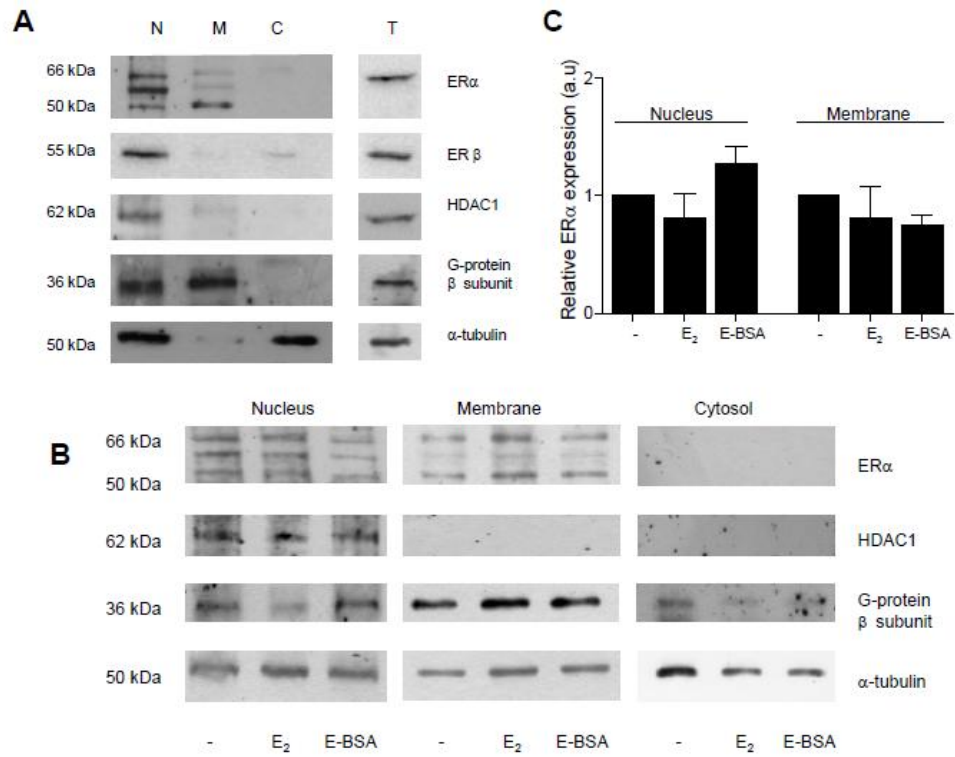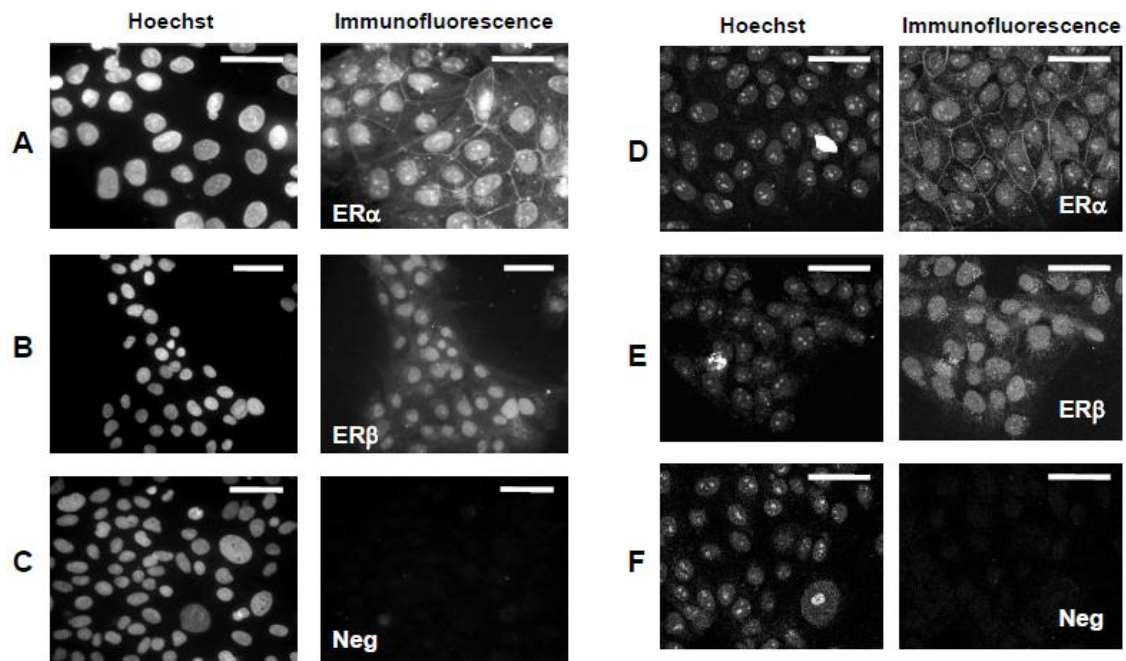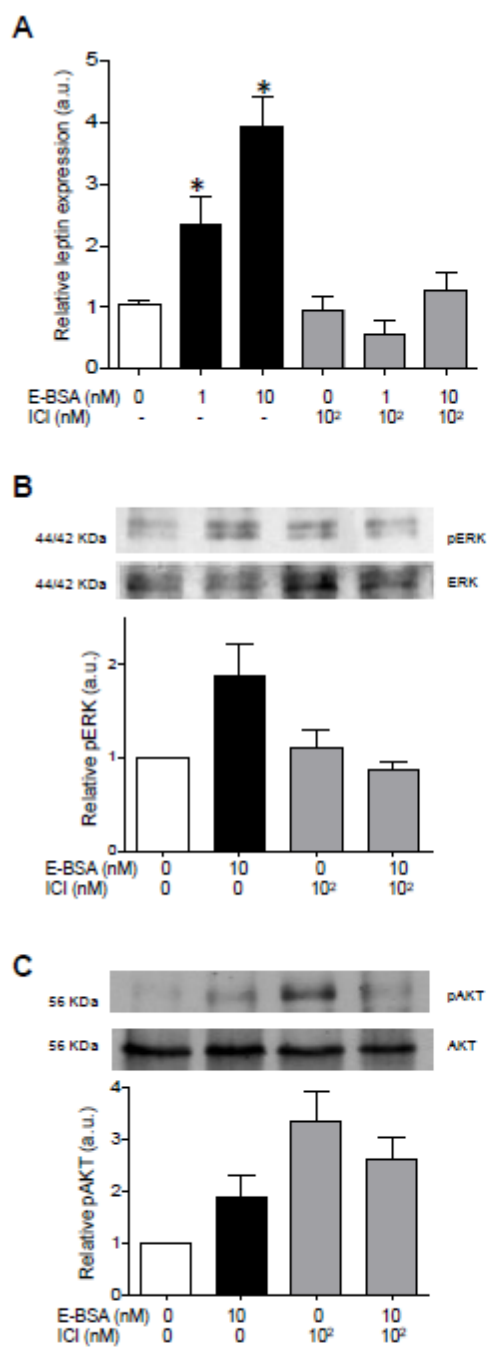
Figure 8

Figure 9

Highlights

- We study estrogen receptor mechanisms that regulate placental leptin expression.

- We use a membrane-constrained E_2 conjugate, E-BSA.

- E-BSA induced leptin expression, involving the activation of MAPK and AKT pathways.

- ERα was detected in the plasma membrane of BeWo cells.

- ERα mediates genomic and nongenomic actions of E_2 involved on leptin expression.