REPRODUCTION
REVIEW

Involvement of leptin in the molecular physiology of the placenta

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Abstract

Leptin is a homeostatic regulator in the placenta where it promotes proliferation, protein synthesis and the expression of tolerogenic maternal response molecules such as HLA-G. Leptin also exerts an anti-apoptotic action in placenta controlling the expression of p53 master cell cycle regulator under different stress conditions. On the other hand, leptin is an integrative target of different placental stimuli. The expression of leptin in placenta is regulated by hCG, insulin, steroids, hypoxia and many other growth hormones, suggesting that it might have an important endocrine function in the trophoblastic cells. The leptin expression is induced involving the cAMP/PKA or cAMP/Epac pathways which have profound actions upon human trophoblast function. The activation of PI3K and MAPK pathways also participates in the leptin expression. Estrogens play a central role during pregnancy, particularly 17β-estradiol upregulates the leptin expression in placental cells through genomic and non-genomic actions. The leptin promoter analysis reveals specific elements that are active in placental cells. The transcription factors CREB, AP1, Sp1, NFκB and the coactivator CBP are involved in the placental leptin expression. Moreover, placental leptin promoter is a target of epigenetic marks such as DNA methylation and histone acetylation that regulates not only the leptin expression in placenta during pregnancy but also determines the predisposition of acquiring adult metabolism diseases. Taken together, all these results allow a better understanding of leptin function and regulatory mechanisms of leptin expression in human placental trophoblasts, and support the importance of leptin during pregnancy and in programming adult health.

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Introduction

The placenta is the organ that establishes fetal maternal interaction during pregnancy. It plays a critical role in conveying water, oxygen, nutrients and growth-regulating signals from the maternal compartment to the fetus. The placenta also produces a broad spectrum of hormones and growth factors that play key roles in maintaining a proper flux of nutrients between the mother and the fetus. Different hormones, growth factors and cytokines may therefore enhance embryo implantation and fetus growth. In this context, leptin has emerged as a key placental hormone that influences various aspects of placental function and fetal development in humans and primates.

Leptin hormone is the product of the *LEP* gene. It was discovered in 1994 by Zhang *et al.* (1994) and is a 16,000-MW non-glycosylated polypeptide of 146 amino acids. Its structure reveals a four-helix bundle similar to the long-chain helical cytokine family (Flier 1995). Leptin was first described to be secreted by the adipose tissue, with the function of food intake and energy expenditure modulation (Houseknecht & Portocarrero 1998). Nevertheless, leptin is now considered as a multifunctional hormone produced by different tissues and organs such as the stomach, skeletal muscle, pituitary cells and placenta (Reitman *et al.* 2001). Leptin exerts pleiotropic effects such us the modulation of thermogenesis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine and immune functions as well as arterial pressure control (Dos Santos *et al.* 2015). Leptin has also been implicated in reproductive functions, including menstrual-cycle regulation, oocyte maturation, implantation, embryo development and lactation (Cervero *et al.* 2005, Henson & Castracane 2006, Israel & Chua 2010). In this regard, leptin is an important player in the regulation of fertility. In fact, the absence of leptin action caused by mutations in the leptin gene or leptin receptor gene (*LEPR*) has been

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linked to infertility in rodents and humans (Hohos & Skaznik-Wikiel 2017).

A relevant role of leptin in implantation has been proposed. Embryo implantation is the most critical step in a successful pregnancy. The blastocyst becomes closely connected and invades the maternal endometrial surface (Aplin 2000, Su & Fazleabas 2015). A large number of mediators are involved in this early fetal–maternal connection, including hormones, adhesion molecules, cytokines, growth factors, lipids and others (Achache & Revel 2006). It was reported that the endometrium and preimplantation embryos possess *LEPR* mRNA, indicating that they are targets for leptin action. Furthermore, leptin mRNA is expressed in the blastocyst suggesting an active leptin role in the fetal–maternal dialog (Cervero *et al.* 2004).

In trophoblasts cells, the synthesis and secretion of leptin as well as its functional receptors were widely demonstrated (Masuzaki *et al.* 1997, Senaris *et al.* 1997), suggesting that leptin may act as a modulator of placental endocrine function (Coya *et al.* 2006). In this way, it was reported that leptin increases the matrix metalloproteinase expression in cytotrophoblasts, facilitating the implantation process (Castellucci *et al.* 2000). Besides, leptin has physiological effects on placenta development including angiogenesis, growth and immunomodulation (Henson & Castracane 2006). Previous studies demonstrated that leptin increases the release of pro-inflammatory cytokines and prostaglandins from human placental explants, stimulates hormone and cytokine production (Cameo *et al.* 2003) and regulates fetal growth and development (Henson & Castracane 2006), further implicating leptin as a modulator of placental function.

The human *LEPR* gene encodes by alternative splicing several transmembrane proteins with different intracellular C-terminal domains and one soluble isoform that lacks the transmembrane region. All leptin receptor isoforms are expressed in the placenta including the soluble form (Gorska *et al.* 2010). Leptin and its receptor have structural and functional similarities with the IL6 family of cytokines (Madej *et al.* 1995, Tartaglia *et al.* 1995). Leptin stimulates Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway by promoting JAK-2 and STAT-3 tyrosine phosphorylation in JEG-3 cell line, as well as in trophoblastic cells from human-term placenta. The STAT-3 activation has been correlated with trophoblast invasiveness (Corvinus *et al.* 2003). We have demonstrated that leptin activates other signal transduction pathways both in cell culture and in human-term placenta explants (Perez-Perez *et al.* 2008) such as the MAPK and PI3K pathways. The activation of the MAPK pathway mediates proliferative responses in different systems (Takahashi *et al.* 1997, Tanabe *et al.* 1997, Bjorbaek *et al.* 2001, Martin-Romero & Sanchez-Margalet 2001). The activation of PI3K, PKB and mTOR has been described to regulate differentiation of human trophoblasts (Pollheimer & Knofler 2005).

This review focuses on current knowledge of leptin as a homeostatic regulator in the placental cells as well as the regulation of leptin expression in trophoblastic cells and the signal transduction pathways involved. Besides, the transcription factors mediating its induction will be discussed. Finally, epigenetic leptin promoter modifications and their consequences in programming will be analyzed.

Leptin is a homeostatic regulator in the placenta

Leptin is implicated in inducing trophoblast cell proliferation and survival. In human pregnancy, trophoblast cells differentiate into two distinct pathways. In the villous pathway, cytotrophoblasts fuse to form a specialized syncytium called syncytiotrophoblast on the outer layer of the placental villi (Carson *et al.* 2000). The syncytiotrophoblast forms a barrier between maternal and fetal circulations and is important for the normal immunological, endocrine and nutritional functions of the placenta (Heazell & Crocker 2008). In the extravillous pathway, cytotrophoblasts proliferate and differentiate into an invasive phenotype that penetrate into the maternal decidua and myometrium and remodel uterine arteries (Su & Fazleabas 2015). We have demonstrated that leptin enhances BeWo, JEG-3 and Swan-71 cell proliferation in a dose-dependent way. After leptin treatment, cyclin D1, one of the key cell cycle signaling protein, is upregulated and cells show a displacement toward G2/M phase. Moreover, leptin induces not only protein synthesis but also the phosphorylation state of EIF4EBP1 and EIF4E in both JEG-3 and trophoblastic cells in a dose-dependent manner (Perez-Perez *et al.* 2009). The translation initiation factor EIF4E binds to the cap structure at the 5′-end of the mRNA and favors the circularization of mRNA via the binding with EIF4G. The EIF4E-binding proteins (EIF4EBPs) such as EIF4EBP1 (Kauma 2000) inhibit the assembly of EIF4E complex but this inhibition is released after EIF4EBPs phosphorylation (Castellucci *et al.* 2000). Leptin also increases protein synthesis by activating the translation machinery via both PI3K and MAPK pathways (Perez-Perez *et al.* 2009, 2010).

The balance between trophoblast proliferation and apoptosis is a mechanism to control normal trophoblast invasion (Heazell & Crocker 2008). We have also demonstrated that leptin increases survival as well (Magarinos *et al.* 2007, Perez-Perez *et al.* 2008). Apoptosis in placental villi changes along normal pregnancy, in the first trimester is low, during the second third increases and beyond 40 weeks of gestation, it is markedly accelerated (Sharp *et al.* 2010).

Placental apoptosis is initiated by various stimuli, including hypoxia and oxidative stress. In trophoblast cells, apoptosis continues through the extrinsic or

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intrinsic pathways concluding in the activation of caspases. We have observed that leptin treatment in Swan-71 cells or human-term placental explants prevents early and late events of the apoptotic pathway, diminishing caspase-3 and PARP-1 cleavage. Moreover, leptin diminished p53 both at mRNA and protein levels (Toro *et al.* 2014).

The p53 protein is a master cell cycle regulator that increases in response to several stress stimuli as heat shock, hypoxia, osmotic shock and DNA damage, resulting in growth arrest, apoptosis promotion and DNA repair (Prives & Hall 1999). In injured cells, p53 is activated by phosphorylation and acetylation at multiple sites to modulate downstream target genes (Meek & Anderson 2009). It was shown that phosphorylation of p53 at serine 46 promotes the expression of proteins like the apoptosis-inducing protein 1 (p53AIP1) which in turn trigger the apoptosis pathway (Oda *et al.* 2000). We have previously reported that leptin treatment in placental cells decreases serine 46 phosphorylation on p53, suggesting a pro-survival effect of this cytokine (Toro *et al.* 2014).

Protein levels of p53 are diminished by the activation of MDM2, an E3 ubiquitin ligase that via a negative feedback loop leads p53 to proteasome degradation (Wade *et al.* 2013). In addition, leptin treatment increased the MDM2 expression both in Swan-71 cells and in human-term placental explants, and concomitantly diminished p53 half-life determined by cycloheximide experiments. It was also demonstrated that leptin diminished apoptosis via MAPK pathway (Toro *et al.* 2015, Perez-Perez *et al.* 2016). Results obtained with the use of pharmacological inhibitors suggest that PI3K activation may mediate other functions of leptin in placenta, and that MAPK pathway is the principal signaling pathway that mediates the anti-apoptotic effect of leptin in placenta (Perez-Perez *et al.* 2008).

During a normal pregnancy, trophoblast differentiation must not only be fairly regulated in a spatial and temporal manner but also requires potent immunomodulatory strategies to prevent trophoblast rejection by maternal alloreactive lymphocytes and natural killer cells present in the decidua (PrabhuDas *et al.* 2015). One of these mechanisms appears to be the particular pattern of histocompatibility antigens expressed by extravillous cytotrophoblasts. They lack the polymorphic human leukocyte antigen (HLA) class Ia surface molecules and express instead the non-classical MHC class IB antigens (Hutter *et al.* 1998). Among these molecules, HLA-G generates great interest due to its immunosuppressive effects increasing apoptosis of activated $CD8^+$T lymphocytes, promoting the generation of tolerogenic antigen presenting cells and preventing NK cell-mediated cytotoxicity (Persson *et al.* 2017). Leptin, as a pro-inflammatory cytokine, was linked to the promotion of immune escape mechanisms. Our data demonstrated a central role of leptin as a paracrine/autocrine signal enhancing trophoblast HLA-G expression (Barrientos *et al.* 2015). Our results place placental leptin as an endogenous regulator of the differentiation process of trophoblast cells, promoting a tolerogenic phenotype to prevent immunological damage; meanwhile, they invade maternal tissues during early stages of pregnancy.

Leptin is an integrative target of different placental stimuli

Leptin acts as an integrative cytokine between different placenta stimuli, such us growth hormones, steroid hormones, hypoxia, pro-inflammatory signals and so on.

During pregnancy, leptin levels in mother circulation are elevated, compared with those in age- and body-mass-index-matched non-pregnant women, reaching a peak in the second and third trimesters. At the end of pregnancy, circulating leptin levels decline to normal values 24 h after delivery (Hardie *et al.* 1997). The placenta is the principal source of maternal-circulating leptin. Although increased mother adiposity during pregnancy might be responsible for the hyperleptinaemia, leptin levels are elevated to an extent that cannot be explained by the increased body mass index (Hardie *et al.* 1997).

Deregulation of leptin function in the placenta may be implicated in the pathogenesis of various disorders during pregnancy, such as gestational diabetes, recurrent miscarriage, preeclampsia and intrauterine growth retardation (Bajoria *et al.* 2002, Sagawa *et al.* 2002). Placental leptin secretion is potentiated by IL1A and IL6 (Hardie *et al.* 1997, Chardonnens *et al.* 1999, Meissner *et al.* 2003, Maymo *et al.* 2011). It was also demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes (Ambrosini *et al.* 2002).

Different placental hormones enhance the leptin expression. We have demonstrated that hCG increased the leptin expression at the transcriptional level and that a minimal promoter region spanning up to −218 bp was enough to evidence this induction (Maymo *et al.* 2009).

On the other hand in cytotrophoblastic cells, leptin secretion is significantly stimulated by GnRH-II (Islami *et al.* 2003). It is probably that among the diverse hormones secreted by the placenta, principally during the first trimester of pregnancy, GnRH, hCG and leptin are involved in an autocrine/paracrine loop-regulating placental function.

The principal hCG function is to maintain progesterone production by corpus luteum during early pregnancy, through the stimulation of LH/HCG receptor. Binding of hCG to its receptor activates signal transduction through the associated heterotrimeric G-proteins. Besides, hCG has many other functions, being one of the earliest embryonic signals, and is probably involved in the embryo-maternal dialog-regulating implantation (Rao 2001).

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The leptin expression is also upregulated in placental cells by estradiol (Chardonnens *et al.* 1999). Steroids play a fundamental role in growth and differentiation of human cells and are required for the development and maintenance of reproductive tissues (Chen *et al.* 2004). Human placenta synthesizes estrogens during pregnancy, in concordance with cytotrophoblast invasion.

We observed a significant upregulation of leptin expression in BeWo cells at the transcriptional level. Moreover, treatment with the antiestrogen ICI 182,780 completely blocked E_2 induction of leptin expression in human placental explants, and also repressed the basal leptin expression, suggesting a role of endogenous estradiol in the autocrine control of leptin synthesis (Gambino *et al.* 2010).

We have also shown that the placental leptin expression is upregulated by insulin (Perez-Perez *et al.* 2013). In this regard, it has been reported that insulin regulates the leptin expression both in adipose tissue and placenta. Insulin administration enhances the synthesis of leptin mRNA transcripts and protein secretion by adipocytes, *in vitro* (Cammisotto *et al.* 2006). We demonstrated that 10 nM insulin enhances the leptin expression and is in agreement with some studies where a positive correlation between insulin and serological levels of leptin in pregnant women was found (Hardie *et al.* 1997, Saltiel & Pessin 2002).

Signal transduction pathways mediating leptin induction

Increments in intracellular cAMP usually lead to the activation of cAMP-dependent protein kinase (PKA), which in turn phosphorylates intracellular substrates, including the cAMP response element-binding protein (CREB). However, several experiments have evidenced that cAMP affects some cellular processes independently on PKA (Renstrom *et al.* 1997, Cass *et al.* 1999, Staples *et al.* 2001). It was demonstrated that in BeWo cells and human-term placental tissue, leptin secretion is augmented by forskolin, an activator of PKA (Yura *et al.* 1998). On the other hand, cAMP is known to alter human trophoblast function as demonstrated in numerous *in vitro* studies of normal and transformed trophoblast cells (Strauss *et al.* 1992).

We demonstrated a significant leptin upregulation by cAMP in BeWo, JEG-3 cells and placental explants. Moreover, cAMP increased leptin promoter activity and leptin mRNA transcription (Maymo *et al.* 2010). The stimulatory cAMP effect on the leptin expression was also reported in several cells such as glioma cells, pituitary tumor cells (Morash *et al.* 2000, Li *et al.* 2001), breast cancer cells (O'Neil *et al.* 2001), gastric cells (Goiot *et al.* 2005) and placental chorionic tissue (Yura *et al.* 1998).

It is known that PKA pathway activation plays an important role in biological signaling of different hormones in the placenta, such as hCG (Strauss *et al.* 1992). The inductive effect of cAMP on the leptin expression might result from the activation of PKA-dependent or PKA-independent events. The downregulation of leptin expression by the transfection of a dominant negative regulatory subunit of PKA (PKI) demonstrated the involvement of PKA pathway in cAMP leptin induction. Besides, specific inhibition of PKA with H89 suppressed cAMP leptin induction both in BeWo cells and in placental explants (Maymo *et al.* 2010). All these data suggest that cAMP stimulates the leptin expression through a PKA-dependent signaling pathway.

On the other hand, it was reported that MAPK pathway is essential for reproduction. It has been involved in oocyte maturation and in the regulation of trophoblast invasion and placental development (Hatano *et al.* 2003, Fitzgerald *et al.* 2005). It has been reported that MAPK cascade can modulate PKA activation by different mechanisms (Gerits *et al.* 2008). Possible crosstalk between these pathways was determined to investigate the molecular mechanisms underlying the cAMP effect on leptin upregulation in trophoblastic cells. We demonstrated that cAMP treatment specifically activated ERK 1/2 phosphorylation in placental cells (Maymo *et al.*2010). Besides, the inhibition of PKA increases ERK phosphorylation in placenta, suggesting that cAMP might activate ERK pathway in a PKA-independent manner. Moreover, using an MEK inhibitor, PD98059 partially blocked leptin induction caused by the overexpression of the catalytic subunit of the PKA.

On the other hand, it is reported that cAMP activates MAPK through members of the Ras superfamily activating Rap1A, which then increases ERK phosphorylation (Lange-Carter *et al.* 1993, Kawasaki *et al.* 1998). Our data demonstrated that the leptin expression is induced in placenta through the cAMP/Epac alternative signaling pathway. It was seen that the overexpression of Epac and Rap1 proteins resulted in a significant increase in leptin promoter activity. Moreover, when trophoblastic cells were incubated with CPT-OMe, a cAMP analog that specifically activates Epac, the leptin expression was increased (Maymo *et al.* 2012). These results demonstrate that the role of cAMP as a second messenger of hCG would be PKA-independent and involves the activation of the alternative cAMP/Epac pathway.

We have also demonstrated the involvement of MAPK signaling pathway in hCG leptin upregulation both by using PD98059 and by the overexpression of a dominant negative mutant of ERK2. Moreover, hCG treatment

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specifically activates MEK and ERK1/2 phosphorylation in placental cells (Maymo *et al.* 2009).

Besides with gene regulation, estradiol also mediates rapid non-genomic effects (Wade *et al.* 2001). These actions are initiated in the cellular membrane and indirectly regulate the gene expression, through the activation of signal transduction pathways. The activation of the MAPK and PI3K signaling pathways by estradiol in different cell types has been reported (Bjornstrom & Sjoberg 2005). Both pathways can also be activated in placental cell explants following estradiol treatment. We have previously demonstrated that the stimulatory effect of E_2 on the leptin expression was blocked by PD98059, an MAPK pharmacological inhibitor or by the overexpression of dominant negative mutant forms of MAPK, suggesting that this pathway is involved in the estradiol-induced leptin expression (Gambino *et al.* 2010).

The nature of the membrane receptors for estrogens is under investigation. However, ERα, ERβ and non-classical ER (e.g. ER-X and GPR30) have been identified at different target cell membranes (Acconcia & Kumar 2006). Particularly, using the conjugated molecule estradiol-BSA, that is unable to enter the cell due to its large size, we demonstrated that estradiol regulation of leptin expression in placental cells was partially mediated by estrogen membrane receptors. The presence of ERα in BeWo plasmatic membranes was also demonstrated (Gambino *et al.* 2012*b*).

Leptin promoter organization and transcription factors involved in its expression

In 1995, the group of J Friedman mapped the human *LEP* gene in a portion of human chromosome 7q (Green *et al.* 1995).

Later on, different regulatory elements were identified along the leptin promoter, e.g., cAMP, estrogen receptors and glucocorticoid response elements, CCATT/enhancer, Sp-1, activator protein 1 (AP1) and nuclear factor kappaB (NFκB) binding sites, among others, suggesting a complex regulation of leptin expression through different transcriptional crosstalks (Zhang *et al.* 1994, He *et al.* 1995, Isse *et al.* 1995, Gong *et al.* 1996). A specific placental enhancer located 1.9-kb upstream the human leptin gene was identified. It is active in choriocarcinoma lines but not in adipose cells (Bi *et al.* 1997). Given the importance of leptin regulation during pregnancy, it is expected that different transcription factors participate in concert to modulate the leptin expression in placenta. In Fig. 1, a schematic representation of *in silico* analysis of leptin promoter can be observed. The cartoon shows both confirmed and putative binding sites to the main transcription factors regulating the leptin expression in the placenta.

In silico analysis of leptin promoter fragment showed consensus elements for different potential transcription factors such as C/EBP and Sp1 with a core similarity of 1, cAMP response elements (CRE) and estrogen

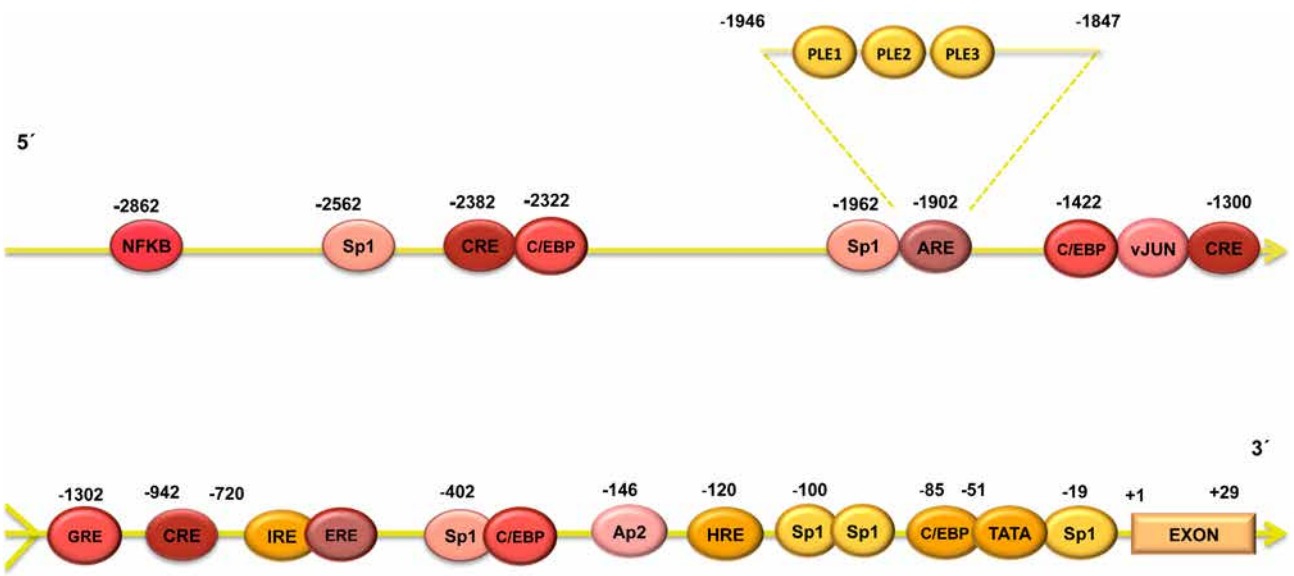

Figure 1 Leptin promoter element analysis. The schematic cartoon shows reported and putative regulatory elements for different transcription factors. The enlargement of the promoter region shows the positions of the placental leptin enhancer (PLE 1, 2 and 3). *In silico* analysis to identify putative elements was performed with the MatInspector Release Professional 8.0.3 program. Ap2, activating protein 2; ARE, androgens response elements; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element-binding protein; ERE, estrogens response element; HRE, hypoxia response element; GRE, glucocorticoid response element; IRE, insulin response element; NFκB, nuclear factor kappaB; Sp1, stimulant protein 1.

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receptor binding sites (ERE), among others. Previous data have demonstrated the involvement of both C/EBP and Sp1 transcription factors in the regulation of leptin gene in adipocytes (Mason *et al.* 1998). Moreover, the C/EBP transcription factor expression was reported to be regulated by hCG in the follicular development (Sirois & Richards 1993), in rat primary cultures of Leydig cells and in human adipose cells (Nalbant *et al.* 1998, Dos Santos *et al.* 2007).

Estrogen, acting through the activation of ERα or ERβ, regulates gene expression through different mechanisms. The classical pathway involves ligand-bound receptor binding at a specific palindromic sequence of DNA (ERE) within the promoters of estrogen-responsive genes (Beato *et al.* 1995). Nevertheless, genomic effects of estrogen can also proceed through ERE-independent mechanism, involving protein–protein interactions with other transcription factors.

Ligand-bound ER mediates gene transcription from AP1 enhancer elements, when binds with AP1 transcription factors FOS and JUN (Gaub *et al.* 1990, Weisz & Rosales 1990, Umayahara *et al.* 1994). It was also reported that genes containing GC-rich sequences in their promoters are regulated through the interaction of ERs with Sp1 transcription factor (Porter *et al.* 1997).

Our results support the participation of ERα in E_2-induced placental leptin expression and demonstrated that a minimal region encompassed between −1951 bp and −1847 bp is sufficient to evidence such induction. Moreover, using *in silico* analysis, we have found that this promoter fragment contains potential consensus half-sites for ERE, a putative binding site for Sp1 transcription factor and a placental enhancer region, PLE, previously described by Gavrilova and collaborators (Bi *et al.* 1997, Gambino *et al.* 2012*a*).

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 (Safe & Kim 2008). Sp1 transcription factor binds the consensus binding site with variable affinities. Previous reports demonstrated the involvement of Sp1 in the modulation of leptin expression in adipocytes (Mason *et al.* 1998).

The overexpression of Sp1 transcription factor on the leptin expression was analyzed in BeWo cells. A significant upregulation was observed. Moreover, estradiol treatment exerted a synergistic effect on Sp1 induction of leptin expression. This effect was dependent on the integrity of the Sp1 binding site, as the estradiol effect was abolished when testing a construction containing a mutated Sp1 binding site and these results suggest a crosstalk between Sp1 and ERs (Schanton *et al.* 2017). These results are shown in Fig. 2. ER and Sp1 transcription factors interact and form a protein–protein complex in the absence of DNA. It was observed that the DNA-binding domain deletion of ER does not prevent

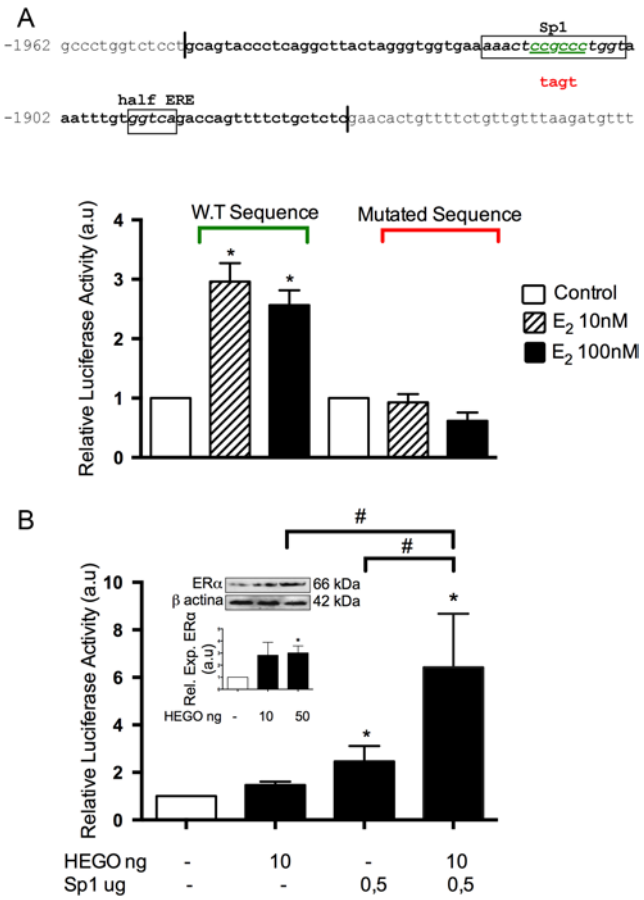

Figure 2 Sp1 is a modulator of estradiol leptin induction in placental cells. (A) Leptin promoter sequence (−2922 to +29) was analyzed by the MatInspector 7.7.3. The enhancer element of placental leptin (PLE) is indicated in bold text and contains the elements related to the action of E_2: Sp1 binding site and half ERE are indicated in black boxes. The mutated Sp1 binding site is indicated below the Sp1 box in red. BeWo cells were transiently transfected with the plasmid wild type, and with the mutated plasmid (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. (B) BeWo cells were transiently transfected with 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. Luc activity was measured in cellular extracts and normalized to β-galactosidase activity ($n = 3$). Statistical analyses were performed by ANOVA followed by the 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 concentrations of the plasmid HEGO. 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 the lower panel ($n = 3$). Statistical analyses were performed by ANOVA follow by the Dunnett *post hoc* test. $^{*}P < 0.05$.

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the synergy of the ER with Sp1, suggesting that ERs can regulate transcription in the absence of a functional ERE (Porter *et al.* 1997). It was reported that ERα preferentially binds to the C-terminal DNA-binding domain of Sp1 protein (Safe 2001). Moreover, it was demonstrated that the binding of ER to Sp1 protein increases Sp1-DNA binding to EREs independently of estrogen, although the transactivation of the gene is enhanced in the presence of the ligand (Porter *et al.* 1997).

We showed data which demonstrate that the overexpression of ERα in BeWo cells synergistically increased Sp1-induced leptin expression. Moreover, this enhancement was not further augmented by estradiol treatment, suggesting a ligand independent effect. These results are in concordance with reported data which showed that the interaction of ER and Sp1 proteins was observed in the presence or absence of E2 and therefore was hormone-independent (Porter *et al.* 1997).

In previous reports, we have determined that cells with decreased expression of ERα through a specific siRNA completely prevented the effect of Sp1 on the leptin expression. On the other hand when using a construct with a mutated Sp1 binding site and intact ERE, the ERα-enhanced leptin transcriptional activity was abolished, demonstrating that the Sp1 binding site contributes to ER signaling.

According to the 'classical genomic model', ligand-activated ERs bind to specific ERE to modulate transcriptional activity of estrogen target genes. Nevertheless, 35% of the categorized human estrogen-responsive genes are expressed via indirect ER-DNA association and require protein–protein interactions with several transcription factors such as Sp1, NF-κB orAP1 (O'Connor *et al.* 2016). Regarding the involvement of NF-κB transcription factor on the leptin expression, unpublished results from our group showed that p65 subunit is entering the nucleus after estradiol treatment and enhances leptin transcription.

Stress is normal during early embryogenesis and during the peri-implantation, embryo is the result of maternal hormones, like leptin, that signal and prepare the new organism for development and implantation. Stress response mechanisms ensure that stem cells in the early embryo and placenta survive and include a small set of stress enzymes like protein kinases that hierarchically regulate nuclear function by modifying transcription factor activity. The transcription factors that are important in the stress response are JunC, JunB, HIFs and NFκB (Puscheck *et al.* 2015). The interaction of leptin, stress enzymes and transcription factors as NFκB in the early embryo and placenta are a continuing central focus of research.

Among PKA intracellular substrates, the CREB transcription factor is one of the most important in regulating the expression of many cAMP-responsive genes in different cell types and in response to several signals (Mukherjee *et al.* 1996). When cAMP concentration rises, it is able to induce CREB phosphorylation not only in choriocarcinoma cells but also in placental explants. Moreover, the overexpression of the catalytic subunit of PKA or the transcription factor CREB exerted an upregulation of leptin promoter activity in a dose-dependent manner (Maymo *et al.* 2010).

On the other hand, the inhibition of MAPK pathway partially blocks cAMP-induced CREB phosphorylation. Initially, the phosphorylation of CREB at serine 133 was attributed exclusively to PKA. However, several studies have demonstrated that other kinases may phosphorylate CREB at the same residue. For example, while ERK 1/2 does not phosphorylate CREB directly, it can activate members of the pp90rsk family of protein kinases (RSK1–3), which, in response, translocate into the nucleus and phosphorylate CREB (Shaywitz & Greenberg 1999).

Estradiol leptin induction in BeWo cells is dependent on the integrity of the PKA signaling. Unpublished results showed that the inhibition of adenylyl cyclase or PKA activities with H89 or SQ22,536, respectively, diminished estradiol effect on the leptin placental expression. On the other hand, the overexpression of the coactivator and acetyltransferase protein CBP enhances estradiol leptin induction, while the overexpression of the histone deacetylase HDAC generates the opposite effect. These results suggest a crosstalk between estradiol and PKA signaling probably involving the acetylation modification of histones present in the leptin promoter regions. In concordance with this hypothesis, it was reported that blocking PKA activation with H89 abrogated the estradiol-induced neuroprogesterone synthesis (Bouskine *et al.* 2008). In Fig. 3, a representative cartoon shows the signal transduction pathways and transcription factors mediating leptin induction in placental cells discussed in previous sections.

Epigenetic regulation of leptin and its consequences on fetal programming

Increasing evidence suggests that the developmental origins of health and disease are dependent on the intrauterine environment and these conditions correlate with the offspring's risk of different metabolic diseases in adult life (Waterland & Michels 2007). Epigenetic marks have been proposed as a mechanism for this developmental programming as they respond to diverse environmental stimuli, and are also stable during the mitosis (Jaenisch & Bird 2003). The placenta, a metabolically active organ that regulates intrauterine environment, is crucial for fetal growth and development (Sferruzzi-Perri & Camm 2016). DNA methylation is an epigenetic modification that involves the addition of methyl group to a cytosine base in the context of a CpG dinucleotide, usually clustered in high density in gene promoters and associated with a decreased expression (Jones 2012). DNA methylation marks are reset during embryonic development, and are then crucial in

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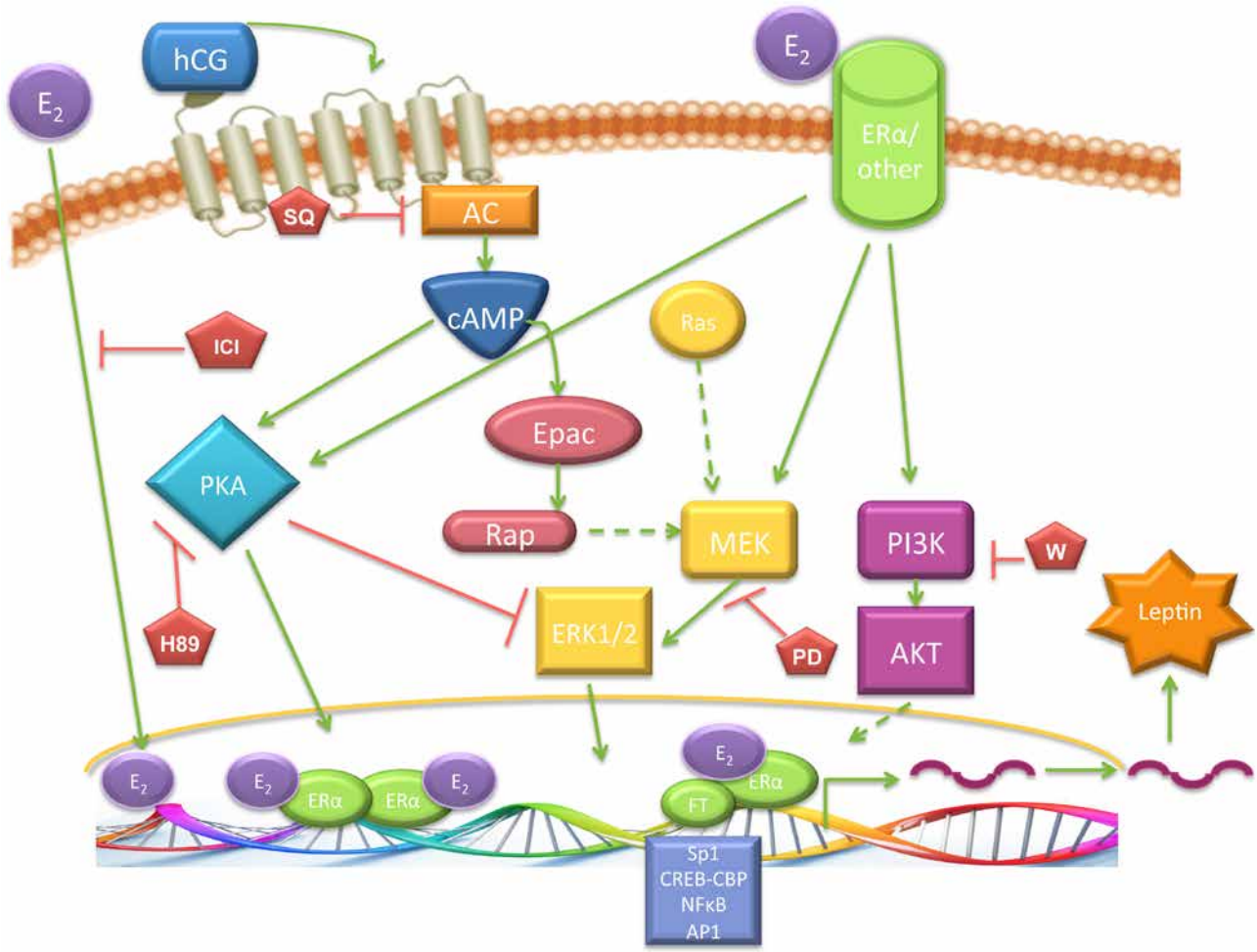

Figure 3 Signal transduction pathways and transcription factors mediating leptin induction. The proposed model shows hCG and ERα mechanisms involved on leptin-induced expression based on current data. hCG stimulates the leptin expression in placenta mainly by the MAPK pathway. ERα directly or through the interaction with different transcription factors may mediate estradiol action on the leptin expression. PKA, protein kinase A; cAMP, cyclic adenosine-3′,5′-monophosphate; MAPK, mitogen-activated protein kinase; MEK, extracellular-signal regulated kinase; ERK1/2, extracellular-signal regulated kinase1/2; PI3K, phosphoinositol-3-kinase; hCG, human chorionic gonadotropin; CREB, element-binding protein; CBP, CREB bond protein; E_2, estradiol; AC, adenylate cyclase; AKT, protein kinase B; NFκB, nuclear factor kappaB; Sp1, stimulant protein 1; ICI, ICI 182,780; PD, PD98059 inhibitor; W, wortmannin H89: PKA inhibitor; SQ, SQ22,536 adenylyl cyclase inhibitor. Open arrows indicate stimulation; flat arrows, inhibition.

establishing cellular differentiation pathways through their restitution in a tissue-specific manner (Novakovic & Saffery 2012).

As discussed earlier during pregnancy, leptin in serum is produced by fetal and maternal adipose tissues and placentas (Tessier *et al.* 2013). It was reported that the leptin gene expression is inversely correlated with promoter DNA methylation (Bouchard *et al.* 2010) and has been proposed as a mediator of metabolic programming (Vickers & Sloboda 2012). Moreover, if the methylation level in fetal or placental leptin promoter is changed during pregnancy, fetal development may be affected. Previous studies have shown that the fetal methylation level of leptin could be affected by the intrauterine environment (Briffa *et al.* 2015). It was reported that gestational high-fat diet decreases promoter methylation of leptin in the offspring (Khalyfa *et al.* 2013). In humans, the concentration of circulating leptin is altered by obesity and the promoter methylation of leptin is also altered in obese individuals (Garcia-Cardona *et al.* 2014). Moreover, previous published results suggest that the maternal metabolic status before and during pregnancy can alter placental *LEP* DNA methylation profile at birth and might contribute to metabolic programming of obesity and related conditions (Lesseur *et al.* 2014*b*).

It was reported that placental *LEP* promoter methylation is accompanied by pregnancy complications such as impaired glucose metabolism (Bouchard *et al.* 2010) and early onset preeclampsia (Hogg *et al.* 2013).

It was also demonstrated that *LEP* DNA methylation is associated with maternal pre-pregnancy obesity and pregnancy smoking and infant factors like small

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for gestational age and genotype in non-pathological pregnancies (Lesseur *et al.* 2013). The same group has found evidence of a placental *LEP* DNA methylation and profiles of male infant neurobehavior with distinct motor disabilities (Lesseur *et al.* 2014*a*).

In conclusion, leptin may be considered a key regulator of placental physiology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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