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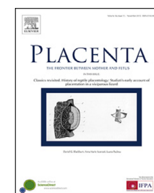

Mechanisms involved in p53 downregulation by leptin in trophoblastic cells

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A B S T R A C T

Leptin, a 16-kDa polypeptide hormone, is produced by the adipocyte and can also be synthesized by placenta. We previously demonstrated that leptin promotes proliferation and survival in placenta, in part mediated by the p53 pathway. In this work, we investigated the mechanisms involved in leptin downregulation of p53 level. The human first trimester cytotrophoblastic Swan-71 cell line and human placental explants at term were used. In order to study the late phase of apoptosis, triggered by serum deprivation, experiments of DNA fragmentation were carried out. Exogenous leptin added to human placental explants, showed a decrease on DNA ladder formation and MAPK pathway is involved in this leptin effect. We also found that under serum deprivation condition, leptin decreases p53 levels and the inhibitory leptin effect is lost when cells were pretreated with 50 μM PD98059 or 10 μM LY29004; or were transfected with dominant negative mutants of intermediates of these pathways, suggesting that MAPK and PI3K signaling pathways are necessaries for leptin action. Additionally, leptin diminished Ser-46 p53 phosphorylation and this effect in placental explants was mediated by the activation of MAPK and PI3K pathways. Finally, in order to assess leptin effect on p53 half-life experiments with cycloheximide were performed and MDM-2 expression was analyzed. Leptin diminished p53 half-life and up-regulated MDM-2 expression. In summary, we provided evidence suggesting that leptin anti-apoptotic effect is mediated by MAPK and PI3K pathways.

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1. Introduction

Human pregnancy successful is accomplished by a functional fetal–maternal interface established at the very initial steps. The placenta allows metabolic exchange along this interface and works as an endocrine tissue that produces steroids, peptide hormones, growth factors and cytokines that are crucial for the maintenance of pregnancy. Several cytokines and growth factors, such as leptin are known to influence trophoblast migration, proliferation, invasion and survival [1]. The apoptotic process is a naturally occurring event in placental cells and is necessary in placenta growth. Apoptotic mechanisms are also associated with cytotrophoblasts fusion and differentiation to multinucleate syncytium and have a major role in maintaining the integrity of villous trophoblast [2,3].

The p53 protein is a master transcription factor that increases in response to different stress stimuli such as heat shock, hypoxia, osmotic shock and DNA damage, leading to growth arrest, apoptosis and DNA repair [4]. Turnover of p53 protein is regulated by diverse post-translational modifications that affect its stability and activity. Post-translational modifications of p53 include phosphorylation, ubiquitination, acetylation, methylation, sumoylation and glycosylation [5]. Modification of p53 by ubiquitination and deubiquitination is an important reversible mechanism that effectively regulates its functions [6]. Mono- or polyubiquitination of p53 by different E3 ligases regulates its nuclear export, mitochondrial translocation, protein stability and transcriptional activity. The protein levels of p53 are negatively regulated by MDM-2, an E3 ubiquitin ligase, via a negative feedback loop that is essential in determining cell survival [7]. The activity of the MDM-2 E3 ligase toward p53 is regulated by multiple parameters: the RING domain, the C terminus, and interactions with MDM-4 [8]. It is known that phosphorylation of p53 at Ser-15 leads to the dissociation of MDM-

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2, and p53 degradation is inhibited [9].

Leptin, the non-glycosylated protein encoded by the *Lep* gene, is a 16,000 molecular weight polypeptide discovered in 1994 by Zhang et al. [10]. This cytokine-type hormone is able to exert multiple functions; the best characterized is the regulation of food intake and energy expenditure [11]. Pleiotropic effects of leptin have been identified, consisting in the modulation of thermogenesis, homeostasis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine and immune functions as well as arterial pressure control [12]. Compelling evidence also implicated leptin in reproductive functions such as the regulation of ovarian function, oocyte maturation, embryo development and recently has emerged as a placental hormone with important regulatory functions during implantation and pregnancy [13,14].

The primary amino acid sequence of leptin indicated that it could belong to the long-chain helical cytokine family [15]. In fact, leptin receptor (Ob-R) shows sequence homology to members of the class I cytokine receptor (gp130) superfamily [16]. Leptin receptors isoforms are generated by splice variance [17]. The full length leptin receptor is membrane bound and is responsible for cell signaling through JAK-STAT signaling pathways [18]. There are three other membrane bound leptin receptor isoforms which vary on the length of the intracellular domain and can signaling through the MAPK pathway. There is also a soluble receptor form that lacks the transmembrane and intracellular domains [19]. In this line, we have studied the signaling pathways activated by leptin receptor in trophoblastic cells, where the long isoform of leptin receptor stimulates the Janus kinase (JAK)-signal transducers and activators of transcription (STAT), PI3K, and MAPK pathways [20].

Placental leptin expression at the fetoplacental unit starts at very early stages of gestation [21] and is delicately modulated by pregnancy-related factors including steroid hormones, insulin, human chorionic gonadotropin (hCG), cAMP, cytokines and also hypoxia [1,22–27].

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 [28,29].

The expression pattern of both leptin and its receptors at the maternal fetal interface argues in favor of a role as an autocrine or paracrine pathway controlling placental development and function. Indeed, among other functions we have found that leptin promotes proliferation, protein synthesis and survival of trophoblastic cells [20,30–32]. Recently we found that leptin exerts its anti-apoptotic effect by increasing the BCL-2/BAX relationship and diminishing the level of BID p53 [33]. All these results reinforce the notion of an anti-apoptotic effect of leptin on trophoblastic cells.

Based on the importance of leptin during pregnancy and previous results from our lab, in the present work we aimed to study the mechanisms involved on leptin effect on trophoblast survival, particularly we analyzed whether MAPK and PI3K pathways were implicated in p53 down-regulation by leptin. Our hypothesis is that leptin is an endogenous component of the proliferation/apoptosis regulatory machinery in trophoblast cells and exerts its action in part by p53 downregulation through different signaling pathways. Our data argue for a central role of leptin as a paracrine/autocrine signal and suggest that could be an endogenous component of the proliferation/apoptosis regulatory machinery of trophoblast cells.

2. Materials and methods

2.1. Ethics statement

Written informed consent was obtained from all subjects and all study procedures were approved by ethical review committees at the Virgen Macarena University Hospital and the Alejandro Posadas National Hospital (Bioethics Committee "Dr. Vicente Federico del Giudice").

2.2. Cell culture and treatments

The human cytotrophoblastic cell line Swan-71 was generously provided by Dr. Gil Mor (Yale University School of Medicine, New Haven, USA). They were generated by the introduction of human telomerase reverse transcriptase, for the immortalization of primary human cells [34,35]. Swan-71 trophoblastic cells were grown in 45% Dulbecco's modified Eagle medium (DMEM) and 45% HAM F-12 (Invitrogen) 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 Chemical Company, St. Louis, MO) at 37 °C in 5% CO_2.

After 24 h of plating, cell culture medium was replaced with DMEM-F12 – 0% FBS to induce apoptosis and cells were treated for 48 h for total protein immunoblotting or during 24 h for protein phosphorylation determinations, in the absence or presence of different concentrations of leptin (from 50 ng/ml to 250 ng/ml). Cells cultured continuously with 10% FBS were used as control. In experiments designed to analyze the signal transduction pathways involved in leptin effect on p53, p53 pSer-46 and MDM-2, the MAPK kinase (MEK) inhibitor PD98059 (50 μM) and the PI3K inhibitor LY29004 (100 nM) (Sigma Chemical Co.) were used. Inhibitors were added 30 min before leptin treatment.

2.3. Placental explants collection and processing

Human placentas (n = 5) were obtained after Cesarean section or vaginal delivery following normal term pregnancies and immediately suspended in ice-cold phosphate buffer saline (PBS) and transported to the laboratory, where they were washed 2–3 times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight).

Placental explants were randomly distributed in tubes containing 1 ml of PBS (1 explant/tube, 5 replicates per treatment), maintained in a shaking water bath at 37 °C during 5 min to equilibrate temperature, pre-incubating during 30 min when indicated with 50 μM PD98059 or 10 μM LY29004 and incubated for 24 h in DMEM-F12 0% FBS in the absence or presence of different concentrations of leptin (from 50 ng/ml to 250 ng/ml). Explants were removed from the bath, centrifuged for 2 min at 2000 g at 4 °C and resuspended in 500 μl of lysis buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml PMSF) during 30 min at 4 °C on an orbital shaker and later centrifuged at 10,000 g for 20 min. Supernatants were analyzed by Western blot.

For real-time PCR, after thoroughly washing with PBS, the tissues were immediately frozen at 80 °C and stored until extraction of total RNA.

2.4. Western blot and half-life studies

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 [36], with bovine serum albumin (BSA) as standard. Lysates were mixed with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond, Amersham

Pharmacia). Membranes were equilibrated in 1x PBS and non-specific binding sites were blocked by 5% non-fat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with monoclonal mouse anti-p53 (1:1000, Santa Cruz), polyclonal rabbit anti-phospho Ser-46 p53 (1:1000, Cell Signaling), monoclonal mouse anti-MDM-2 (1:1000, Oncogene), monoclonal rabbit anti-pERK (1:1000, BD Biosciences Pharmingen) or monoclonal rabbit anti-pAKT (1:1000, BD Biosciences Pharmingen). Loading controls were performed by immunoblotting the same membranes with monoclonal mouse anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000, Calbiochem) or polyclonal rabbit anti-β-actin (1:1000, Santa Cruz). The antibodies were detected using horseradish peroxidase-linked goat anti-rabbit IgG (1:10,000, Santa Cruz) or goat anti-mouse IgG (1:10,000, Santa Cruz) and visualized by the Amersham Pharmacia ECL Chemiluminescence signaling system and a Bio-Imaging Analyzer Fujifilm LAS-1000 in all cases except in blots showed in Figs. 4 and 5, that were exposed to X-ray films. Quantification of protein bands was determined by densitometry using Image J 1.47 software (Wayne Rasband National Institute of Health, USA).

In half-life studies cells were treated with 10 μg/ml of cycloheximide (Sigma) 30 min before leptin treatment (100 ng/ml) during 0–24 h to inhibit protein synthesis. Whole cell extracts were prepared as above and protein levels were determinate by Western blot. Proteins were detected with antibodies against p53 and band intensities for each treatment condition were normalized. Linear regression was performed and the half-life calculated from de fitted line equation.

2.5. DNA fragmentation assay

Apoptosis was evaluated by examining the characteristic pattern of DNA laddering to assay DNA fragmentation. Placental explants (40 mg of tissue per treatment) were preincubated during 30 min when indicated with 50 μM PD98059 or 10 μM LY29004 and incubated in 1 ml of DMEM-F12 in the presence or absence of FBS containing 100 ng/ml of leptin in a shaking water bath at 37 °C. After 24 h of treatment, placental explants were harvested, washed in PBS and homogenized in 500 μl of lysis buffer containing 1% of SDS, 50 mM EDTA, 50 mM Tris HCl and 50 mM NaCl. Then, proteinase K (10 mg/mL) was added to the homogenates and incubated at 55 °C during 2 h. Afterwards, 5 M NaCl was added, mixed by inversion and centrifuged at 15,000 rpm for 15 min at 4 °C. Supernatants were transferred and cold ethanol was added, mixed by inversion and the mixtures were left at −20 °C overnight. Samples were centrifuged at 15,000 rpm for 15 min at 4 °C and DNA pellet was air dried and resuspended in 100 μL of sterile water. DNA fragments were separated by a 2% agarose gel electrophoresis at 80 V for 1.5 h. The gels were stained with 0.5 mg/ml ethidium bromide and low DNA fragments visualized on a UV-illuminator. Quantification of low molecular weight bands was determined by densitometry using Image J 1.47 software.

2.6. Plasmids

The pcDNA1 plasmids encoding the kinase inactive mutant of MAPK (designated MAPK kd), the kinase inactive mutant of MEK (designated MEK kd) and the kinase inactive mutant of PI3K pathway (designed AKT kd) were kindly supplied by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). 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 vector was used as a control. Plasmids were purified using the Midipreps Wizard kit (Promega Co.), and DNA concentration was estimated spectrophotometrically.

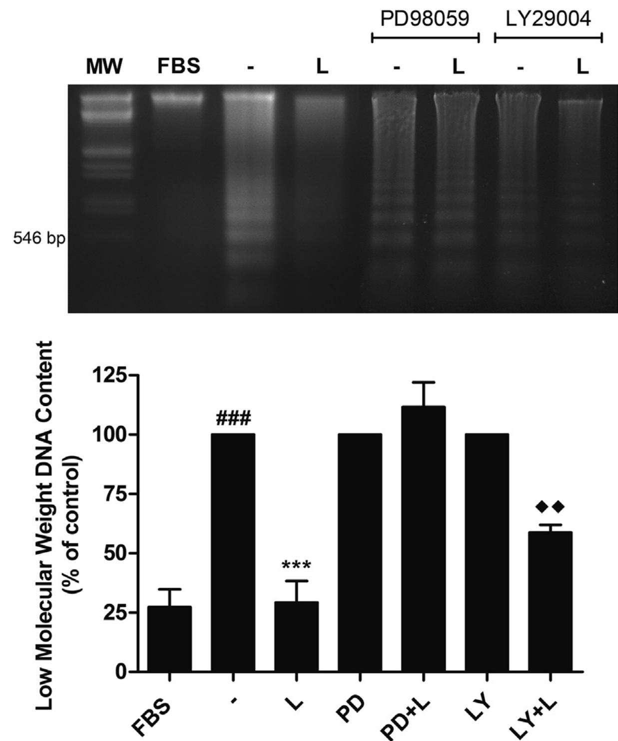

Fig. 1. Signaling through the MEK/Erk pathway is required for leptin to prevent apoptosis in human placental explants. Gel electrophoresis of apoptotic DNA fragmentation. Inspection of electrophoretic profiles revealed a lower ladder formation in presence of 100 ng/ml of leptin (L). Pretreatment with 50 μM of the pharmacological specific inhibitor PD98059 (PD) abolished leptin antiapoptotic effect, since 10 μM LY29004 (LY) not. Quantification of low molecular weight (MW) bands is shown. Results were relativized to the 100% of apoptosis in the sample of cells cultivated with DMEM in the absence of serum (−), with PD or with LY. DNA fragmentation assay was performed as indicated in Materials and Methods. Results are expressed as mean ± SD for three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison *post hoc* test, relative to FBS 10% (#), FBS 0% (*) or pretreatment with LY (◆). ###p < 0.001, ***p < 0.001, ◆◆ p < 0.01.

2.7. Transient transfection experiments

For transient transfection experiments, Swan-71 cells were plated at a density of 2 × 10^5 cells/ml onto six-well dishes containing 2 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. Typically 5 μg of expression vector and 5 μg of pRSV-βgal internal control construct were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc.). The medium was replaced after 5 h with DMEM-F12 0% FBS with the addition of 100 ng/ml of leptin. Transfection analysis was performed by duplicate in each of at least three independent experiments. To perform a transfection efficiency control we verify the reduction of p-ERK when cells were transfected with MEK kd and MAPK kd and p-AKT when cells were transfected with AKT kd.

2.8. Assays for β-galactosidase activity

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

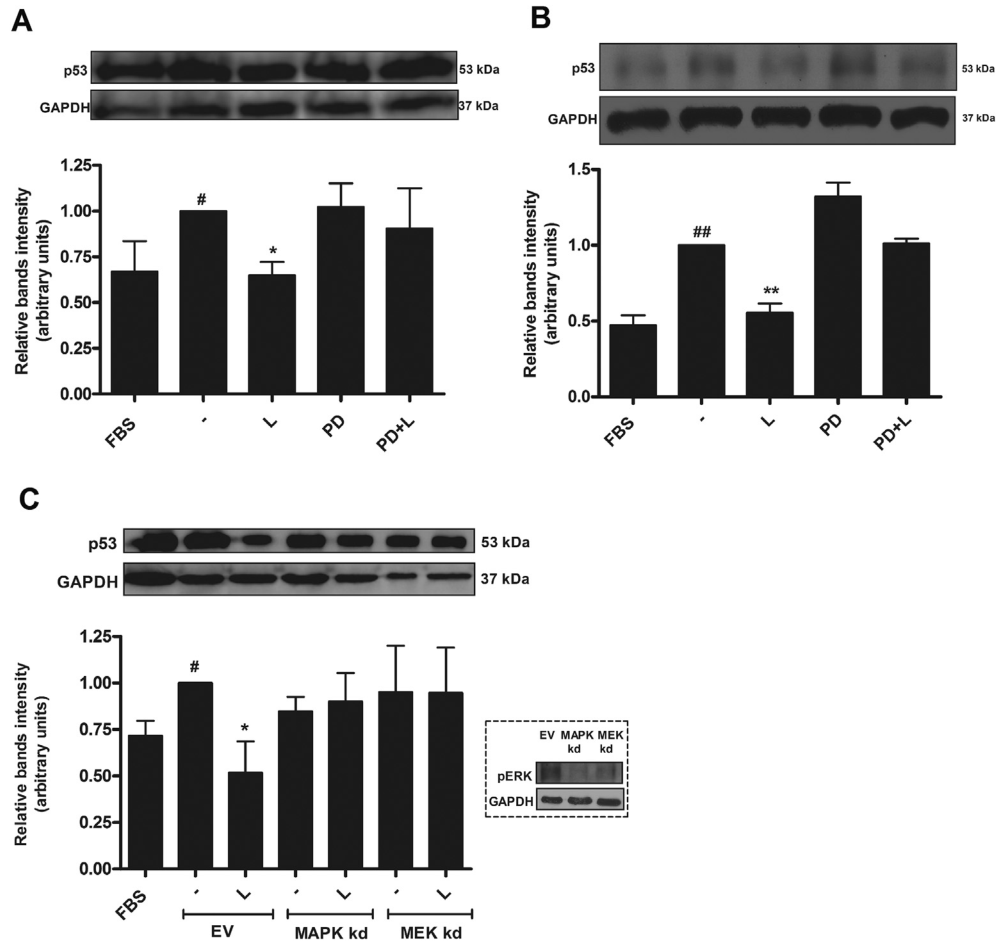

Fig. 2. The MEK/Erk pathway mediates p53 diminution by leptin in placental cells. **A)** Swan-71 cells were plated in DMEM-F12 media in the absence of serum (−), pretreated with 50 μM PD98059 (PD) and incubated during 48 h with 100 ng/ml of leptin (L). DMEM-F12 10% FBS (FBS) was used as a control. Cell extracts were prepared as indicated in Materials and Methods. Proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. **B)** Placental explants were processed as described in Materials and Methods, pretreated with 50 μM PD98059 (PD) and incubated during 24 h in DMEM-F12 media supplemented with 100 ng/ml of leptin (L). DMEM-F12 10% FBS (FBS) was used as control. Placental extracts were prepared and proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. **C)** Swan-71 cells were transiently transfected with plasmids encoding the kinase inactive mutant of MAPK (MAPK kd), the kinase inactive mutant of MEK (MEK kd) or an empty vector (EV). After transfection, cells were incubated for 48 h in DMEM-F12 and treated with 100 ng/ml of leptin (L). Cell extracts were prepared as indicated in Materials and Methods and β-galactosidase activity was measure for a transfection control. Proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. The inset shows representative blots confirming reduced p-ERK level in transiently transfected cells. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Bands densitometry is shown in lower panels, results are expressed as mean ± SD for three independent experiments. Statistical analyzes were performed by ANOVA. Symbols indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to FBS 10% (#) or FBS 0% (*). #p < 0.05, ##p < 0.01, *p < 0.05, **p < 0.01.

staining. The product was determined by absorption at 420 nm. This value was used to correct variations in transfection efficiency. Duplicate samples were analyzed for each data point.

2.9. Quantitative real-time RT-PCR assay

Abundance of Mdm-2 mRNA was determined by quantitative real time RT-PCR reaction (*q*RT-PCR). Total RNA was extracted from placental explants using TRISURE reagent, according to the manufacturer's instructions (Bioline Co). Concentration and purity of the isolated RNA were estimated by spectrophotometry at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse-transcribed at 50 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). Quantitative real time PCR reaction was performed using the following primers based on the sequences of the NCBI GenBank database: Mdm-2, forward, 5′TTACCCAGGCTGGAGTGCAG3′; reverse, 5′GAGAATGGTGCGAACCCG3′; and cyclophilin, forward, 5′CTTCCCCGATACTTCA3′; reverse, 5′TCTTGGTGCTACCTC 3′. *q*RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green) and PCR reactions 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 (C_T), from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method [37]. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin), and relative to the untreated control.

2.10. Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as the mean ± standard deviation (S.D.). The statistical significance was assessed by ANOVA followed by different post hoc tests indicated in

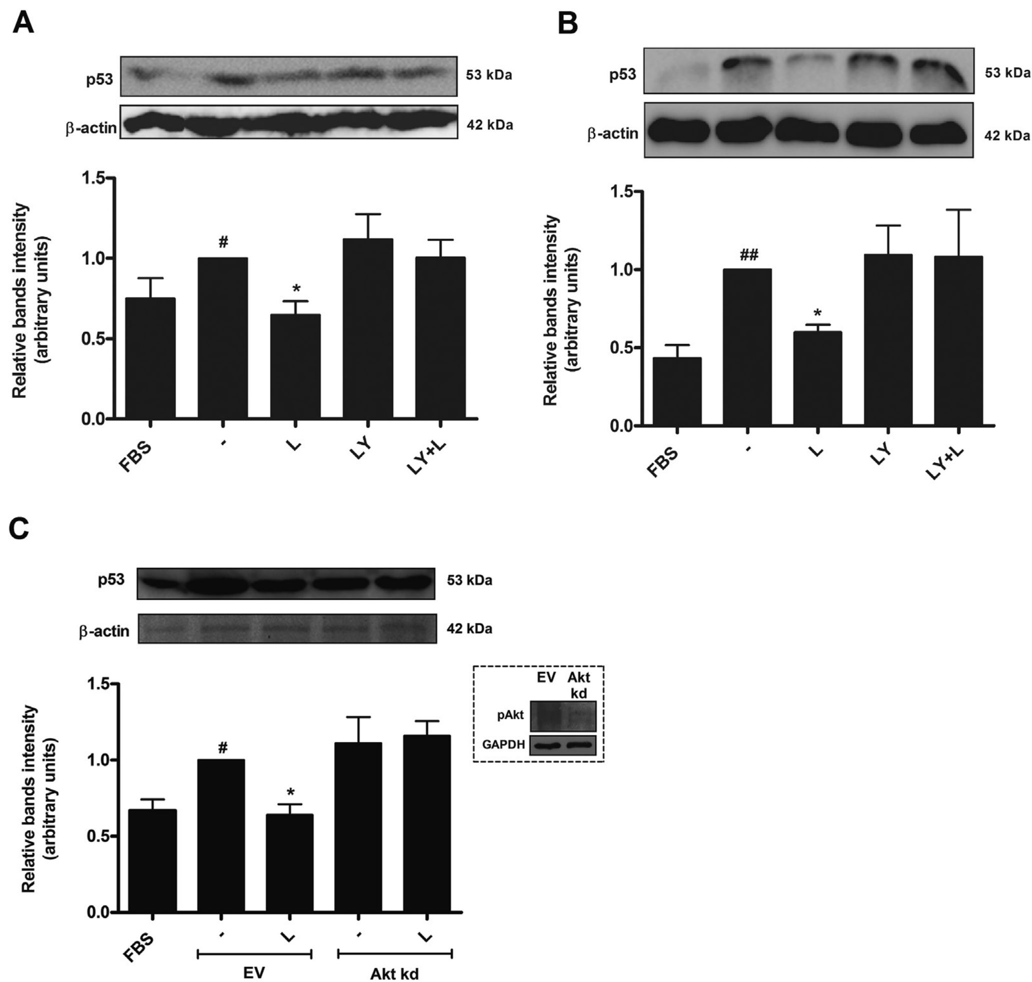

Fig. 3. PI3K activity is necessary for leptin inhibitory effect on p53 expression. **A)** Swan-71 cells were plated in DMEM-F12 media in the absence of serum (−), pretreated with 10 μM LY29004 (LY) and incubated during 48 h with 100 ng/ml of leptin (L). DMEM-F12 10% FBS (FBS) was used as a control. Cell extracts were prepared as indicated in Materials and Methods. Proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. **B)** Placental explants were processed as described in Materials and Methods, pretreated with 10 μM LY29004 (LY) and incubated during 24 h in DMEM-F12 media supplemented with 100 ng/ml of leptin (L). DMEM-F12 10% FBS (FBS) was used as control. Placental extracts were prepared and proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. **C)** Swan-71 cells were transiently transfected with a plasmid encoding the kinase inactive mutant of PI3K pathway (Akt kd) or an empty vector (EV). After transfection, cells were incubated for 48 h in DMEM-F12 (−) and treated with 100 ng/ml of leptin. Cell extracts were prepared as indicated in Materials and Methods and β-galactosidase activity was measure for a transfection control. Proteins were separated on SDS-PAGE gels and p53 expression was determined by Western blot analysis. The inset shows representative blots confirming reduced p-AKT level in transiently transfected cells. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-β-actin or anti-GAPDH. Bands densitometry is shown in lower panels, results are expressed as mean ± SD for three independent experiments. Statistical analyzes were performed by ANOVA. Symbols indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to FBS 10% (#) or FBS 0% (*). #p < 0.05, ##p < 0.01, *p < 0.05.

each figure and was calculated using the Graph Pad Instat computer program (San Diego, CA). A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. MAPK signaling pathway is involved in leptin anti-apoptotic effect in placental explants

Leptin diminished the presence of low DNA fragments, product from apoptotic cleavage in placental explants [33], for this reason in this section we aimed to study signal transduction pathway involved using the DNA fragmentation assay. Human placental explants were pretreated with specific pharmacological inhibitors for MAPK kinase (MEK) (PD98059) and for PI3K (LY29004). As it is seen in Fig. 1, serum deprivation increased the apoptotic cleavage of DNA meanwhile 100 ng/ml of leptin significantly reduced low weight population of DNA fragments. Pretreatment with 50 μM PD98059 abolished leptin anti-apoptotic action, but leptin partially sustained its anti-apoptotic effect in the presence of 10 μM LY29004.

3.2. MAPK is involved in leptin down-regulation of p53 in placental cells

We aimed to study whether MAPK signaling pathway is involved in leptin reported effect on p53 level [33]. For this purpose we pretreated Swan-71 cells with the specific pharmacological inhibitor PD 98059. As it is shown in Fig. 2A, serum deprivation increased p53 level in Swan-71 cells meanwhile treatment with 100 ng/ml leptin diminished its expression. Pretreatment with 50 μM PD98059 blocked leptin effect on p53 level. Similar experiments were carried out in placental explants, a more physiological system. Fig. 2B shows that 50 μM PD98059 abolished leptin

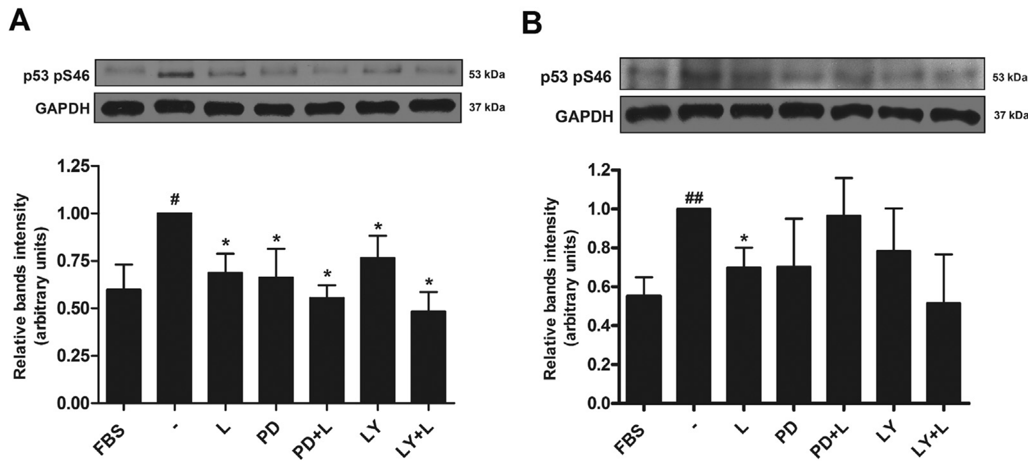

Fig. 4. Leptin Ser-46 p53 phosphorylation level diminution in human placenta could be mediated by MAPK and PI3K pathways. **A)** Swan-71 cells were plated in DMEM-F12 10% FBS. After 24 h cells were pretreated with 50 μM PD98059 (PD) or 10 μM LY29004 (LY) and incubated during 24 h with 100 ng/ml of leptin (L) in DMEM-F12 in the absence of serum (−). Cell extracts were prepared as indicated in Materials and Methods and proteins were separated on SDS-PAGE gels. **B)** Placental explants were processed as described in Material and Methods, pretreated with 50 μM PD98059 (PD) or 10 μM LY29004 (LY) and incubated in DMEM-F12 0% FBS media supplemented 100 ng/ml of leptin (L) doses during 8 h. Placental extracts were prepared and proteins were separated on SDS-PAGE gels. In both cases (A and B) Phospho Ser-46 p53 was determined by Western blot analysis. DMEM-F12 media supplemented with 10% FBS (FBS) was used as control. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Bands densitometry is shown in lower panels. Results are expressed as mean ± SD for three independent experiments. Statistical analyzes were performed by ANOVA. Symbols indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to FBS 10% (#) or FBS 0% (*). #p < 0.05, ##p < 0.01, *p < 0.05.

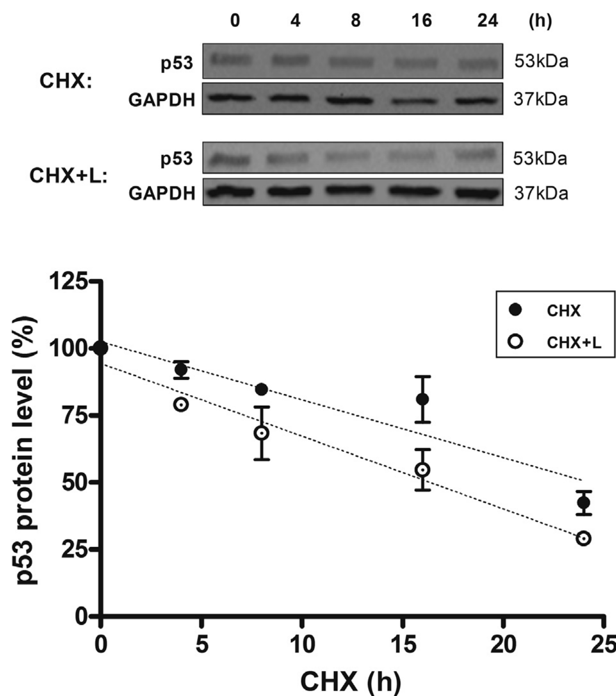

Fig. 5. Leptin decreases p53 half-life. Swan-71 cells were treated with 10 μg/ml of cycloheximide (CHX) and with or without 100 ng/ml leptin (L) during the indicated time periods (0–24 h). Proteins were isolated, separated by SDS-PAGE and detected by Western blot. Protein levels were quantified and the relative protein level of p53 was blotted against the duration of CHX treatment for estimation of the half-life. The dashed line indicates the linear regression obtained. Representative Western blots are shown above.

inhibitory effect in p53 level.

To further study MAPK signaling pathway role on leptin action, Swan-71 cells were transiently transfected with plasmids expressing dominant negative mutants for MAPK and MEK activities (MAPKkd and MEKkd respectively). As it is seen in Fig. 2C, leptin decreased p53 level when a control empty vector was transfected. The over-expression of each dominant negative mutant inhibited leptin effect, since no changes in p53 levels were detected. As it is seen in the inset, the over-expression of MAPKkd or MEKkd completely blocked ERK phosphorylation. All these findings suggest that MAPK signaling pathway is involved in leptin action on p53 in placental cells.

3.3. PI3K mediates the reduction of p53 by leptin in placental cells

We also studied the possible participation of phosphatidylinositol 3 kinase (PI3K) dependent signaling pathway on the inhibitory effect of leptin on p53 expression. For this purpose, we pretreated cells with the pharmacological inhibitor LY29004. In Fig. 3A it could be seen that 10 μM LY29004 completely blocked leptin action on p53 levels in Swan-71 cells. Similar results were obtained in placental explants (Fig. 3B). To confirm the PI3K signaling pathway participation, Swan-71 cells were transiently transfected with a plasmid expressing a dominant negative mutant for AKTkt (AKTkd). As it is shown in Fig. 3C, leptin decreased p53 level when a control empty vector was transfected. The over-expression of AKTkd blocked leptin effect on p53 level. All these results suggest that PI3K signaling pathway participates in leptin effect on p53 downregulation in placental cells.

3.4. MAPK and PI3K are involved in the reduction of Ser-46 p53 phosphorylation in trophoblastic cells and placental explants

The phosphorylation of Ser-46 p53 plays a pivotal role in apoptotic signaling by p53. We aimed to study if MAPK and PI3K pathways are involved in leptin regulation of this posttranslational modification. The effect of pretreatment with PD98059 or LY29004 was assessed. As seen in Fig. 4, serum starvation increases Ser-46 p53 phosphorylation and treatment with 100 ng/ml leptin diminished it both in Swan-71 cells and placental explants. When Swan-71 cells were pretreated with pharmacological inhibitors, no changes in Ser-46 p53 phosphorylation levels were detected in the presence of leptin (Fig. 4A). Nevertheless, pretreatment with each

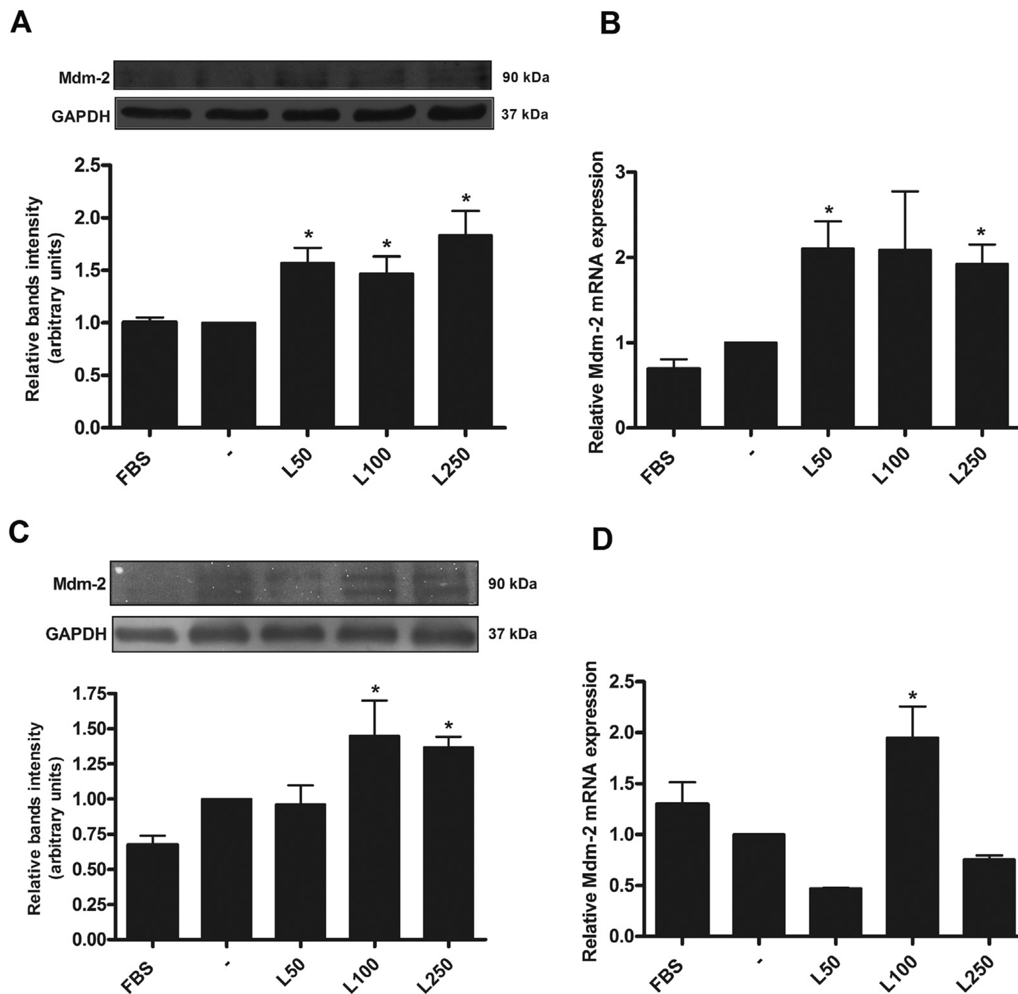

Fig. 6. Leptin enhances Mdm-2 levels in human placenta. **A)** Swan-71 cells were plated in DMEM-F12 media in the absence of serum (−) and incubated during 48 h with different leptin concentrations (50 ng/ml: L50, 100 ng/ml: L100 and 250 ng/ml: L250). Cell extracts were prepared as indicated in Materials and Methods and proteins were separated on SDS-PAGE gels. **B)** Mdm-2 expression in Swan-71 cells was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. **C)** Placental explants were processed as described in Material and Methods and incubated in DMEM-F12 media supplemented with increasing leptin doses (L50, L100 and L250) during 24 h. Placental extracts were prepared and proteins were separated on SDS-PAGE gels. **D)** Mdm-2 expression in human placental explants was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. In **A** and **C** MDM-2 was determined by Western blot analysis. DMEM-F12 10% FBS (FBS) was used as a control. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Bands densitometry is shown in lower panels. Results are expressed as mean ± SD for three independent experiments. Statistical analyzes were performed by ANOVA. Symbols indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to FBS 0% (*). *$p < 0.05$.

specific pharmacological inhibitor abolished leptin effect on Ser-46 p53 phosphorylation level in placental explants (Fig. 4B), suggesting that MAPK and PI3K pathways might be involved in leptin effect in this model.

3.5. Leptin diminished p53 half-life in placental cells

In view of determining how leptin affects p53 protein stability, we estimated p53 half-life. For this aim, Swan-71 cells were treated with the protein synthesis inhibitor cycloheximide during 0–24 h in the presence or absence of leptin and p53 level was quantified by Western blot. As shown in Fig. 5, while p53 half-life was 24.03 ± 0.39 h in control, cells treated with leptin exhibited a p53 half-life of 15.85 ± 1.52 h. These data show that leptin significantly decreases p53 protein stability ($p < 0.5$).

3.6. Leptin enhances Mdm-2 expression in placental cells

As it was shown previously, leptin treatment in serum free media significantly reduces p53 half-life in Swan-71 cells. An important regulator of p53 protein level is MDM-2, an E3 ubiquitin ligase that promotes p53 degradation. For this reason, our next purpose was to study leptin effect on Mdm-2 expression. As it is seen in Fig. 6, serum starvation did not modify Mdm-2 mRNA or protein levels in Swan-71 cells. Meanwhile leptin treatment significantly increased MDM-2 protein level measured by Western blot (Fig. 6A) and mRNA level measured by *q*RT-PCR (Fig. 6B). Human placental explants were also analyzed. As it is shown in Fig. 6C, leptin significantly increased MDM-2 protein. Leptin treatment significantly increased Mdm-2 mRNA level determined by *q*RT-PCR (Fig. 6D). These results evidence that leptin regulates Mdm-2 expression and strongly suggest a possible mechanism to explain leptin diminution of p53 half-life in placental cells.

4. Discussion

The original view of leptin solely as an adipocyte-derived factor acting centrally to control energy expenditure and food intake

[38,39] changed after the identification of leptin synthesis at the fetoplacental unit. Leptin is now consider an important local factor in placenta that function as a trophic molecule, promoting growth and preventing the apoptotic process [1]. These effects have physiological relevance since invasive extravillous trophoblast cells are a main source of this hormone during the first trimester of normal pregnancies [40]. The localization of leptin and its receptor in human placental indicates that leptin may have both autocrine and paracrine activities as a local immunomodulatory signal [41]. Deregulation of the autocrine/paracrine function of leptin at feto-placento-maternal interface may be implicated in the pathogenesis of recurrent miscarriage gestational diabetes, pre-eclampsia and intra-uterine fetal growth retardation [14,28,42–45]. In this study, we employed Swan-71 human trophoblastic cells. Swan-71 cell line represents a model of first trimester trophoblast cells. It was generated by the introduction of telomerase that generally do not produce karyotypic and/or phenotypic abnormalities. They secrete fetal fibronectin and hCG and exhibit a cytokine and growth factor profile that is similar to primary trophoblast cells, representing a valuable model for *in vitro* trophoblast studies. On the other hand these cells are considered a good model of extravillous cytotrophoblasts as they express vimentin and HLA-G These cells maintain many characteristics of human trophoblast cells and have been used to study placental physiology [34,35]. Normal trophoblastic explants from healthy donors were also used to confirm our results in a more physiological model. Recently we found that leptin treatment in Swan-71 cells triggers an anti-apoptotic response [33]. On the other hand, previous results from our group have demonstrated that leptin exerts an anti-apoptotic action in placenta mediated by the mitogen-activated protein kinase (MAPK) pathway [20]. In this work we aimed to study signal transduction pathways involved in leptin survival effect on Swan-71 and human placental explants. DNA fragmentation assay was used to determine the presence of low weight DNA fragments, products from apoptotic DNA cleavage. Our results showed that leptin treatment diminished the population of small weight DNA fragments involving MAPK signaling pathway, since the pharmacological inhibition of MAPK can block the anti-apoptotic effect of leptin. Leptin activation of MAPK pathway has been previously found to be the mechanism whereby leptin promotes cell survival preventing apoptosis [46,47] moreover, the anti-apoptotic effect of leptin on blood monocytes is mediated by the activation of MAPK [48]. Now, in trophoblastic Swan-71 cells we have found that leptin prevents the apoptotic process triggered by the deprivation of serum by means of the activation of MAPK pathway. However, PI3K inhibition did not prevent the presence of low weight DNA fragments.

The p53 tumor suppressor protein is a key component of cellular mechanisms that are activated by different cellular stress signals [49]. Protein expression of p53 is significantly increased in pregnancies complicated by preeclampsia or fetal growth restriction in which occur an exaggerated trophoblast apoptosis [50,51].

Previously, we have investigated whether this key cell cycle-signaling protein was involved in the leptin anti-apoptotic effect. We have determined a significant decrease of p53 expression in the presence of leptin in a model of serum deprivation condition, both in Swan-71 and human placental explants [33]. In this work we aimed to study MAPK and PI3K transduction pathways activation in on leptin dependent p53 diminution. We take advantage of the use of specific pharmacological inhibitors of each pathway, PD98059 and LY29004 respectively. We also performed a different strategy in which dominant negative mutants of intermediates of these pathways were over-expressed by transient transfection. Our results showed that leptin diminishes p53 protein level in serum deprivation conditions by means of the activation of both MAPK and PI3K pathways.

After stress, such us serum deprivation, p53 is activated mostly at the post-translational level by a complex series of modifications that include the phosphorylation and acetylation of specific residues in the amino-terminal and carboxyterminal domains. Among these post-translational modifications p53 is phosphorylated at Ser-46 to regulate apoptosis after DNA damage. Moreover, there is evidence that Ser-46 of p53 is phosphorylated in response to DNA damage in vivo, and it plays a pivotal role for apoptotic signaling by p53 through regulating the transcriptional activation of an apoptosis-inducing gene, p53AIP1 [52]. We previously have studied the effect of leptin on this post-translational modification, demonstrating that leptin significantly diminishes Ser-46 phosphorylation of p53 under stress condition [33]. In the present work we analyzed signal transduction pathways involved in leptin action. We used the same experimental approaches described above. Our data might suggest that leptin decreases Ser-46 p53 phosphorylation by the activation of both MAPK and PI3K signal transduction pathways.

Immunohistochemical studies of first-trimester trophoblast have shown that p53 is detectable in the nucleus of cytotrophoblasts and faintly in syncytiotrophoblasts [53]. Under normal conditions, p53 is a short-lived protein that is highly regulated and maintained at low or undetectable levels; [54]. In this work we analyzed whether leptin destabilizes p53, with this purpose we determined p53 half-life in cells treated with cycloheximide in the presence of leptin after serum deprivation. Our data demonstrates that under stress condition such us serum deprivation, leptin significantly diminished p53 half-life.

The p53 protein is stabilized after damage and must be destabilized for cell survival. MDM-2 regulates this transition. On the other hand, Mdm-2 is itself a transcriptional target of p53. The P2 promoter of Mdm-2 has two distinct p53-binding sites by which the p53 induced by damage binds and activates Mdm-2 transcription [55,56]. This mechanism results in a feedback loop between p53 and MDM-2 in which p53 induces the expression of its inhibitor after the cell recovers from stress. Variations in MDM-2 levels are important determinants of p53 function [57]. For example, low MDM-2 levels induce monoubiquitination and nuclear export of p53, while higher levels promote its poly-ubiquitination and degradation [58]. As MDM-2 is known to negatively regulate p53 by mediating its ubiquitination and subsequent degradation in the proteasome and leptin diminished p53 half-life in placental cells, we speculated that leptin might regulate MDM-2 expression, triggering the degradation of p53. Indeed, we demonstrated that leptin enhanced MDM-2 protein and mRNA expression determined by Western blot and *q*RT-PCR both in Swan-71 and placental explants.

Importantly, our findings suggest that leptin has biological activities greater than simply being a regulator of protein level since it modulates the steady-state mRNA levels of p53 [33] and MDM-2 in human trophoblasts. Whether these responses involve alterations in transcription rates, RNA stability, or both, remains a topic for further investigation.

In summary our data demonstrated that leptin protects human trophoblasts from serum deprivation-induced cell death. This protective effect associates with decreased expression of p53 and decrease phosphorylation of Ser-46 p53, mediated by MAPK and PI3K signaling pathways. Moreover, under serum deprivation, leptin decreased p53 half-life and increased MDM-2 levels suggesting a reinforcing regulatory loop.

Conflict of interest

The authors declare that no conflict of interest exists.

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