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Leptin promotes HLA-G expression on placental trophoblasts via the MEK/Erk and PI3K signaling pathways

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ABSTRACT

Introduction: The development of the human haemochorial placenta requires complex regulatory mechanisms to protect invasive trophoblast cells from cytotoxic responses elicited by maternal immune cells. Leptin, the adipocyte derived hormone encoded by the *Lep* gene, is synthesized by placental trophoblasts and exerts pleiotropic effects on the immune system, including the promotion of inflammation and the activation of T cell responses.
Methods: To address its possible involvement in the modulation of maternal immune responses during pregnancy, we investigated the effect of leptin on the expression of the class Ib histocompatibility antigen HLA-G as one of the chief immunosuppressive strategies used by trophoblast cells.
Results: In vitro incubation of the trophoblast derived Swan 71 and JEG-3 cell lines with 25–50 ng/ml recombinant leptin significantly boosted HLA-G mRNA and protein expression, and this effect was abrogated upon pharmacological inhibition of the PI3K-Akt and MEK-Erk signaling pathways. A similar stimulatory effect of leptin was observed in term placental tissue explants, though 10-fold higher doses were required for stimulation. Further, JEG-3 cells treated with a leptin antisense oligodeoxynucleotide displayed decreased HLA-G expression levels, which were partially recovered by addition of stimulating doses of exogenous hormone. Immunofluorescence and qPCR analysis confirmed leptin biosynthesis in placental tissue, further showing that invasive extravillous trophoblast cells were a main source of this hormone during the first trimester of normal pregnancies.
Discussion: Taken together, our results show that leptin acts as an autocrine/paracrine signal promoting HLA-G expression in placental trophoblasts suggesting an important role in the regulation of immune evasion mechanisms at the fetal maternal interface.

1. Introduction

The establishment of a functional maternal-fetal interface is critical to determine the success of human pregnancy. The placenta allows metabolic exchange along this interface and is an important regulator of fetal growth, with many pregnancy complications (e.g., pre-term delivery, preeclampsia) being often linked to defective placental development and function. During the process of placentation, villous cytotrophoblast (CTB) precursors differentiate along two possible pathways: either they fuse giving rise to the syncytiotrophoblast (STB), the outermost layer of the placenta bathed in maternal blood; or they detach and migrate from the anchoring villi as extravillous trophoblast (EVT), which infiltrate the decidua basalis and breach the maternal spiral arteries [1]. To ensure normalcy of pregnancy, trophoblast differentiation must not

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only be precisely regulated in a spatiotemporal manner but also requires potent immunomodulatory mechanisms to prevent rejection of STB and invasive EVTs by alloreactive lymphocytes and natural killer (NK) cells present in maternal blood and the decidua. One of the most important among these strategies appears to be the particular pattern of histocompatibility antigens expressed by EVTs, which in contrast to most somatic cells lack polymorphic human leukocyte antigen (HLA) class Ia surface molecules [2]. They express instead the non-classical MHC class IB antigens, of which HLA-G has raised a great interest due to its potent immunosuppressive effects promoting apoptosis of activated $CD8^+$ T lymphocytes, the generation of tolerogenic antigen presenting cells and the prevention of NK cell-mediated cytotoxicity [3–5]. Of the seven splice variants identified to date, the full length HLA-G1 is the predominant isoform expressed on the surface of trophoblast cells and its expression is considered as a molecular signature of differentiation along the invasive pathway [6]. Indeed, recent reports indicate that aside from its well-described immunomodulatory properties, HLA-G may play a significant role in regulating placental function due to effects on trophoblast invasiveness [7,8] and angiogenic responses [9,10].

Leptin, the non-glycosilated protein encoded by the *Lep* gene, has recently emerged as a placental hormone with important regulatory functions during pregnancy [11]. The original view of this hormone solely as an adipocyte-derived factor acting centrally to control energy expenditure and food intake [12,13] changed after the identification of leptin synthesis at the fetoplacental unit. Placental leptin expression starts at very early stages of gestation (i.e., seven weeks) [14,15] and is delicately modulated by pregnancy-related factors including steroid hormones, human chorionic gonadotropin (hCG), cytokines and also hypoxia [16–19]. The expression pattern of both leptin and its receptors at the maternal fetal interface, localizing mainly to CTB, STB and fetal endothelial cells [14], argues in favor of a role as an autocrine or paracrine pathway controlling placental development and function. Indeed, among other functions leptin has been found to promote the proliferation and survival of trophoblast cells [20,21], to enhance hCG production [22,23], induce vascular-like features in EVT cells [24] and modulate the expression of adhesion molecules and metalloproteinases involved in the invasive process [25,26]. Additionally, leptin is regarded as a cytokine-like hormone with pleiotropic effects on the immune system including the promotion of inflammation and the modulation of innate and adaptive immunity [27–29], a functional aspect that remains poorly investigated in the context of pregnancy. A pioneering study from Laivouri and co-workers reported that placental leptin mRNA and protein were higher in the preterm preeclampsia (PE), whereas maternal leptin is up-regulated in term PE patients [30]. In line with this finding, gene array studies shown that leptin placental expression is upregulated in preeclampsia patients [31], suggesting that expression of leptin at the feto-maternal interface is sensitive to the complications of pregnancy, especially PE.

As a pro-inflammatory cytokine, leptin has been linked to the promotion of immune escape mechanisms in certain types of tumors [32,33], which led us to hypothesize that it could participate in the immune modulation associated with trophoblast differentiation by regulating HLA-G expression. Our *in vitro* data argue for a central role of leptin as a paracrine/autocrine signal enhancing trophoblast HLA-G expression, which appears to involve the MEK-Erk and PI3K signaling cascades. Given the well-known immunosuppressive properties of HLA-G, our results place placental leptin as an endogenous component of the differentiation machinery of trophoblast cells, conferring them a tolerogenic phenotype to prevent immunological damage as they invade maternal tissues during early stages of pregnancy.

2. Materials and methods

2.1. Isolation of placental CTB and EVTs

The analysis of placental leptin expression was performed in choriodecidual samples obtained from patients carrying normal pregnancies after elective termination during the first trimester (8–12 weeks of gestation, n = 10) or at delivery (n = 12). These same RNA samples have previously been analyzed for galectin-1 induced HLA-G expression as described [34]. All patients provided informed written consent before their inclusion in the study, which was approved by the local ethics committee of Geneva University Hospital (Switzerland). For qRT-PCR analysis, trophoblast cells were isolated by immunopurification as described previously [35]. Briefly, isolated fresh placental tissue specimens were digested with an enzyme cocktail (0.25% trypsin, 0.25 mg/ml Dnase I, Roche Diagnostics GmbH, Germany) for 20 min, followed by centrifugation of the resulting suspension on a 70-5% Percoll gradient for 25 min at 1200 g. For the isolation of CTB, cells collected from the 30–45% Percoll layer were incubated with immobilized anti-CD45 antibodies for depletion of immune cells. Approximately 95% of the immunopurified cells were positive for cytokeratin 7 and negative for vimentin and thus identified as villous CTB. The EVTs were obtained by seeding the cells on Petri dishes for 15 min, collecting the supernatants and incubating in 6- or 96 well plates for 24 h in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Switzerland). Cultured cells were identified as > 95% EVT based on the presence of cytokeratin 7 and HLA-G and absence of vimentin expression.

2.2. Collection and processing of placental explants

Placental explants were processed following our standard protocol [18]. In brief, placentas (n = 9) from voluntary donors carrying normal pregnancies were obtained after vaginal delivery at term. Samples were obtained under written informed consent and all procedures were approved by the ethical committee at Hospital Nacional Prof. Alejandro Posadas (Buenos Aires, Argentina). After washing in sterile PBS to remove excess blood, villous tissue from at least five healthy cotyledons (i.e., with no signs of necrosis or calcification) was dissected at a distance midway between the chorionic and basal plates. The dissected pieces were further cut into cubic segments (10–15 mg wet weight), which were rinsed and collected in cold DMEM-F12 medium. Placental explants were pre-incubated in DMEM-F12 for 30 min at 37 °C, followed by overnight stimulation with human recombinant leptin (Cat. L4146, Sigma Aldrich, Argentina; 50–250 ng/ml).

2.3. Cell culture and treatments

The human trophoblast cell lines JEG-3 (ATCC No. HTB-36, USA) and Swan 71 [kindly provided by Dr. R Ramhorst, [36,37]] were cultured in DMEM-F12 medium (Invitrogen/Life Technologies, Argentina) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen/Life Technologies, Argentina), and 1 mM sodium pyruvate (Sigma Aldrich, Argentina) at 37 °C in 5% CO_2. These cell lines reproduce many features of human primary cytotrophoblast (particularly in terms of HLA-G expression), and have been extensively used as model systems in cases of limited availability of first trimester placental specimens. To test the effects on HLA-G expression, cells were serum starved for 24 h and incubated with human recombinant leptin (Cat. L4146, Sigma, Argentina) at doses of 10–250 ng/ml during 48 h. Leptin doses producing the maximal effect were chosen for subsequent experiments designed to characterize the mechanisms involved in the modulation of HLA-G, which were performed by co-incubating the cells with the hormone and pharmacological inhibitors of the PI3K/Akt (LY29002, 10 μM) and MEK/Erk (PD98059, 100 μM; all purchased from Sigma) pathways for 48 h.

2.4. Inhibition of leptin expression with an oligodeoxynucleotide

Endogenous leptin expression in JEG-3 cells was inhibited by incubating with an antisense oligodeoxynucleotide (Lep AS) as previously described [21]. The Lep AS sequence (5′GCACAGGGTTCCCCAATGCAT3′) was chosen to span the first five codons of *Lep* mRNA. Twenty-four hours prior to the experiments, the FCS concentration in the cultures was reduced to 2%. Cells were then treated with 1 μM Lep AS or a random oligodeoxynucleotide (control) for 72 h. To investigate the substrate specificity of the treatment, some experiments were performed co-incubating the oligodeoxynucleotides in the presence of 50 ng/ml human recombinant leptin. After incubation, cells were processed for protein isolation and cytospin preparation following standard procedures.

2.5. Quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol (15596-026, Invitrogen/Life Technologies, Argentina) according to the manufacture instructions. After DNase I digestion (Invitrogen, Germany), RNA samples (2 μg) were reverse transcribed using random primers and the Superscript II RT System. Quantitative real time PCR was performed using the TaqMan 7500 System (Applied Biosystems/Life Technologies, Germany). Each reaction had a total volume of 11 μl containing 1 μL cDNA, 6.25 μl *Power* SYBR® Green PCR master mix (4367659, Applied Biosystems, Germany), 3.75 μl DEPC water and 900 nM of the appropriate forward and reverse primers (HLA-G forward: 5′-TTG

CTG GCC TGG TTG TCC TT-3′, reverse: 5′-TTG CCA CTC AGT CCC ACA CAG-3′; Leptin forward: 5′-GAACCCTGTGATTCTT-3′, reverse: 5-′CCAGGTCGTTATTTGG-3′; Cyclophilin forward: 5′-CTTCCCCGATACTTCA-3′, reverse, 5′-TCTTGGTGCTACCTC-3′). All primer pairs were purchased from Tib Mol Biol GmBH (Germany). The following PCR program was used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A melting curve analysis was performed which consisted of 70 cycles of 10 s with a temperature increment of 0.5 °C/cycle starting at 60 °C. PCR efficiencies were 98.59% (leptin), 98.64% (HLA-G) and 99.78% (cyclophilin A) as calculated from standard curves according to the equation $E = -1 + 10^{(-1/\text{slope})}$. Relative expression (RE) for each gene was calculated as follows: $RE = 2^{-dCt}$, where $Ct = Ct_{\text{gene of interest}} - Ct_{\text{reference gene}}$.

2.6. Western blot

Total cell lysates were prepared using the Total Protein Isolation kit (Fisher Scientific GmbH, Germany). Protein concentrations were assessed by Bradford. For the analysis of HLA-G expression, 40 μg total proteins were loaded onto 12% polyacrylamide gels and resolved by SDS-PAGE under reducing conditions (5% β-mercaptoethanol). Separated proteins were then electro-transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia, Buckinghamshire, UK), followed by blocking of nonspecific binding sites using 5% nonfat milk in PBS for 1 h at RT. Blots were incubated with biotinylated anti-HLA-G antibody (Ab) (MEM/G1 Exbio, Germany; 1:1500) o.n. at 4 °C, which was then detected with an HRP-streptavidin conjugate (E2886, Sigma Aldrich, Germany; 1:3000) for 1 h at RT. Reactions were developed using the enhanced chemiluminescence (ECL) detection reagent on a Bioimaging analyzer (LAS-1000; Fujifilm, Tokyo, Japan). Equal loading was confirmed by blotting the same membranes with an HRP-conjugated anti-β actin Ab (Sigma Aldrich, Germany; 1:50000). Densitometric analysis of the protein bands was performed using the Gel Analyzer Tool of the Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/), and relative HLA-G expression was calculated by normalizing to β actin expression as loading control.

2.7. Immunofluorescence

Leptin expression in first trimester placental tissue was analyzed following a standard protocol. Briefly, 8 μm cryosections were washed in Tris-buffered saline (TBS) and blocked with 2% normal serum for 20 min. Slides were then incubated with rabbit anti-leptin Ab (Y-20 sc-843, Santa Cruz Biotechnology; 1:200) overnight at 4 °C. For negative controls, the primary Ab was replaced by irrelevant IgG. After washing, stained sections were incubated 1 h at RT with TRITC-conjugated secondary antibodies (Code 111-026-003, Jackson ImmunoResearch, Germany; 1:200). Cell nuclei were stained by incubating 5 min in a 0.1% DAPI solution. Samples were then washed, mounted on Shandon Immu-Mount™ (99-904-02, Thermo Scientific, Germany) and stored at −20 °C until analysis.

For the analysis of HLA-G expression, cytospins were prepared by loading equal amounts of JEG-3 or Swan 71 cells on a Cellspin I cytocentrifuge (Tharmac, Germany). Slides were fixed in ice-cold acetone, and then incubated with avidin- and biotin blocking solution (Vector, USA) for 15 min at RT. After 20 min blocking with a protein solution (Immunotech, Germany), slides were incubated with biotin-conjugated mouse IgG1 anti-HLA-G mAb (MEM-G/1, Exbio, AXXORA GmbH, Germany; 1:500) o.n. at 4 °C. Control reactions were established by replacing the HLA-G Ab with irrelevant mouse IgG1 (BD Biosciences, Germany) at the same concentration. Slides were then washed and incubated with TRITC-conjugated steptavidin (Code 016-020-084, Jackson ImmunoResearch, Germany; 1:100) for 1 h at RT. Nuclei were stained by incubating for 5 min in DAPI solution, followed by washing and mounting on Shandon Immu-Mount™. Photodocumentation and analysis were performed on a Keyence BZ-9000 microscope, using the Hybrid Cell Count protocol of the BZ-II Analyzer software (Keyence, Japan).

2.8. Statistical analysis

Results correspond to at least three independent experiments and are expressed as mean ± standard error (SEM). Comparisons for leptin dosing experiments or qRT-PCR data (i.e., for leptin mRNA) were performed using one-way ANOVA followed by appropriate post-hoc tests as stated in each figure. Data for the antisense and pharmacological inhibition of MEK/Erk and PI3K signaling experiments were analyzed by two-way ANOVA, followed by Bonferroni post-hoc test comparing between individual means for one factor within each level of the second factor for graphical representation. All calculations were performed using the Prism V 5.01 software for Windows (Graph Pad Software Inc., 2007). A p value lower than 0.05 was considered statistically significant.

3. Results

3.1. Leptin is highly expressed by EVT cells during the first trimester of normal pregnancy

As a first step to elucidate the role played by leptin in the modulation of HLA-G expression, we sought to confirm placental leptin expression with focus on the identification of leptin-producing trophoblast lineages. For this aim, we analyzed the distribution of leptin protein in choriodecidual samples obtained from normal pregnant patients during the first trimester by immunofluorescence. As shown in Fig. 1A (left panel), immunoreactive leptin localized to villous syncytiotrophoblast, while no expression was found on the villous stroma or the decidual compartment. Interestingly, a high fluorescence signal was found on EVT cells (Fig. 1A, right panel). To further identify leptin-producing cells during pregnancy, we assessed *Lep* mRNA levels on CTB and EVT cells purified from normal 1st trimester and term placental tissue. Fig 1B displays qRT-PCR results showing that during the first trimester, *Lep* expression was significantly higher in EVT cells when compared to placental CTB ($p < 0.05$, Bonferroni post-hoc test). At term, *Lep* mRNA levels on CTB cells were significantly up-regulated respect to CTB isolated during the first trimester.

3.2. Exogenous leptin treatment up-regulates HLA-G expression on trophoblast cells

As leptin is synthesized and secreted by the placenta and we found high levels of expression on invasive EVT cells, our next aim was to investigate its effect as a paracrine signal modulating the expression of HLA-G as a chief immune evasion strategy induced on trophoblast cells. For these experiments we employed the well

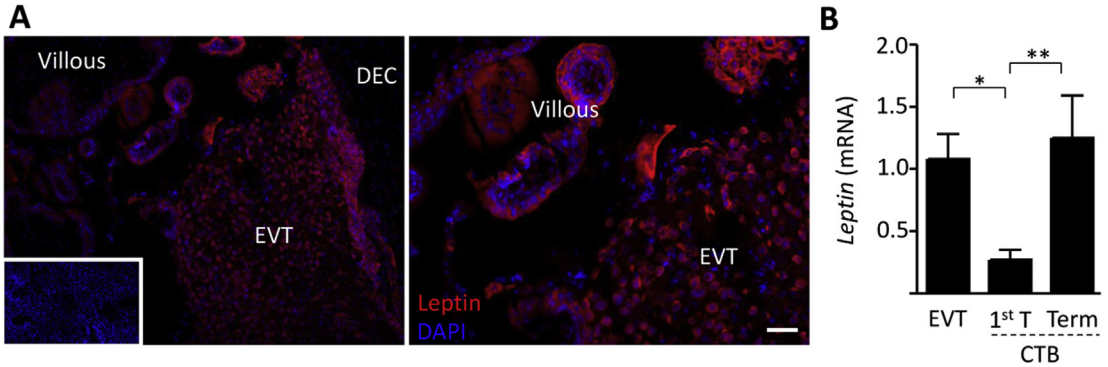

Fig. 1. Normal first trimester extravillous trophoblasts express high levels of leptin. (A) Representative examples of immunofluorescence staining for leptin on first trimester normal choriodecidual tissue. Leptin expression (red staining) localized mainly to syncytiotrophoblast and EVT cells, but was absent from decidual (DEC) cells. Left inset: Negative control using irrelevant IgG. Scale bar: 50 μm. (B) Leptin expression on trophoblast cells isolated from normal pregnancies, as analyzed by qRT-PCR. Leptin mRNA levels during the first trimester were significantly higher in EVT compared to CTB cells. Results are expressed as mean ± SEM of three replicate reactions. *$p < 0.05$, **$p < 0.01$; as assessed by ANOVA, Bonferroni multiple comparisons test.

described choriocarcinoma cell line JEG-3 as well as Swan 71 cells, which derive from telomerase-transduced normal first trimester trophoblasts [37]. After 48 h treatment with recombinant leptin, both cell lines displayed up-regulated protein and mRNA levels of HLA-G expression (Fig. 2A–B, Fig. S1A–B). Western blot analysis showed that HLA-G protein levels in JEG-3 cells were ~2.5 fold increased after stimulation with 50 ng/ml leptin ($p < 0.05$, Fig. 2A) whereas Swan 71 cells displayed a 3 fold increase expression when stimulated with 25 ng/ml leptin ($p < 0.005$, Fig. 2B). These results were confirmed by immunofluorescence, which showed that in both trophoblast-derived cell lines stimulation with leptin resulted in significantly increased cytoplasmic HLA-G levels (expressed as mean fluorescence intensity, Fig. 2C–D) and up-regulated percentage of HLA-G$^+$ cells (Fig. S1C–D). Dose effects of exogenous leptin on HLA-G expression were also analyzed in villous explants obtained from normal placentas at term. In these experiments, concentrations of leptin within the physiological range during pregnancy (i.e. 50 ng/ml) did not alter HLA-G expression whereas a significant up-regulation of HLA-G mRNA ($p < 0.005$, Fig. S1E) and protein ($p < 0.005$, Fig. S1F) was observed after 24 h treatment with 250 ng/ml leptin.

3.3. Blockade of endogenous leptin expression abolishes HLA-G induction on trophoblast cells

Having found that EVT cells synthesize leptin during the invasion period, the next series of experiments dealt with its possible role as an autocrine factor modulating HLA-G expression. To test this hypothesis, we aimed at blocking endogenous leptin expression on JEG-3 cells by means of a specific antisense oligodeoxynucleotide (Lep AS) [21]. As shown in Fig. 3A, 72 h treatment with Lep AS led to an almost complete inhibition of leptin expression in JEG-3 cells. Such Lep AS treated cells displayed significantly decreased levels of HLA-G protein respect to control cells, as evidenced in western blot experiments ($p < 0.05$ Two way ANOVA + Bonferroni post-hoc test; Fig. 3A) and by a decreased HLA-G fluorescence signal intensity in cytospin preparations stained with the MEM-G1 antibody ($p < 0.001$, Fig. 3B). Next, we tested whether administration of recombinant leptin could compensate for the defective induction of HLA-G observed when blocking endogenous leptin in Lep AS treated cells. Western blot analysis revealed that stimulation with 50 ng/ml leptin partially restored HLA-G expression in Lep AS treated cells, as these cells showed significantly increased protein levels compared to cells treated with the AS alone but lower than those observed upon leptin stimulation of control cells (two-way ANOVA AS effect: $p = 0.0001$, Leptin effect: $p < 0.0001$, interaction $p = 0.1443$; Fig. 3A). Similar results were obtained in immunofluorescence experiments which confirmed a partial recovery of HLA-G expression in Lep AS treated cells upon addition of recombinant leptin (two-way ANOVA AS effect: $p < 0.0001$, Leptin effect: $p = 0.0006$ interaction $p = 0.8632$; Fig. 3B), though in this case the fluorescence signal observed upon leptin treatment did not differ significantly from basal levels observed in control cells.

3.4. Signaling through the MEK/Erk and PI3K pathways are required for leptin induction of HLA-G on trophoblast cells

To gain insight into the mechanisms involved in the modulation of HLA-G by leptin, we next performed stimulation experiments in the Swan 71 and JEG-3 cell lines treated with pharmacological

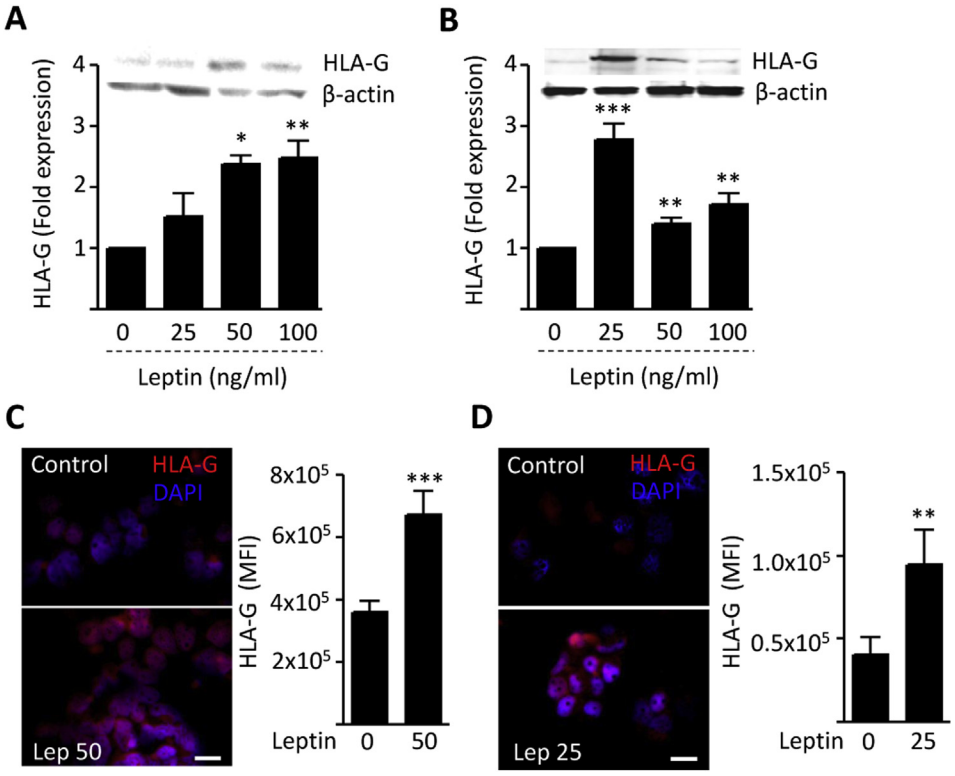

Fig. 2. Exogenous leptin stimulates HLA-G expression on trophoblast cell lines. (A) Analysis of HLA-G expression on JEG-3 cells upon treatment with hrLeptin for 48 h. Leptin caused a significant, dose-dependent increase on HLA-G levels, as assessed by western blot. (B) Western blot analysis of HLA-G expression in Swan 71 cells treated with hrLeptin for 48 h. Leptin treatment (25 ng/ml) significantly up-regulated HLA-G protein levels. (C) Representative examples of JEG-3 cytospin preparations confirming leptin-induced expression of HLA-G (red), as analyzed by IF. Scale bars: 25 μm. Right panel represents mean fluorescence intensity (MFI) for HLA-G, which was significantly increased by treatment with 50 ng/ml Leptin (D) IF analysis of Swan 71 cytospin preparations, showing up-regulated HLA-G signal in response to stimulation with 25 ng/ml hrLeptin. Scale: 25 μm. Results are expressed as mean ± SEM for 3 independent experiments. In all panels *, **, *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$ compared to untreated control respectively, as determined by ANOVA followed by Newman–Keuls post-hoc test.

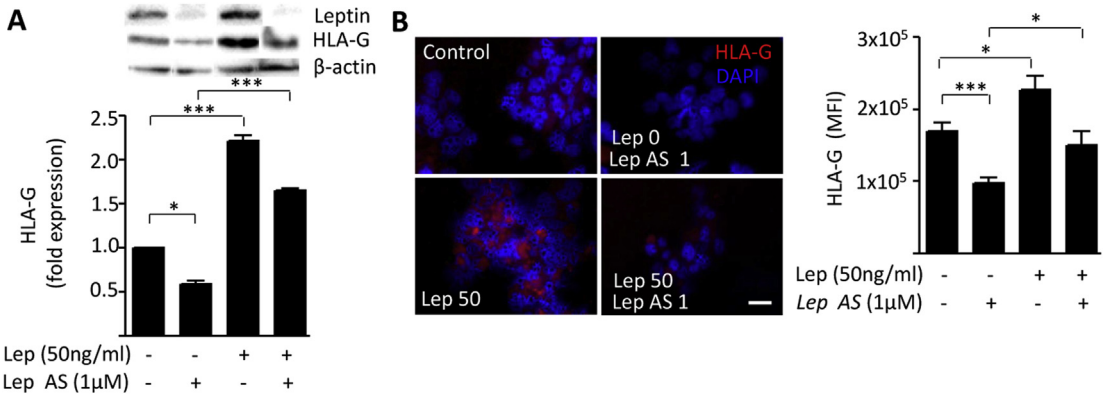

Fig. 3. Blockade of endogenous leptin impairs HLA-G induction in JEG-3 cells. (A) Western blot analysis of HLA-G expression in leptin stimulated JEG-3 cells after treatment with (+) or without (−) Lep AS oligonucleotides for 72 h. Upper panel shows representative blots confirming reduced leptin expression in AS treated cells. Basal levels of HLA-G expression were significantly decreased in Lep AS treated cells, and this effect was partially reversed upon coincubation with 50 ng/ml hrLeptin. (B) Representative pictures of JEG-3 cytospins stained for HLA-G (red) to evaluate effects of endogenous leptin blockade in AS treated cells. Scale bar: 25 μm. Quantitative analysis (left) confirmed a significant reduction of HLA-G fluorescence intensity in AS treated cells, which was partially recovered following stimulation with hrLeptin. Results are presented as mean ± SEM of 3 independent experiments. In all panels, $^{*}p < 0.05$ and $^{***}p < 0.001$ according to two-way ANOVA and Bonferroni posttest.

inhibitors of different signaling cascades. We focused on PI3K and Ras-MEK-Erk signaling, since these have been reported as the main pathways activated upon LepR ligation to mediate trophic effects in trophoblast cells [20,38]. In these experiments (Fig. 4A–B), treatment of Swan 71 cells with a specific PI3K/Akt inhibitor (LY294002, 10 μM) did not affect steady-state levels of HLA-G but led to a complete blockade of leptin-mediated up-regulation of expression (two-way ANOVA Inhibitor effect p = 0.002, Leptin effect p = 0.0641, interaction p = 0.0064; Fig. 4B). Similarly, inhibition of the Ras-MEK-Erk pathway by treatment with 100 μM PD98059 (Fig. 4C) produced no alteration in basal levels of expression, but prevented leptin induction of HLA-G in Swan cells (two-way ANOVA Inhibitor effect p = 0.0722, Leptin effect p = 0.2996, interaction p = 0.0006; Fig. 4D). Swan cells treated with 25 ng/ml leptin combined with LY294002 or PD98059 showed no differences in HLA-G signal intensities when compared to control cells. Similar results were observed in leptin-treated JEG-3 cells, which showed a significant decrease of HLA-G expression upon treatment with

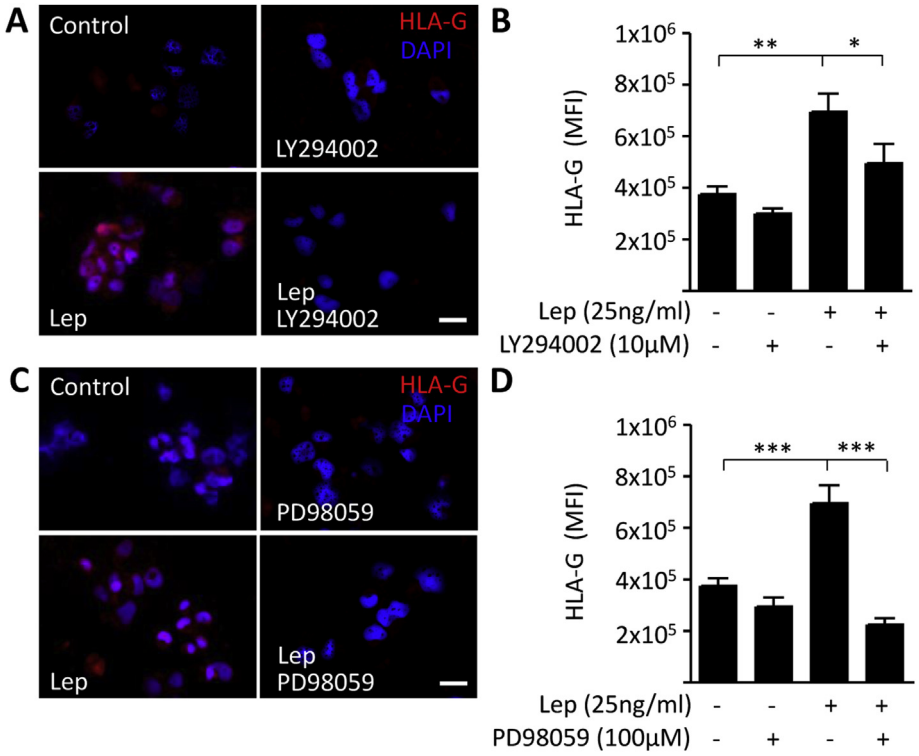

Fig. 4. Leptin induction of HLA-G in Swan 71 trophoblast cells involves the PI3K and MEK/Erk signaling pathways. (A) Cytospin preparations analyzing HLA-G expression in cells treated with leptin and the PI3K inhibitor LY294002. (B) Quantitative analysis of HLA-G mean fluorescence intensities (MFI). Pharmacological inhibition of PI3K signaling significantly down-regulated leptin-induced HLA-G expression in Swan 71 cells. (C) Examples of HLA-G IF results in cells treated with leptin and the specific MEK inhibitor PD98059. Scale bar: 25 μm. (D) Statistical analysis of mean fluorescence intensities. Leptin induced HLA-G expression was completely abrogated upon inhibition of MEK/Erk signaling by PD98059. Quantitative results are expressed as mean ± SEM for 3 independent experiments. In all panels, *, **, *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively, as determined by two-way ANOVA + Bonferroni posttest.

10 μM LY294002 (two-way ANOVA Inhibitor effect $p = 0.0177$, Leptin effect $p < 0.0001$, interaction $p = 0.2634$, Fig.S2A) or 100 μM PD98059 (two-way ANOVA Inhibitor effect $p < 0.0001$, Leptin effect $p = 0.0061$, interaction $p < 0.0001$; Fig. S2B).

4. Discussion

Despite its identification more than 15 years ago as a placenta-derived factor in humans [39], the precise function of leptin during pregnancy is only emerging. Here, we demonstrated that leptin is synthesized by the placental EVT during the early stages of pregnancy and that this expression is important for the modulation of the class Ib molecule HLA-G on trophoblast cells. These evidence, together with our previous *in vitro* studies showing an enhanced proliferation and decreased apoptosis in response to leptin stimulation [20–22], point to an important role of this hormone as an autocrine mediator influencing placental growth and immune modulation during pregnancy.

Our immunofluorescence analysis of normal first trimester choriodecidual samples confirmed the presence of leptin protein, localizing to the villous CTB and syncytium as reported in most of the previous studies analyzing leptin expression in placental tissue [14,15,25]. Of note, the highest leptin fluorescence signal was observed on interstitial EVT cells and cell columns, a finding that was only occasionally reported most probably due to the limited availability of samples covering these regions used for previous studies [25]. Furthermore, our study compared isolated villous CTB and EVT cells in terms of leptin expression, clearly demonstrating that EVT are an important source of leptin biosynthesis during early stages of pregnancy. Since isolated EVT in this study were identified based on HLA-G expression, our results imply that leptin biosynthesis might represent a feature of trophoblast cells differentiating along the invasive pathway.

Placenta derived factors and microenvironmental signals are believed to be most important in the modulation of HLA-G expression. When exogenously added to trophoblast cells at concentrations within the physiological range typically observed during early pregnancy [40,41], leptin showed the ability to up-regulate HLA-G expression at the mRNA and protein levels. Given that leptin is actively secreted by the placenta contributing to local and systemic concentrations during pregnancy [42], these observations point to a functional role of the hormone as a paracrine mediator controlling HLA-G expression on trophoblast cells. Interestingly, a differential dose response to leptin stimulation was observed in JEG-3 cells compared to the Swan 71 line in terms of HLA-G induction. This most probably reflects a different degree of differentiation resulting either from tumoral transformation (i.e., JEG-3 derive from choriocarcinoma whereas Swan 71 were developed from telomerase-transduced normal first trimester cells) or from lineage differences in the primary trophoblast populations giving rise to these cell lines. Indeed, Apps et al. [43] have suggested that with respect to HLA-G surface expression (which is considered the definitive EVT-specific HLA class I molecule), JEG-3 display a phenotype more comparable to primary EVT than that of Swan 71 cells. Besides these differences, our qRT-PCR and western blot results in normal term placental tissue reinforce the concept that leptin is a physiological modulator of HLA-G expression, though in this case much higher hormonal doses were required for stimulation. Of note, a similar effect was described in a recent study comparing adrenomedullin 2 (ADM2)-induced HLA-G expression in HTR-8SV/neo cells *versus* normal villous explants collected at term [44]. This decreased sensitivity to leptin could be related either to the fact that villous explants comprise different cell types that may exhibit a differential response to hormonal stimulation, or to the reduced invasive potential of term CTB compared to first trimester cells [6] that may translate into a decreased induction of HLA-G expression. In previous studies analyzing trophic effects of leptin on trophoblast cells, we observed that term villous explants shared the same dynamics of dose-dependent stimulation of protein synthesis than leptin-treated trophoblast cell lines [38]. Thus, an indirect corollary of these observations is that leptin exerts pleiotropic functions at the fetal-maternal interface, some of which may be stage-specific: while its anti-apoptotic and growth regulatory effects would be necessary for placental homeostasis throughout pregnancy, the ability to stimulate HLA-G expression may be restricted to early stages reflecting the need of protective mechanisms to avoid immunological damage towards invading trophoblasts during placentation. In this regard, it is well known that HLA-G is the specific ligand of multiple cellular receptors present in maternal cells including CD8, leukocyte immunoglobulin-like receptor (LILR) B1, LILRB2, killer cell immunoglobulin-like receptor (KIR) 2DL4, and possibly CD160 [45]. These HLA-G - receptor specific interactions limit potentially harmful maternal anti-paternal immune responses by impairment of decidual NK cell cytotoxicity [46], inhibition of CD4+ and CD8+ T-cell proliferation [47], B-cell proliferation [48], and induction of apoptosis of activated CD8+ T cells [49,50]. In this way, while leptin acts as a systemic mediator of inflammation and immune cell activation [28], it might indirectly play an immunosuppressive role by stimulating HLA-G expression locally at the maternal-fetal interface and subsequently protect trophoblast cells from immunological damage during placentation.

On the other hand, our results showing that JEG-3 cells, which in terms of their HLA repertoire can be considered an EVT-like cell line [43], decreased their HLA-G expression upon treatment with the Lep AS point out the importance of autocrine mechanisms for the induction of HLA-G molecules in EVT cells. This is consistent with previous studies showing that uterine vs. tubal implantations display similarly up-regulated HLA-G levels [51], which implies that HLA-G expression may be more dependent on intrinsical mechanisms and the state of differentiation of trophoblast cells rather than on maternal environmental signals.

As for the mechanisms modulating HLA-G expression in trophoblast cells, we found that pharmacological inhibition of the PI3K/Akt and Ras-MEK-Erk pathways abolished the stimulatory effect of leptin treatment in both Swan 71 and JEG-3 cell cultures. Our previous studies have shown that specific binding of Lep-R on trophoblast cells activates the Jak/STAT, PI3K and MAPK signaling cascades, which mediate leptin's effects on cell proliferation, apoptosis and protein synthesis [20,38,52]. While the direct involvement of these pathways in the modulation of HLA-G has not been fully investigated so far, PI3K and MEK-Erk signaling are generally regarded as major regulators of EVT invasion due to their influence on processes related to cell motility such as integrin signaling, cytoskeletal dynamics and nuclear functions [53]. In this context, recent *in vitro* studies showing that leptin significantly enhanced the migration of EVT-like cells via the PI3k/Akt pathway [54] support the notion that HLA-G induction may as well be a consequence of pro-invasive effects exerted by the hormone. This may also be the case for other HLA-G modulators such as ADM2, which has been shown to promote HTR-8SV/neo cell migration via a RAF1/MEK/Erk-dependent mechanism [44].

What would be the functional consequences of leptin induced HLA-G expression? During placentation, trophoblast invasion poses a challenge in terms of immune modulation as throughout this process EVT become exposed to maternal immunocompetent cells in the decidual milieu. While leptin itself exhibits a wide variety of immunomodulatory functions, its reported effects on innate and adaptive responses are consistent with a role promoting inflammation and immune cell activation [28,55]. Indeed, up-regulation

of leptin is often linked to chronic inflammatory conditions and has been suggested to enhance tumor growth by promoting mechanisms to evade the host's immune response [32,33]. Thus, induction of HLA-G expression would represent a protective mechanism triggered by leptin to avoid damage of invading trophoblast associated with the pro-inflammatory environment typical of early stages of pregnancy. By up-regulating HLA-G, leptin would confer a tolerogenic phenotype to trophoblast cells protecting them from inflammatory condition, as observed in other physiopathological settings (i.e., after pathogen clearance or at the tumor microenvironment) in which exacerbated or chronic inflammation gives rise to immune regulation and silencing to avoid unnecessary tissue damage [56,57]. Leaving interspecies differences aside, a similar protective mechanism may explain the recent observations that *in vivo* infusion of leptin during mid-pregnancy did not produce any overt alterations in pro-inflammatory cytokines and leukocyte infiltration to the mouse placenta [58]. In summary, our results clearly demonstrate that leptin enhances placental HLA-G expression and strongly suggest an important role of this hormone as an autocrine modulator of trophoblast cell physiology. Further studies assessing the functional consequences of leptin induced HLA-G expression on maternal immune cells would greatly improve our understanding of the hormonal regulation of placental development with important implications in the field of reproductive medicine.

Conflict of interest

The authors declare that no conflict of interest exists.

Acknowledgments

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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.2015.01.006.

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