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Sp1 transcription factor is a modulator of estradiol leptin induction in placental cells

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ABSTRACT

Introduction: Pleiotropic effects of leptin have been identified in reproduction and pregnancy, particularly in the placenta, where it functions as an autocrine hormone. 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 upregulates placental leptin expression through genomic and nongenomic mechanisms.
Methods: To improve the understanding of estrogen receptor mechanisms in regulating leptin gene expression, we examined Sp1 transcription factor effect on estradiol leptin induction in human BeWo cell line.
Results: We demonstrated that Sp1 induces leptin expression determined by *q*RT-PCR, Western blot and transient transfection experiments. We also found that estradiol induction effect on leptin expression is enhanced by the over expression of Sp1 factor. Moreover, estradiol effect was not evidenced when Sp1 binding site on leptin promoter is mutated, suggesting that estradiol action is dependent on Sp1. On the other hand we showed data that demonstrate that Sp1 induction of leptin expression is insensitive to the antiestrogen ICI 182 780. By over expression experiments, we have also found that Sp1 effect on leptin expression could be mediated by estrogen receptor alpha. Supporting this idea, the downregulation of estrogen receptor alpha level through a specific siRNA, abolished Sp1 effect on leptin expression.
Discussion: Taken together all these evidences suggest a cooperative behavior between estrogen receptor alpha and Sp1 transcription factors to induce leptin transcription.

1. Introduction

The major role of the placenta is to establish the fetal maternal interface generating a crosstalk between maternal and fetal circulation. In addition, the placenta works as an endocrine tissue that produces steroids, peptide hormones, growth factors and cytokines that are crucial for the establishment and maintenance of pregnancy. Several cytokines and growth factors, such as leptin, are known to influence trophoblast migration, proliferation and invasion [1].

Leptin, the product of the *LEP* gene, is a small non-glycosylated pleiotropic peptide of 146 aminoacid residues (16 kDa), firstly found to be secreted by adipose tissue [2], with the function of modulating satiety and energy homeostasis [3]. Compelling evidence also implicated leptin in reproductive functions such as the regulation of fertility, ovarian function, oocyte maturation, embryo development and implantation [4–6]. The synthesis and secretion of leptin as well as its functional receptors by trophoblast cells have been widely demonstrated [7,8], suggesting that leptin may act through a paracrine or autocrine mechanism. In this way, previous

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studies have demonstrated the interactions between leptin and some placental hormones, implicating leptin as a modulator of placental endocrine function [9]. Moreover, leptin stimulates the process of proliferation and protein synthesis, and inhibits apoptosis [10–13] in human trophoblastic cells.

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 restriction, and preeclampsia [14,15].

Placental leptin production is strictly regulated and there are differences between the regulation of transcription of human placental and adipose leptin [16]. In fact, the human leptin gene has an enhancer located at −1.9 kb that is activated by a placental-specific transcription factor [17]. In this context, we have previously reported that estradiol, a key hormone in pregnancy, upregulates placental leptin expression [18–20].

Steroids play an important role in the development and maintenance of reproductive tissues [21]. The most potent and dominant estrogen in humans is 17β-estradiol (E_2) [22]. Human placenta synthesizes estrogens during pregnancy [23], in association with the cytotrophoblast invasion [24,25]. Moreover, placenta derived estrogen plays an autocrine role in trophoblast differentiation [26,27]. Estrogen receptors (ER) ERα and β proteins have been localized in nuclei of cultured human syncytiotrophoblast cells using immunohistochemistry [27]. Estradiol mediates its effect through genomic and nongenomic actions. Estradiol binding to its ER generates in the classical response, the dimerization of the receptor and the association with coactivators or corepresors that regulate gene transcription through binding to target genes [28]. The regulation of gene expression by ER activation might also involve the interaction with other DNA-bound transcription factors [29]. In our previous work [18,30], we have shown that E_2 indeed upregulates leptin expression in human placental cells involving genomic and nongenomic actions by a crosstalk between ERα and the MAPK and PI3K signal transduction pathways.

Although these findings suggest the importance of estrogens in leptin biosynthesis, the mechanisms that mediate leptin expression by estrogen in human placenta remain undefined. To improve the understanding of estrogen receptor mechanisms in regulating placental leptin expression, we examined here the effect of specific protein 1 (Sp1) transcription factor on leptin expression in human placental cells.

Sp1 is a transcription factor that has been found to be present in all mammalian cell types. However, Sp1 is now known to be involved in the regulation of tissue-specific, cell cycle, and signaling pathway response genes [31]. Sp1 is a member of the Specificity protein/Kruppel-like factor (Sp/KLF) family of transcription factors, which has a total of 26 proteins [32]. Sp1 functions by recruiting the basal transcription machinery and interacting with the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs) [33].

Sp1 also regulates gene expression by modulating the chromatin state. It has been found to interact with histone modifying enzymes, including the histone acetyl transferase p300. Thus, gene expression can be enhanced both by changing the chromatin modifications at the promoter towards a more permissive structure and by the binding of a transcription activator such as Sp1 [34]. Moreover, other proteins can bind to Sp1 and activate transcription synergistically, such as ER proteins. When ER binds to Sp1 increases Sp1-DNA binding to estrogen responsive elements independently of estrogen, but the transactivation of the gene is only enhanced in the presence of estrogen [29].

Since estradiol plays a critical role in the control of numerous placental hormones and seems to mediate leptin gene expression in placenta, we reasoned that Sp1 transcription factor may regulate estradiol effect on leptin expression.

Our study demonstrates that Sp1 enhances leptin expression induced by estradiol in placenta involving a Sp1 binding site. At the same time, ERα is necessary to evidence Sp1 effect.

2. Materials and methods

2.1. Cell culture and treatments

The human choriocarcinoma cell line BeWo was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in Dulbecco Modified Eagle Medium (DMEM-F12) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml, streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma) at 37 °C in 5% CO_2.

Maximal effect of 17β-estradiol (E_2; Sigma) on leptin was previously determined, and these concentrations (10 or 100 nM) were used on subsequent experiments [18]. The antiestrogen ICI 182 780 (AtraZeneca) was used to assay the specificity of estrogen effects. All E_2 treatments were performed in DMEM/F-12 media without phenol red and supplemented with 1% charcoal stripped FBS unless indicated. Serum in the incubated medium was reduced from 10% to 1% to lower the nonspecific effects.

2.2. Western blot

Cells were seeded at 50–60% confluence in DMEM-F12 medium supplemented with 10% FBS. Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10,000 g for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by the Bradford staining method [35], with bovine serum albumin (BSA) as a standard. Lysates were mixes with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and eletrophoretically transferred to nitrocellulose membrane (Hybond, Amersham Pharmacia). Membranes were equilibrated in 1× PBS and non-specific binding sites were blocked by 5% non-fat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal antibody rabbit anti-Sp1 (1:1000, Millipore) with polyclonal rabbit anti-human leptin Y20 (1:1000, Santa Cruz Biotechnolgy, Inc.), or with polyclonal rabbit anti-ERα HC20 (1:500, Santa Cruz Biotechnology, Inc) antibodies. Loading controls were performed by immunoblotting the same membranes with monoclonal mouse anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10 000, Calbiochem) or with polyclonal rabbit anti-β-actina (1:1000, Sigma) antibodies. The antibodies were detected using horseradish peroxidase-linked goat anti-rabbit IgG (1:10 000, Sigma.) or peroxidase-linked goat anti-mouse IgG (1:1000, Sigma) and visualized by the Amersham Pharmacia enhancing ECL chemiluminescence signaling system and a Bio-Imaging Analyzer Fujifilm LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Quantification of protein bands was determined by densitometry using Image J 1.47 software (Wayne Rasband National Institute of Health, USA).

2.3. Plasmids

The luciferase reporter constructs based on the PGL-3 basic vector and PGL-3 promoter vector, contained fragments of leptin promoter regions upstream the luciferase (Luc) reporter gen that codify for the luciferase enzyme. They were all kindly provided by Dr. Oksana Gavrilova (NIDDK, National Institute of Health, Bethesda, MD) [17]. To simplify the notation we renamed plasmids indicating the number of base pairs upstream of transcription

initiation as follows: pL1951/42 (−1951 to +42 bp of leptin promoter). The pGL-3 basic is the empty construct. Plasmid derived from pGL-3 promoter vector contained fragments of leptin promoter upstream of the SV40 minimal promoter, are pL1951/1847 (−1951 to −1847 bp of leptin promoter), pL1951/1887m (−1951 to −1887 bp of leptin promoter containing a mutation at Sp1 binding site), pGL-3promoter (empty construct). The expression plasmids Sp1 and HEGO contain respectively the cDNA of the Sp1 human protein [36] and the cDNA of the ERα (kindly provided by Dr. Adalí Pecci, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina). 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) [37]. 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 normalize the efficiency of individual transfections, pRSV-βgal containing the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) was used. In experiments using expression plasmids, the empty vectors were use as control. To perform transient transfection assays, plasmids were purified using the Maxipreps Wizard kit (Promega), and DNA concentration of was estimated spectrophotometrically.

2.4. Transient transfection experiments

For transient transfection experiments, BeWo cells were plated at a density of 2.5×10^5 cell/ml onto six-well dishes containing 1 ml of DMEM-F12 plus 10% FBS. Cells were incubated for 24 h. Medium was replaced and transfection of cells was performed according to the standard liposome-mediated method. In order to determine the sensitivity of the method in this cell type, a standard dose of reporter plasmid vs. light emission was performed (data not shown). Typically 5 μg of the luciferase reporter, 5 μg of the pRSV- βgal internal control construct and 5 μg of each expression plasmid were transfected using 5 μl of Lipofectamine (Life Technologies, Inc.). The medium was replaced after 5 h with DMEM-F12 1% FBS with the addition of the different effectors. Transfection analysis was performed by duplicate in each of 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 FBS containing 5 mg/ml doxycycline (Pfizer) during 48 h.

2.5. Assays for luciferase and β-galactosidase activities

Luciferase activity in cell lysates was measured using the Luciferase Assay System (Promega). Cells were washed with PBS and harvested 72 h after transfection using 50 μl of lysis buffer. Luciferase activity was measured with the GloMax-Multi + Microplate Multimode Reader luminometer (Promega). β-galactosidase activity was assayed using 1 mg of *o*-nitrophenyl β-D-galactopyranoside (AmResco) 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 °C until yellow staining. The product was determined by absorption at 420 nm. This value was used to correct variations in transfection efficiency. Luciferase results were calculated as the ratio of luciferase activity per unit of β-galactosidase activity. Duplicate samples were analyzed for each data point.

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

Abundance of leptin mRNA was determined by *q*RT-PCR. Total RNA was extracted from BeWo cells using TRISURE reagent, according to the manufacture instructions (Bioline). 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 °C 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′ GAACCCTGTGCGGATTCTT 3′; reverse, 5′ CCAGGTCGTTG- GATATTTGG 3′; and cyclophilin: forward, 5′ CTTCCCCGATGAGAACTTCA 3′; reverse, 5′ TCTTGGTGCTCTCCACCTTC 3′. Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (BioRad). 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 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 s at 95 °C and 1 min annealing and extension 1 min at 59 °C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the 2-ΔΔCT method [38]. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a house-keeping gene (cyclophilin), and relative to the untreated control. Melting curve analysis was performed to confirm specificity of amplification. Reaction mixtures without reverse transcriptase or RNA were run in parallel to ensure the absence of sample contamination.

2.7. Data analysis

For Western blots analysis, representative images of at least three independent experiments are shown along with quantification of immunoreactive bands. Quantitative RT-PCR experiments were repeated separately at least three times to ensure reproducible results. Transient transfection experiments were repeated at least three times and each treatment performed by duplicates. Results are expressed as the mean ± standard deviation (SD). The statistical significance was assessed by ANOVA followed by Bonferroni's or Dunnet's multiple comparisons post hoc tests and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. The Sp1 transcription factor enhances leptin expression in placental cells

The choriocarcinoma cell line BeWo was used as a model for trophoblastic cells as previously reported [7,39]. Published data have demonstrated that leptin and leptin receptor are expressed in this line, suggesting that leptin is probably exerting both paracrine and autocrine effects [11]. We have previously shown that estradiol stimulates leptin expression in BeWo cells as well as in human

placental explants [18,19]. By serial deletion analysis, we have demonstrated that the region encompassed between −1951 bp to −1847 bp in leptin promoter is sufficient to evidence E_2 induction on its expression. Moreover, using Matinspector *in silico* analysis, we found that this region contains close a putative half ER element (ERE) and a Sp1 binding site. In this regard, we aimed to study Sp1 effect on leptin expression in placental cells. Fig. 1A shows that the over expression of the human transcription factor Sp1 (1 and 5 μg plasmid), significantly enhanced endogenous leptin expression in BeWo cells, measured by Western blot analysis. No doses response curve was observed probably due to system saturation. To further study leptin regulation by Sp1, transient transfection analysis with leptin promoter reporter plasmids were performed. Fig. 1B shows that the over expression of the human transcription factor Sp1 with 0.5 and 1 μg plasmid, enhanced leptin expression in BeWo cells, measured by transient transfection

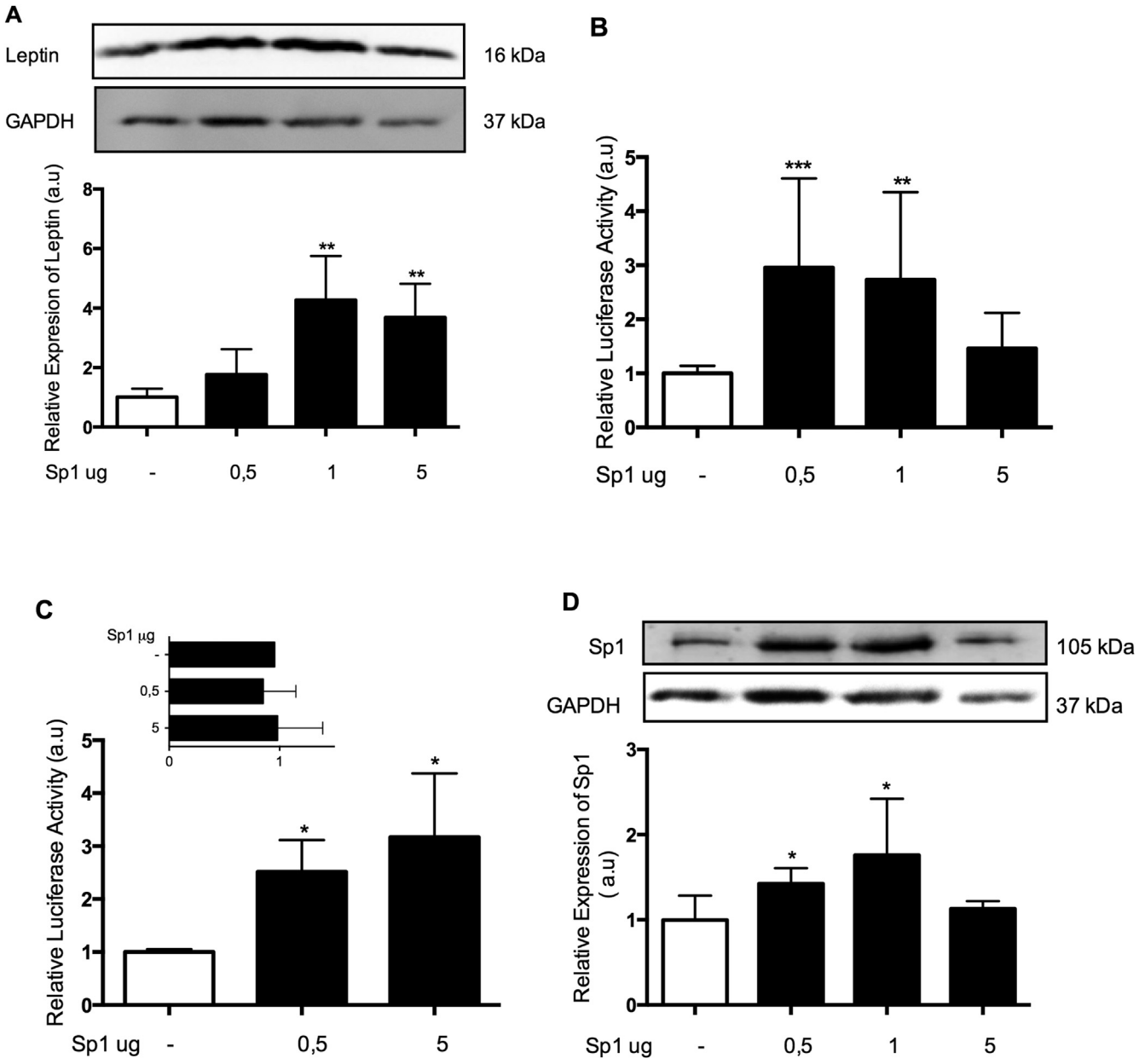

Fig. 1. Sp1 transcription factor enhances leptin expression. **A)** BeWo cells were transiently transfected with Sp1 expression plasmid in three different concentrations as indicated, during 48 h in DMEM-F12 media supplemented with 1% FBS. Leptin expression was determined by Western Blot as indicated in Materials and Methods. Loading control was performed by immunoblotting the same membrane with anti-GAPDH as indicated. Samples were loaded in a 12% SDS-PAGE. Band densitometry is shown in lower panel in arbitrary units. Results from representative experiment are shown. Statistical analyses were performed by ANOVA followed by Dunnett hoc test $^{**}p < 0.01$ **B)** BeWo cells were transiently transfected with the pL1951/42 and Sp1 expression plasmid different concentrations as indicated, during 48 h in DMEM-F12 media supplemented with 1% FBS. Cell extracts were prepared as indicated in Materials and Methods and Luc activity was normalized to β-galactosidase activity. Significant differences from control were determined by ANOVA followed by Dunnett post hoc test $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. **C)** Cells were transiently cotransfected with the reporter vector pL1951/1847 and different concentrations of Sp1 expression plasmid as indicated, during 48 h in DMEM-F12 media supplemented with 1% FBS. The inset shows the empty vector pGL-3promoter used as control. Luc activity was normalized to the β-galactosidase activity. Significant differences from control were determined by Bonferroni test $^{*}p < 0.05$. n = 3. a.u. = arbitrary units. **D)** BeWo cells were transiently transfected with Sp1 expression plasmid. Sp1 expression was determined by Western blot. Proteins were separated on 12% SDS-PAGE gels. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Band densitometry is shown in the lower panels. Representative result is shown (n = 4). Significant differences from control were determined by ANOVA followed by Dunnett hoc test $^{*}p < 0.05$ n = 4. a.u. = : arbitrary units.

analysis with the pL1951/42 reporter plasmid construction that contains leptin promoter region from −1951 to +42 bp. To further investigate Sp1 effect on leptin expression, we performed transient transfection analysis using the pL1951/1847 construction. This reporter plasmid contains the minimal estradiol responsive region from −1951 bp to −1847 bp of leptin promoter cloned upstream a minimal SV40 promoter directing luciferase expression. As displayed in Fig. 1C the co-transfection of this construction with the Sp1 expression vector from 0.5 to 5 μg significantly increased luciferase expression. Negative control using empty reporter vector showed no response to Sp1 over expression (Fig. 1C, inset). It can be observed in transfection analysis, that the Sp1concentrations required to enhance leptin expression are different than the observed in Western blot analysis. This effect is probably due both to differences in the chromatin state between the endogenous leptin promoter and the fragments cloned in the reporter plasmids and also to the presence of diverse binding elements to other transcription factors in the two constructions. No dose response curves were observed in any case, suggesting that leptin regulation by Sp1 is probably saturated at high transcription factor level or competence with other modulators are occurring in these conditions. In Fig. 1D a control of over expression of Sp1 factor, measured by Western blot is shown. It can be observed a significant increase in Sp1 signal with 0.5 and 1 μg of expression plasmid. All these results suggest a regulatory effect of Sp1 transcription factor on leptin expression.

3.2. The induction effect of estradiol on leptin expression is dependent of Sp1

In order to establish if Sp1 effect is related to E_2 enhancement of leptin expression, we decided to perform transient transfection experiments with different amounts of Sp1 expression vector and 100 nM E_2 treatment. Endogen leptin mRNA was measured by *q*RT-PCR. As it is seen in Fig. 2A, Sp1 over expression with 0.5 and 1 μg plasmid enhanced leptin expression. Treatment with 100 nM E_2 also increased leptin expression. The combination of 0.5 μg of Sp1 plasmid plus 100 nM E_2 significantly enhanced leptin mRNA level.

To further study Sp1 and E_2 effect on leptin expression, a different approach was carried out using reporter assays in transient transfection experiments. In Fig. 2B results obtained with pL1951/42 reporter plasmid are shown. As it can be seen both Sp1 over expression and E_2 treatment enhanced leptin expression. When added in a concentration of 100 nM E_2 and 0.5 μg Sp1

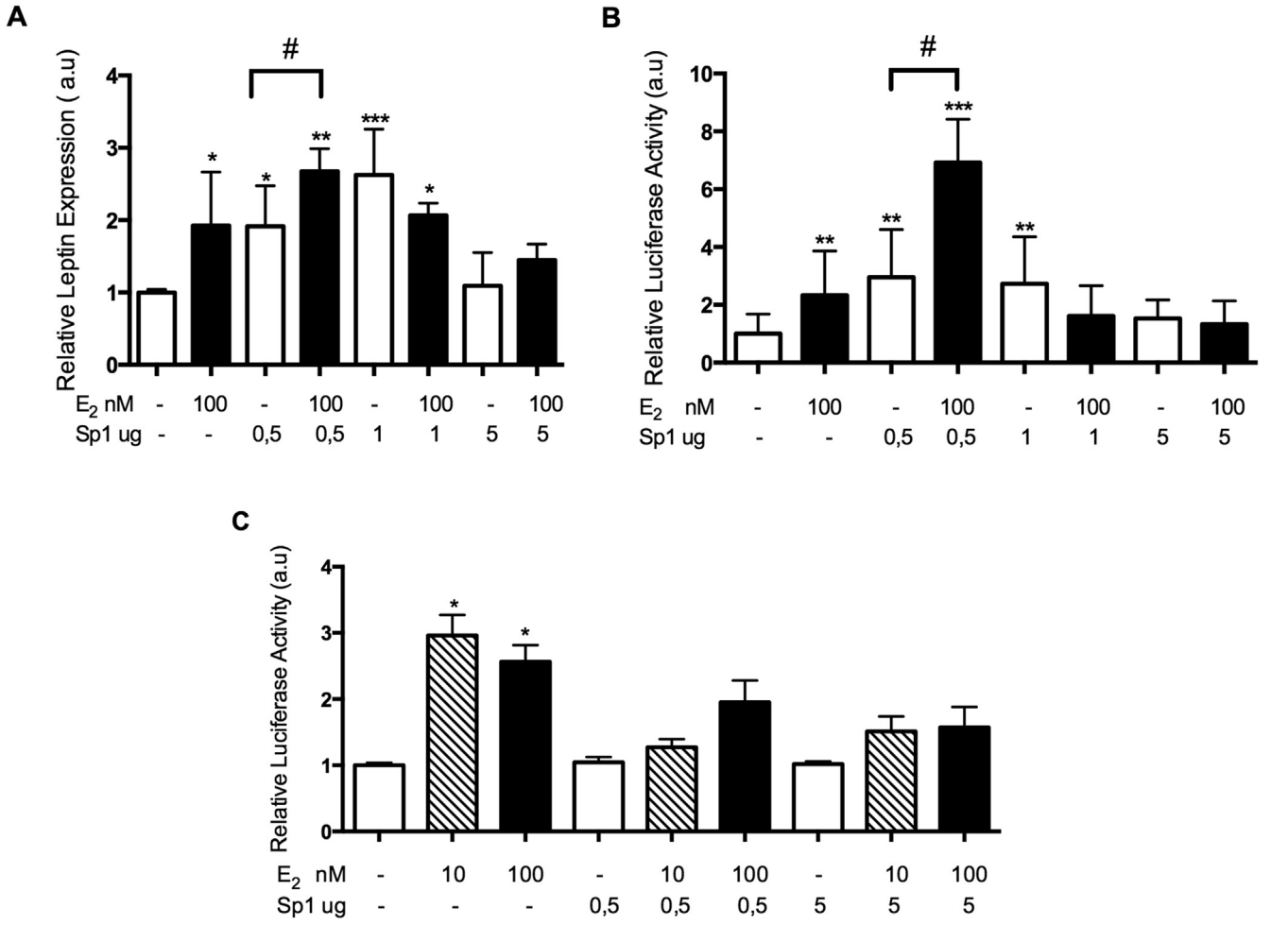

Fig. 2. Leptin induction by E_2 is increased by Sp1. BeWo cells were transiently transfected with pL1951/42 plasmid construction and Sp1 expression plasmid and treated with E_2 as indicated, during 48 h in DMEM-F12 media supplemented with 1% FBS**. A)** Leptin mRNA was quantified by *q*RT-PCR. Cyclophilin was used as an internal standard. (n = 3). **B)** Luc activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 6). **C)** Cells were transiently transfected with pL1951/1847 plasmid construction containing the promoter region of leptin gene from −1951 to −1847 bp upstream the minimal SV40 promoter and Sp1 expression plasmid and treated with E_2 in different concentrations as indicated, during 48 h in DMEM-F12 media supplemented with 1% FBS. Luciferase activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 4). Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control. #p < 0.05 between indicated treatments. a.u = arbitrary units.

plasmid the enhancement was synergistic.

In Fig. 2C the reporter plasmid pL1951/1847 was used. This construction contains the leptin promoter region from −1951 bp to −1847 bp upstream a minimal SV40 promoter. As it is seen, 10 and 100 nM E_2 significantly increased leptin expression as already published [18]. When Sp1 transcription factor was over expressed, the addition of 10 nM E_2 showed no significant effect on induced leptin expression. Probably in the minimal E_2 responsive region Sp1 overexpression is sufficient to evidence leptin induction effect.

With the aim of characterizing the role of Sp1 effect on E_2 enhancement of leptin expression, we decided to study the effect of mutated Sp1 recognition sequence in reporter transfection assays. To perform these experiments, a plasmid containing a mutation CGCC for TAGT in the core Sp1 binding site was used as it is represented in Fig. 3A. As it is shown in Fig. 3B, when using the mutated Sp1 binding site construction, E_2 effect on leptin expression was completely abolished (compare with Fig. 2C). These results demonstrated the need of this intact Sp1 site to evidence E_2 effect on leptin expression in placental cells.

3.3. Sp1 induction of leptin expression is insensitive to the antiestrogen ICI 182 780

To further characterize Sp1 and E_2 effect on leptin expression experiments the antiestrogen ICI 182 780 (ICI) was used. We have already reported that this antiestrogen diminished E_2 effect on leptin expression in a dose dependent manner demonstrated by Western blot and reporter analysis [18]. In Fig. 4A it can be seen that ICI treatment exerted no significant effect on Sp1 induction of endogenous leptin expression, determined by Western blot. Similarly, in transient transfection experiments, the addition of this antiestrogen did not modify Sp1 effect on the reporter gene. All these results suggest that Sp1 induction of leptin expression is insensitive to the antiestrogen ICI.

3.4. The upregulation of leptin by Sp1 is dependent on estrogen receptor alpha

It is known that estrogen receptors can bind to Sp1 transcription factor to regulate the transactivation of different genes [29]. On the other hand we reported data that demonstrate estradiol induces leptin expression in placental cells principally through genomic and nongenomic actions involving the isoform ERα [19]. To analyze the cooperation between ERα and Sp1 transcription factors on placental leptin transcription, different approaches were performed. First, the over expression of ERα was evaluated in transient transfection assays in BeWo cells. As seen in Fig. 5A the over expression of 0.5 μg of Sp1 expression vector significantly increased leptin expression as already discussed. The cotransfection with 10 ng of the expression vector for ERα expression vector synergistically increased Sp1 induced leptin expression. It is worthy to notice that very small quantities of HEGO plasmid were used to not saturate the system. In the inset of Fig. 5A, it could be seen ERα expression in BeWo cells transfected with HEGO, determined by

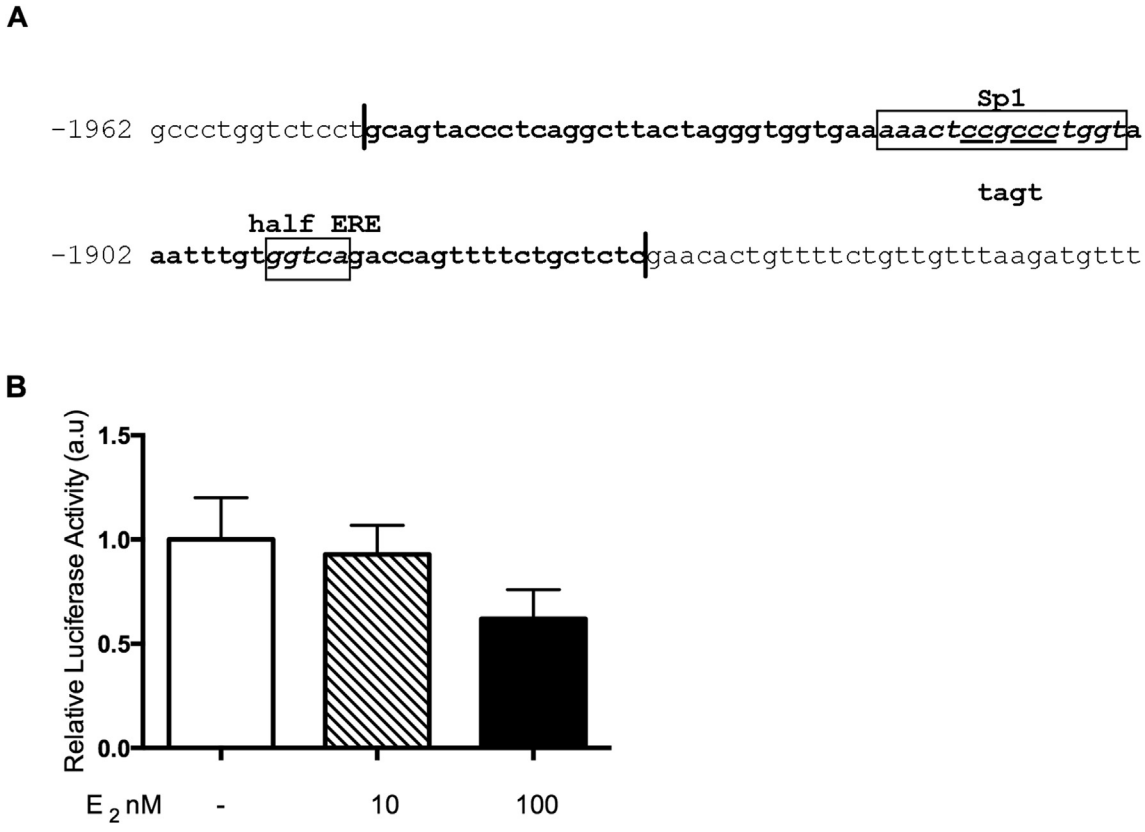

Fig. 3. Estradiol effect on leptin expression is dependent on Sp1. A) Leptin promoter sequence (−2922 to +29) was analyzed by the MatInspector 7.7.3. The enhancer element of placental leptin (PLE) is indicated in bold text and contains the elements related with the action of E_2: Sp1 binding site and half ERE are indicated in black boxes. The mutated Sp1 binding site present in the pL1951/1887m construction is indicated below the Sp1 box. **B)** BeWo cells were transiently transfected with the plasmid pL1951/1887m (leptin promoter region from −1951 to 1887 bp, with a mutation in the Sp1 binding site), and treated with E_2 as indicated during 48 h in DMEM-F12 media supplemented with 1% FBS. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 3) Statistical analyses were performed by ANOVA followed by Dunnett's post hoc test vs. control. a.u = arbitrary units.

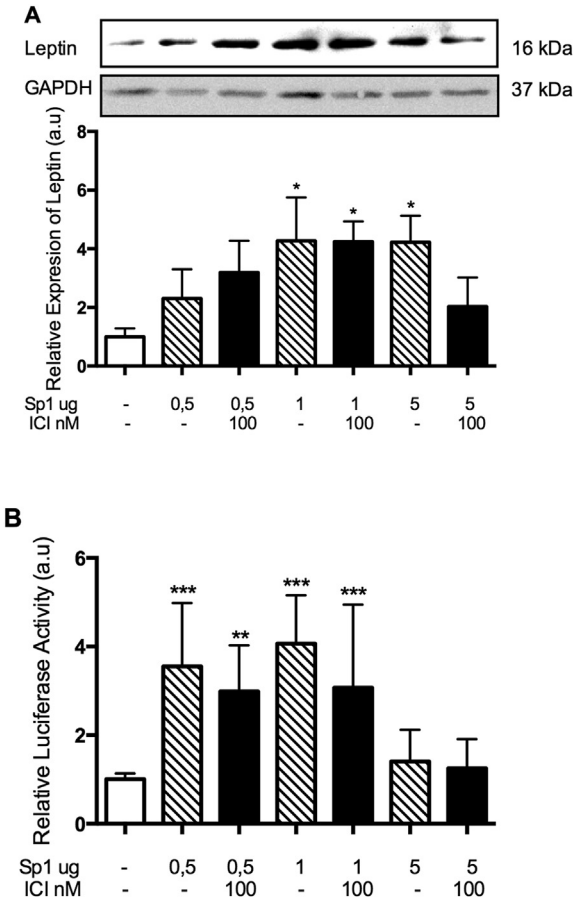

Fig. 4. Sp1 induction of leptin expression is insensitive to the antiestrogen ICI 182 780. BeWo cells were transiently transfected with Sp1 expression plasmid and treated with different doses of the antiestrogen ICI 182 780 during 48 h in DMEM-F12 media supplemented with 1% FBS. **A)** Leptin expression was determined by Western Blot as indicated in Materials and Methods. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Band densitometry is shown in the lower panel. Results from a representative experiment are shown. (n = 2). **B)** BeWo cells were transiently transfected with pL1951/42 plasmid construction, Sp1 expression plasmid and treated with different doses of the antiestrogen ICI 182 780 during 48 h in DMEM-F12 media supplemented with 1% FBS. Cell extracts were prepared as indicated in Material and Methods and Luc activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 7). Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ vs. control. a.u. = arbitrary units.

Western blot as control. To further characterize ERα on Sp1 induction of leptin expression, experiments with E_2 activation were performed. As it is seen in Fig. 5B the addition of 10 or 100 nM E_2 was unable to enhanced ERα and Sp1 leptin induction. In a second approach BeWo cells with ERα downregulated expression were generated using siRNA strategy. Cells were stably transfected with an inducible expression plasmid encoding a precursor RNA that after processing maturates in a siRNA against ERα. This cell line called BeWo-Sh2 was cotransfected with the pL1951/42 reporter vector and Sp1 expression plasmid, siRNA for ERα was induced with doxycycline and Luciferase activity was determined after 48 h. Fig. 5C shows that the downregulation of ERα abolished Sp1 induction of leptin expression suggesting that ERα is necessary to evidence Sp1 effect. As expected, E_2 induction of leptin expression is lost. As a positive control an experiment was performed in BeWo-Sh2 cells in which doxycycline was omitted corroborating the inductive effect of E_2 on the leptin reporter plasmid pL1951 (Fig. 5C inset). Finally, experiments using the reporter plasmid pL1951/1887m, that contains leptin promoter region from −1951 to 1887 bp with Sp1 binding site mutated, was used. As it is shown in Fig. 5D even the transfection with 50 ng of HEGO expression plasmid, was unable to induce the activity of the pL1951/1887m reporter plasmid suggesting that an intact Sp1 binding site is necessary to evidence ERα leptin induction. A positive control is shown in the inset of Fig. 5D. It can be observed that ERα overexpression increased leptin expression when using the pL1951/1847 reporter plasmid without the mutation at the Sp1 binding site. All these results strongly suggest a cooperative effect between ERα and Sp1 transcription factors to induce leptin expression.

4. Discussion

During pregnancy leptin level raises in the mother serum [40] and is principally synthesized by the placenta [7]. Although little is known about the exact physiological role of leptin during human pregnancy, recent observations suggest that this hormone could be a key player in the regulation of placental function and development, embryo implantation and growth [4,30]. The regulation of leptin expression in placenta is not completely known. In this way, it has been reported that leptin synthesis is modulated by steroid hormones [41,42], glucocorticoids and insulin [16,43]. It was also demonstrated that the human leptin gene is actively enhanced by hypoxia through mechanisms that are common to other hypoxia-inducible genes [44]. Moreover, we have previously shown that leptin expression in placenta is upregulated by some important pregnancy signals such as hCG, cAMP and E_2 [18,45,46]. In particular, in the present work, we have studied the mechanisms involved in the regulation of leptin expression by E_2 and Sp1 transcription factor in BeWo human choriocarcinoma cells. These cells express both leptin and its receptor [11]. They maintain many characteristics of human trophoblast cells and have been widely used to study placental cellular signaling [47–49].

The molecular actions of E_2 coordinating leptin expression in trophoblasts are not fully elucidated. During human pregnancy, the production of E_2 raises eighty fold, from 0.75 nM pre-ovulatory peak to 60 nM at term. Estradiol influences different aspects of placental function and fetal development in humans and primates [50], and plays a crucial role in the regulation of fetal growth, onset of parturition, placental steroideogenesis, release of neuropeptides and glycoproteins and also the secretion of leptin [51]. Moreover, it was demonstrated that E_2 enhanced leptin promoter activity in JEG-3 cells [52]. These investigations suggest that E_2 could be an important regulator of leptin expression in placental cells. Our previous results indicate 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 [18]. Our reported data also showed that the leptin promoter region encompassed between −1951 bp to −1847 bp is sufficient to evidence E_2 effect on its expression. Moreover, using *in silico* analysis, we have found that this region contains a putative half ERE and a Sp1 binding site. These binding sites are very close and immersed in a leptin placental enhancer already described by Gavrilova and collaborators [17]. Estrogen, acting via ERα or ESRβ, modulates gene expression through multiple mechanisms. The traditional activation of an ERE involves ligand-bound receptor binding at a specific palindromic sequence of DNA within the promoters of estrogen-responsive genes [53]. However, genomic effects of estrogen could also be explained by an ERE-independent mechanism, because ERs can regulate gene expression by modulating the function of other classes of transcription factors through protein-protein interactions in the nucleus. It was reported that genes containing GC-rich promoter sequences are regulated in a similar manner through the interaction of ERs with the Sp1 transcription factor [29].

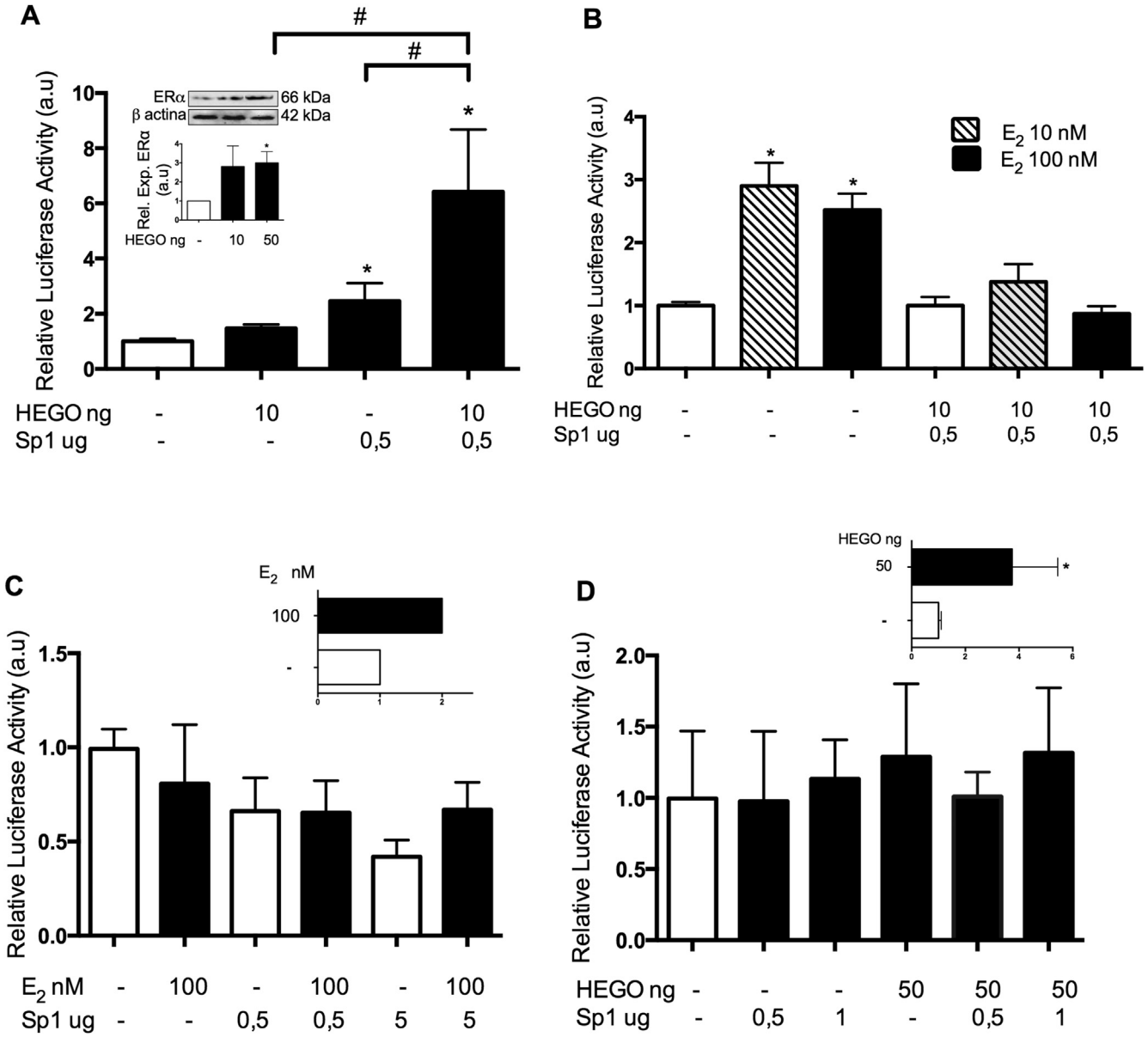

Fig. 5. Sp1 effect on leptin expression is dependent on ERα. A) BeWo cells were transiently transfected with pL1951/1847 plasmid construction containing the promoter region of leptin gene from −1951 to −1847 bp and different amounts of plasmid expressing human ERα (HEGO) and Sp1 expression plasmid. After transfection, BeWo cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS. Cell extracts were prepared as indicated in Materials and Methods. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 3). Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test * $p < 0.05$, vs. control, and #$p < 0.05$ between treatments. The inset figure shows BeWo cells transiently transfected with different concentration of the plasmid HEGO. After 48 h of incubation, cell extracts were prepared as indicated in Material and Methods. ERα was determined by Western blot. Loading controls were performed by immunoblotting the same membrane with anti-β-actin as indicated. Band densitometry is shown in lower panel (n = 3) Statistical analyses were performed by ANOVA follow by Dunnett post hoc test. *$p < 0.05$. **B)** BeWo cells were transiently transfected with pL1951/1847 plasmid construction, different amounts of plasmid expressing human ERα (HEGO) and Sp1 expression plasmid, and treated with E_2 as indicated. After transfection, cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS. Cell extracts were prepared as indicated in Materials and Methods; Luc activity was measured in the cellular extract and normalized to β-galactosidase activity (n = 3). Statistical analyses were performed by ANOVA followed by Dunnett post hoc test * $p < 0.05$ vs. control. **C)** BeWo-Sh2 cells were transiently transfected with pL1951/42 plasmid construction and Sp1 expression plasmid and treated with different doses of E_2, and with 5 μg/ml of doxycycline, to induce the expression of the interference RNA of the ERα. Cells were incubated for 48 h in DMEM-F12 media supplemented with 1% FBS. As control, doxycycline was omitted, corroborating the inductive effect of E_2 on the pL1951/42 vector (inset figure). Cell extracts were prepared as indicated in Materials and Methods, Luc activity was measured in the cellular extract and normalized to β-galactosidase activity (n = 2). **D)** BeWo cells were transiently transfected with the plasmid pL1951/1887m that contains leptin promoter region from −1951 to 1887 bp, with a mutation at the binding site of Sp1, different amounts of plasmid expressing human ERα (HEGO) and Sp1 expression plasmid as indicated. After transfection, BeWo cells were incubated during 48 h in DMEM-F12 media supplemented with 1% FBS. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity (n = 4). The inset shows BeWo cells transiently transfected with pL1951/1847 plasmid construction containing the promoter region of leptin gene from −1951 to −1847 bp with a plasmid expressing human ERα (HEGO) as a control (n = 2). Statistical analyses were performed by ANOVA followed by Bonferroni post hoc test * $p < 0.05$. a.u = arbitrary units.

In this regard, we aimed to study the regulation of leptin expression by Sp1 transcription factor. The effect of Sp1 transcription factor overexpression on leptin transcription was analyzed in BeWo cells. A significant upregulation was observed using Western blot, *q*RT-PCR and reporter assays with either the pL1951/42 or pL1951/1847 constructions. We observed an increase of leptin protein level after addition of Sp1 5 μg but leptin mRNA level was unchanged in the same experimental conditions. This difference might be due to posttranscriptional regulation. Further experiments will be needed to understand the mechanisms involved. Moreover E_2 treatment showed a synergistic effect on Sp1 induction of leptin expression both in *q*RT-PCR and transient transfection assays. Sp1 transcription factor as well as other members of its family, binds the consensus Sp1 binding sequence and also non-consensus sequences with variable affinities. However the importance of specific GC-rich sequences in a gene promoter in mediating transactivation is highly variable and dependent on cell context and the relative expression of nuclear coactivators and other cofactors [29]. Previous reports have demonstrated the involvement of Sp1 in the regulation of the leptin gene expression in adipocytes [54]. It was also reported that a mutation at the Sp1 binding sites in a leptin promoter construct significantly reduced promoter activity in 3T3-L1 adipocytes, suggesting that Sp1 is important in its regulation [55].

To further characterized Sp1 effect on leptin expression we decided to perform transient transfection experiments with different amounts of Sp1 expression vector and E_2 treatment. We demonstrated that E_2 significantly enhanced Sp1 leptin induction. This effect was dependent on the integrity of the Sp1 binding site, as no E_2 effect was observed when testing the construction containing the mutated Sp1 binding site suggesting a crosstalk between Sp1 and ER.

Some proteins can bind to Sp1 and activate transcription synergistically, such as ER proteins [31]. ER and Sp1 proteins physically interact and form a protein-protein complex in the absence of DNA. It was observed that the deletion of the DNA-binding domain of ER does not abolish the synergy of the ER with Sp1, indicating that ERs can regulate transcription in the absence of a functional ERE [56]. Recent studies also show that ER alpha preferentially binds to the *C*-terminal DNA-binding domain of Sp1 protein. Moreover, it was seen that ERα/Sp1 can activate transcription from a consensus GC-rich Sp1 binding site in transient transfection studies in MCF-7 human breast cancer cells, and this response is also observed with ERα variants that do not contain the DNA-binding domain [57].

Moreover, it was reported that the binding of ER to Sp1 increases Sp1-DNA binding to estrogen responsive elements independently of estrogen, but the transactivation of the gene is usually enhanced in the presence of estrogen [56]. Sp1-mediated transcription can be altered to respond to signaling pathways, alternatively, transcription factors can overactivate Sp1-dependent transcription by interacting with DNA-bound Sp1, but not binding to DNA directly [31].

In this work, our data support a mechanism activated by Sp1 to induce leptin expression in coordination with ERα in placenta. We show data that demonstrate that the overexpression of ERα in BeWo cells synergistically increased Sp1 induced leptin expression. Moreover this enhancement was not further augmented by E2 treatment, suggesting a ligand independent effect. These results are in concordance with reported data that showed that the interaction of ER and Sp1 proteins was observed in the presence or absence of E_2 and therefore was hormone-independent [56].

Many E_2 responsive gene promoters, such as leptin, contain both GC-rich and ERE half sites that cooperatively interact to activate gene expression. According to the "classical genomic model" ligand-activated ERα binds to specific ERE to modulate transcriptional activity of E_2 target genes. Nevertheless, 35% of the categorized human E_2 responsive genes are transcribed via indirect ER-DNA association through protein–protein interactions with several trans factors such as Sp1, nuclear factor-kappaB (NF-κB), or activator protein-1 (AP-1) [31]. For instance, Sp1 and ERα can form a complex *in vivo* to mediate E_2 induced activation of kisspeptin 1 promoter in hypothalamic GT-1 cells [58], the integral membrane protein Claudin-5 [59] among other genes. The actions of ERs at Sp1 binding sites depend on the ligand, the cell type, and the receptor subtype [29]. On the other hand, it was reported that Sp1/Sp3 compound heterozygous mice are not viable and manifest severe placental defects [60].

We have seen that cells with decreased expression of ERα through a specific siRNA completely abolished the effect of Sp1 on leptin expression. On the other hand the construct with the mutated Sp1 binding site and the intact ERE, also prevented ERα enhanced leptin transcriptional activity. These results demonstrated that Sp1 binding site contributes to E_2-ERα signaling.

On the other hand we show evidence that Sp1 effect is insensitive to ICI treatment. ICI is an estrogen analogue compound with an alkylsulphinyl side-chain at position 7α. In the classical model, estradiol binds and stimulates receptor dimerization and subsequent activation of two activation domains (AF1 and AF2). The activated receptor complex then undergoes nuclear localization and subsequent binding to EREs. Upon binding to these sequences, transcriptional coactivators are recruited to the complex, resulting in activation of gene expression. ICI is capable of binding to the ER but the receptor dimerization is impaired, in this way receptor degradation is accelerated by increase of receptor protein turnover, and AF1 and AF2 remain inactive. Ultimately, this results in disrupted nuclear localization and a failure to recruit transcriptional coactivators [61]. We propose that in our system ICI is not able to block ERα-Sp1 crosstalk to induce leptin expression.

In summary, in this study, we provide evidence that Sp1 induces leptin expression and that this effect cooperates with E_2 action through ERα. The mechanism of regulation of placental leptin expression by E_2 could possibly be a multifactorial process, and may depend on the presence, concentration and localization of ERs, transcription factors such as Sp1 factor, co-regulatory proteins and signal transducers, among others. Our results provide new evidence of the regulatory mechanisms of placental leptin expression.

Declaration of interest

All the authors declare that there is no financial or other potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2017.07.005.

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