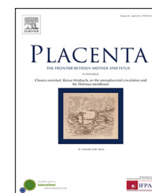
Placenta 42 (2016) 106–113

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta



Leptin reduces apoptosis triggered by high temperature in human placental villous explants: The role of the p53 pathway

Antonio Pérez-Pérez [a], Ayelén R. Toro [b], Teresa Vilarino-Garcia [d], Pilar Guadix [c], Julieta L. Maymó [b], José L. Dueñas [c], Cecilia L. Varone [b], Víctor Sánchez-Margalet [a, *]

[a] Department of Medical Biochemistry and Molecular Biology, Virgen Macarena University Hospital, University of Seville, Spain
[b] Department of Biological Chemistry, School of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina
[c] Department of Obstetrics and Gynecology, Virgen Macarena University Hospital, University of Seville, Spain
[d] Department of Medical Biochemistry and Molecular Biology, Virgen Macarena University Hospital, University of Seville, Spain

A R T I C L E I N F O

Article history:
Received 9 November 2015
Received in revised form
17 March 2016
Accepted 20 March 2016

Keywords:
Placenta
Leptin
Apoptosis
P53
Temperature
CK-18(M30)

A B S T R A C T

Maternal fever is common during pregnancy and has for many years been suspected to harm the developing fetus. Whether increased maternal temperature produces exaggerated apoptosis in trophoblast cells remains unclear. Since p53 is a critical regulator of apoptosis we hypothesized that increased temperature in placenta produces abnormal expression of proteins in the p53 pathway and finally caspase-3 activation. Moreover, leptin, produced by placenta, is known to promote the proliferation and survival of trophoblastic cells. Thus, we aimed to study the possible role of leptin preventing apoptosis triggered by high temperature, as well as the molecular mechanisms underlying this effect.

Fresh placental tissue was collected from normal pregnancies. Explants of placental villi were exposed to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin in DMEM-F12 medium. Western blotting and qRT-PCR was performed to analyze the expression of p53 and downstream effector, P53AIP1, Mdm2, p21, BAX and BCL-2 as well as the activated cleaved form of caspase-3 and the fragment of cytokeratin-18 (CK-18) cleaved at Asp396 (neoepitope M30).

Phosphorylation of the Ser 46 residue on p53, the expression of P53AIP1, Mdm2, p21, as well as caspase-3 and CK-18 were significantly increased in explants at 40 °C and 42 °C. Conversely, these effects were significantly attenuated by leptin 10 nM at both 40 °C and 42 °C. The BCL2/BAX ratio was also significantly decreased in explants at 40 °C and 42 °C compared with explants incubated at 37 °C, which was prevented by leptin stimulation.

These data illustrate the potential role of leptin for reducing apoptosis in trophoblast explants, including trophoblastic cells, triggered by high temperature, by preventing the activation of p53 signaling.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Maternal fever is common during pregnancy and has for many years been suspected to harm the developing fetus [1,2]. 1 in 5 women report having experienced fever on at least one occasion while being pregnan [1–5]. Therefore, given the high proportion of pregnant women who are exposed to fever, a small increase in the risk of these outcomes would make maternal fever a public health concern. In animal models it was studied, as a marker of maternal fever, the effect of raising body temperature during pregnancy and it has been reported that prenatal exposure to elevated body temperature leads to increased prevalence of adverse health outcomes in the offspring. It was evidenced that even a short exposure to elevated maternal body temperature is able to lead to cell death, membrane and vascular disruptions, as well as placental infarction. These disturbances may alter the integrity of the offspring [1,6] causing growth retardation, malformations and to longer-term outcomes behavioral alterations and impaired cognitive functioning [6,7]. In this sense, several mechanisms have been proposed through which fever interferes with fetal development. Particularly all of them include heat shock proteins expression [6] and

* Corresponding author. Department of Medical Biochemistry and Molecular Biology, School of Medicine, Virgen Macarena University Hospital, University of Seville, Spain Av. Dr. Fedriani 3, 41071 Seville, Spain.
E-mail address: margalet@us.es (V. Sánchez-Margalet).

http://dx.doi.org/10.1016/j.placenta.2016.03.009
0143-4004/© 2016 Elsevier Ltd. All rights reserved.

interruption of protein synthesis and enzyme production, which ultimately results in altered or dysfunctional cellular processes, such as apoptosis [6]. In this line, it has been reported that apoptosis could be determinant for normal placental development and its increase and early appearance may also be involved in the pathophysiology of pregnancy-related diseases [8,9].

Intriguingly, it has been reported that leptin, produced by placenta, as well as their receptors seem to play a possible role in the apoptotic process acting through an autocrine mechanism [10]. Physiological leptin effects in placenta include angiogenesis, growth and immunomodulation [11] as well as increase protein synthesis [12–14] and anti-apoptotic actions [12]. Furthermore placental leptin levels are increased under stressful condition, such as preeclampsia or intra-uterine growth restriction (IUGR) [15], where apoptosis is also boosted [16]. It has been suggested that this overproduction of leptin may be helpful to prevent the highly stress-mediated apoptosis of the trophoblastic cells. Although the role of leptin in preventing the apoptotic process triggered by the deprivation of serum in trophoblastic JEG-3 and Swan-71 cells is well established [12,17], the role during cellular responses to high temperature is still unknown. For this reason we aimed to investigate the effect of leptin in the apoptosis triggered by elevated temperature (40 °C and 42 °C) in human placental explants [18,19]. More specifically, we investigated the leptin effect on apoptosis by studying the protein cleavage of caspase-3 as well as the p53 expression, the master key regulator of death signaling, and several of its downstream proteins such as Mdm-2, p21, Bax and Bcl-2.

2. Materials and methods

2.1. Placental explants collection and processing

Term placentas from uncomplicated pregnancies ($n = 10$) were obtained after cesarean section delivery following normal term pregnancies in the Virgen Macarena University Hospital. None of the patients had previous history of diabetes mellitus or any known endocrinopathy. Subject characteristics were mean maternal age at delivery (26.0 years ± 7.0), mean infant birth weight (3095 g ± 69), mean placenta weight (525 g ± 81). Subject characteristics were similar with regards to gestational age (39.5 weeks ± 0.7).

Human placentas were obtained after cesarean section and immediately suspended in ice-cold PBS and transported to the laboratory, where they were washed two to three 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- to 15-mg wet weight) and thoroughly rinsed with cold DMEN-F12 medium pH 7.4 (137 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, and 4 mM NaHCO3). None of the donor patients suffered from anomalous pregnancy. This study was approved by the local ethical committee (Comité Local de Etica en Investigación del Hospital Universitario Virgen Macarena), and the patients' written consent was obtained. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

2.2. Treatments of placental explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium 0% FBS ($n = 1$ explant/tube, three replicates per treatment). Placental explants were maintained in a shaking water bath at 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin (Sigma Chemical Co.). Explants were removed from the bath, centrifuged for 2 min at 2000 × g at 4 °C, and resuspended in 500 µl of lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 50 mM NaF, 10 mM pyrophosphate and protease inhibitor cocktail) 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 analysis.

2.3. Western blot analysis

Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10 000 × g for 10 min to remove cellular debris. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard. 50 µg protein were loaded in each lane. Lysates were mixed with Laemmli's sample buffer containing 2% sodium dodecyl sulfate 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 Biotech, Piscataway, NJ) thereafter. Membranes were equilibrated in 1 × PBS, and nonspecific binding sites were blocked by 5% nonfat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal rabbit anti-Caspase-3 (8G10) (1:1000, Cell Signaling; #9665), monoclonal mouse anti-cytokeratin-18 neoepitope M30 (1:1500, Peviva prod.10700), monoclonal mouse anti-p53 (1:1000, Santa Cruz; sc-126), polyclonal rabbit anti phospho Ser46 p53 (1:1000, Cell Signaling; #2521), polyclonal goat anti-p53AIP1 (1:3000, Santa Cruz; sc-14095), polyclonal rabbit anti-p21 (1:1000, Santa Cruz; sc-756), polyclonal rabbit anti-Mdm-2 (1:1000, Santa Cruz; sc-965), polyclonal rabbit anti-Bax (1:1000, Santa Cruz; sc-493), or polyclonal rabbit anti-BCL-2 (1:1000, Epitomics; #1017-1).

Membranes were stripped and loading controls were performed by immunoblotting the same membranes with monoclonal mouse anti-β tubulin (1:2500, Santa Cruz; sc-5274), except for the p53 and phospho Ser46 immunoblots, whose loading control was determined loading the same amount of samples in a different gel. The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (1:12,000, Amersham; NA934/NA931), anti-goat immunoglobulin (1:15,000, life technologies; QJ226103) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (ScienceLab, Fuji Photo Film Co., Ltd.).

2.4. Quantitative real-time RT-PCR assay

Abundance of p53 mRNA was determined by quantitative real time RT-PCR reaction (qRT-PCR). Total RNA was extracted from placental explants using TRISURE reagent, according to the manufacture'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: p53, forward, 5′GGAAGAGAATCTCCGCAA3′; reverse, 5′AGCTCTCGGA ACATCTCGAAG3′; cyclophilin, forward, 5′CTTCCCCGATACTTCA3′; reverse, 5′TCTTGGTGCTACCTC 3′; p53AIP1, forward, 5′GGGGACTTC TCAGGTCGTGT3′; reverse, 5′TGGACTTCTTCATGCCCCGA3′; p21, forward, 5′GATGGCACCAGAGGTGGTTA3′; reverse, 5′TCCCGAAA TATTGGGGAAAG3′; Mdm-2, forward, 5′TTACCCAGGCTG

GAGTGCAG3′; reverse, 5′GAGAATGGTGCGAACCCG3′

qRT-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 [20]. 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. Melting curves in the qPCR experiments showed a single PCR product (p53 or p53AIP1 at a different temperature than the reference gene (supplementary data).

2.5. Data analysis

In placental explants, immunoblot are a representative experiment from the 10 placentas studied. Results are expressed as the mean ± SD. The statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison post hoc test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Leptin diminishes apoptotic effect of high temperature in human placental explants

We have previously reported that leptin has a trophic effect (promoting cellular growth and survival) in trophoblastic JEG-3 and BeWo cells, preventing the apoptosis promoted by serum deprivation [12,17]. Now, we have further analyzed the anti-apoptotic effect of leptin on the trophoblast inducing apoptosis by high temperature. We incubated placental explants at 37 °C, 40 °C and 42 °C, in the presence or absence of leptin (10 nM) to study the possible role of leptin preventing the high-temperature induced apoptosis in the trophoblast. 10 nM leptin is the leptin concentration with maximal effects in trophoblast as previously reported [13]. Time-response experiments (30 min, 1 h, 2 h, 2.5 h, 3 h, 4 h, 5 h and 6 h incubation) at high temperature yielded 3 h incubation as optimal to study the effect on apoptosis (data not shown). Apoptosis was investigated by determination of Caspase-3 activated form by Western blot. As shown in Fig. 1A, placental explants incubated at 40 °C and 42 °C increased Caspase-3 activated form compared with placental explants incubated at 37 °C. Moreover, treatment with leptin 10 nM significantly reduced Caspase-3 activation in placental explants similarly to previously reported leptin effects preventing the apoptosis promoted by serum deprivation [12].

In order to investigate whether the increased apoptosis and leptin effects are, indeed, occurring in trophoblasts cells, we also tested one of neoepitopes of Cytokeratin-18 (CK-18) (the cleavage site on CK-18 of caspases), identified by a monoclonal antibody (M30), which has been proposed as an specific apoptosis marker of trophoblast cells in human placenta [21]. As shown in Fig. 1B, placental explants incubated at 40 °C and 42 °C increased the 21 KDa fragment of CK-18 activated form compared with placental explants incubated at 37 °C. Moreover, treatment with leptin 10 nM significantly reduced CK-18 activation in placental explants similarly to the results obtained from caspase 3 experiments.

3.2. Leptin impairs the increase in p53 expression and downstream effectors produced by high temperature in explants of placental villi

To further explore the effect of temperature-induced apoptosis, we next focused on the expression of p53, a pivotal regulatory protein in the apoptotic pathway, which accumulates in cells in response to DNA damage, oncogene activation and other stressful stimuli [22]. Moreover, we also analyzed the phosphorylation of p53 at Ser-46, which has been shown to be involved in the regulation of apoptosis by transactivating apoptosis genes such as p53AIP1 [23], and the expression of Mdm-2 (a negative feedback regulator of p53) and p21 (a marker of p53 activity) [17,24].

As it is shown in Fig. 2A, the high temperature (40 °C and 42 °C) did not significantly increased the p53 protein amount. However p53 mRNA levels were increased by high temperature incubation (Fig. 2B). Moreover an increased p53 phosphorylation at Ser-46 (Fig. 2A), as well as, P53AIP1 (Fig. 3), Mdm-2 (Fig. 4) and p21 (Fig. 5) expression were observed. Treatment with leptin 10 nM significantly prevented the increase in p53 expression at mRNA levels, as well as the p53 phosphorylation at Ser-46 (Fig. 2A). Moreover, leptin significantly prevented the increase of P53AIP1 (Fig. 3), Mdm-2 (Fig. 4) and p21 (Fig. 5) expression promoted by both 40 °C and 42 °C incubation. These results suggest a strong anti-apoptotic response of leptin to high temperature in human placental explants.

3.3. Leptin enhances Bcl-2/Bax relationship in placental explants incubated at 40 °C and 42 °C

p53 also promotes Bax, a pro-apoptotic mitochondrial pore protein [25]. That is why, we next investigated the effect of high temperature, 40 °C and 42 °C in the Bax expression as well as Bcl-2 expression, an anti-apoptotic member of the Bcl-2 family which function as major regulator of the intrinsic apoptotic pathway [26]. Similarly, placental explants were incubated with or without leptin 10 nM to further characterize anti-apoptotic effect of leptin in placental explants.

As showed in Fig. 6B, high temperature decreased the Bcl-2/Bax ratio, both at 40 °C and 42 °C. Leptin treatment at 10 nM significantly increased Bcl-2/Bax ratio, consistent with the antiapoptotic effect of leptin.

4. Discussion

Normal placentation and placental development are critical for a successful pregnancy and mediate important steps necessary for fetal development [27]. However, foetus may be especially vulnerable to chemical and physical insults during defined stages of development. In particular, it has been reported that exposure to maternal fever during pregnancy can cause abortion, growth retardation and developmental defects [28]. It is known that placenta locally releases a broad spectrum of hormones and growth factors that play key roles in compensatory changes to its pathophysiological alterations. Regulators of apoptosis are now considered to have a major role in maintaining the integrity of villous trophoblast, so the study of the molecular mechanisms that regulate placental cell death is important for understanding normal development and a variety of diseases of the placenta. In this regard, leptin has been described as an important cytokine regulating trophoblast survival, promoting growth and preventing the apoptotic process in serum deprived trophoblastic cells [29]. This leptin anti-apoptotic effect seems to be mediated by p53 pathway [17]. In this work, we aimed to study the leptin effect in preventing the apoptotic process triggered by high temperature (40 °C-42 °C), as a marker of maternal fever, in human placental explants. They

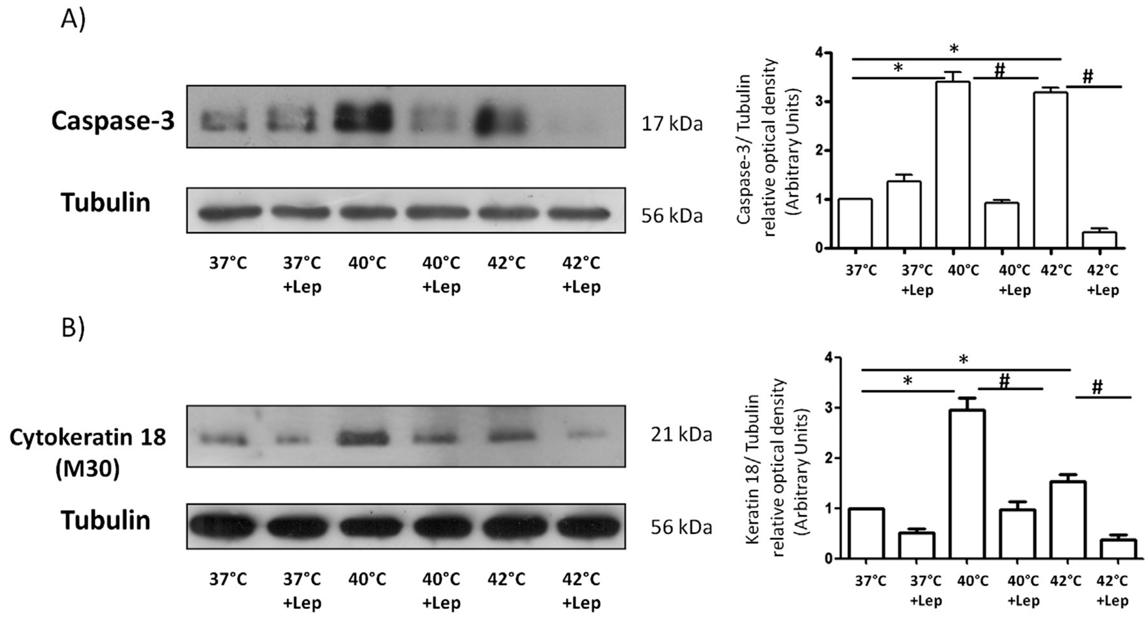


Fig. 1. Leptin diminishes apoptosis in human placental explants incubated at 40 °C and 42 °C. Caspase and CK18 cleavage were determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin. Caspase-3 cleaved fragment (p17 and p12) and the 21 KDa fragment of Cytokeratin-18 (CK18Asp396) was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. 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 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.001, *p < 0.001.

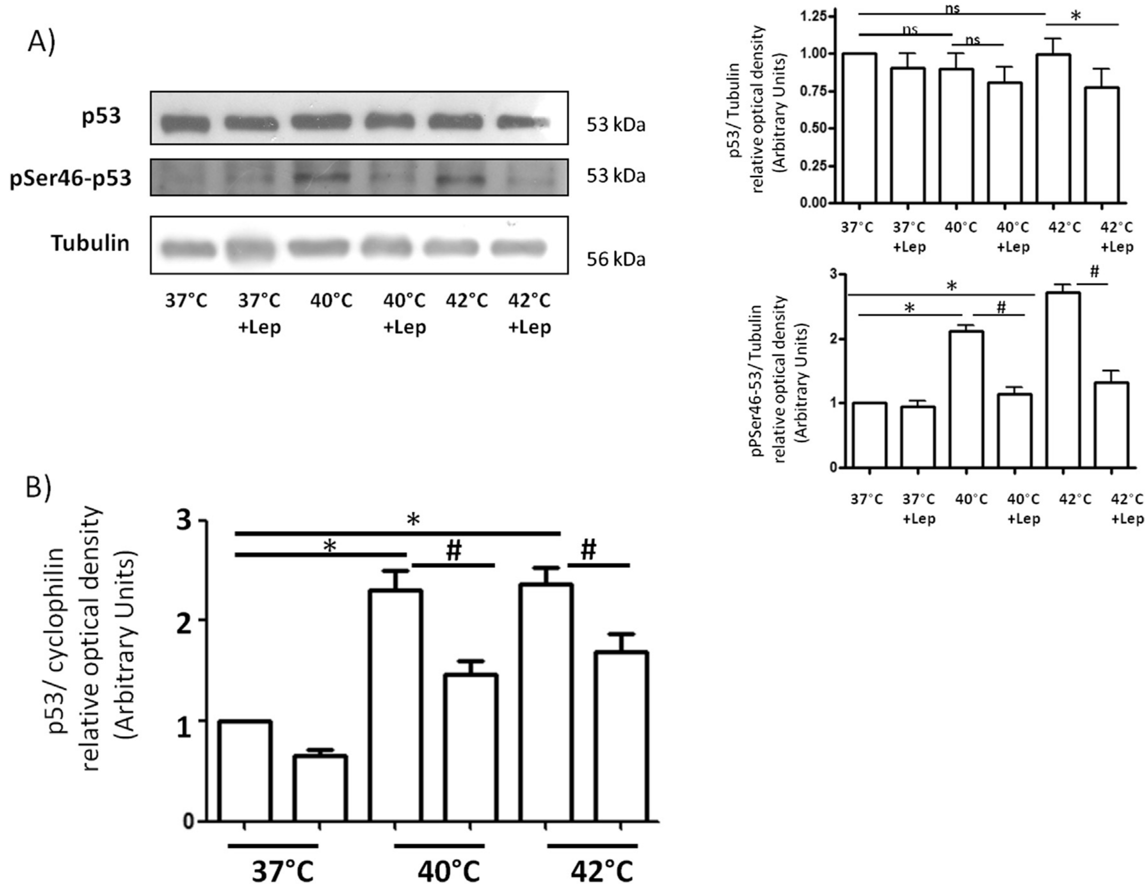


Fig. 2. Leptin diminishes p53 expression as well as Ser-46 p53 phosphorylation in human placental explants incubated at 40 °C and 42 °C. A) Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin. P53 as well as phospho Ser-46 p53 were determined by Western blot analysis. Placental explants to 37 °C were used as a control. Loading controls were performed by immunoblotting with anti-tubulin antibodies. Results are expressed as mean ± SD for three independent experiments. B) p53 expression in human placental explants was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.01, *p < 0.01.

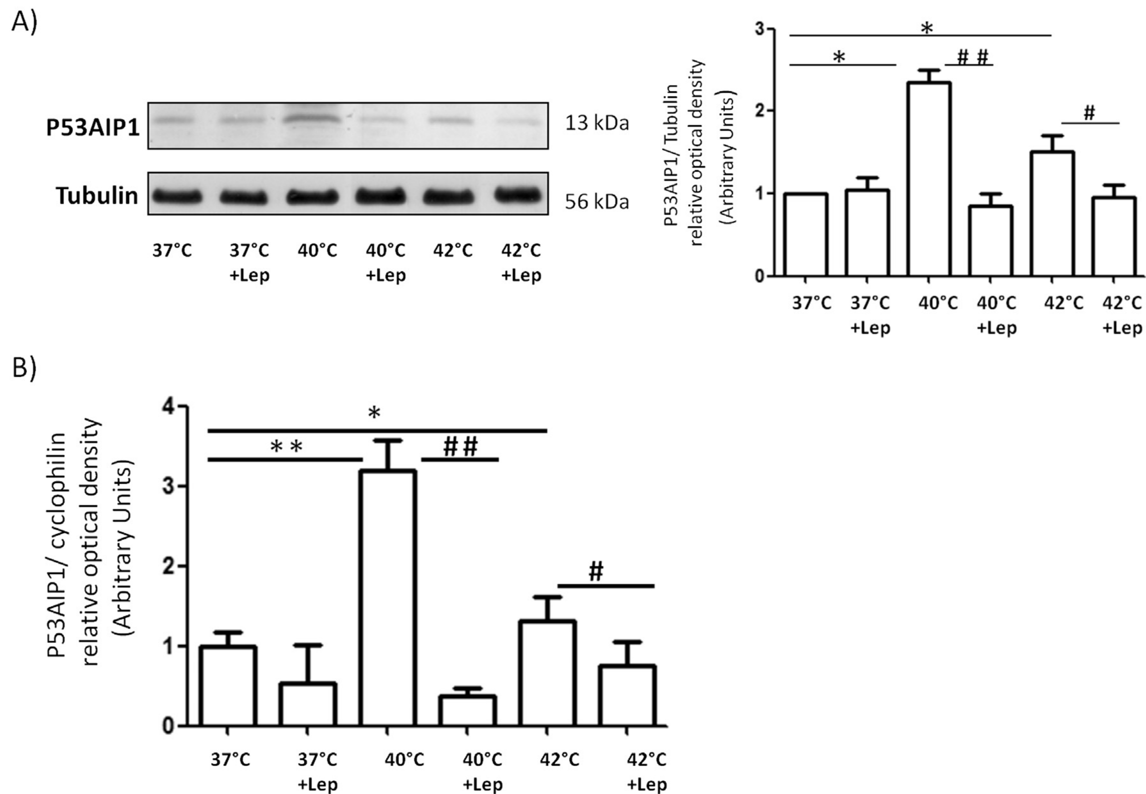


Fig. 3. Leptin reduces P53AIP1 expression in placental explants incubated at 40 °C and 42 °C. P53AIP1 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 3 h. A) P53AIP1 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. 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 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.001, *p < 0.001. B) p53AIP1 expression in human placental explants was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

that represent a valuable model to confirm the physiological relevance of leptin in trophoblastic survival. We showed that high temperature induced apoptosis by activating caspase-3, as previously reported in the apoptotic process triggered by serum deprivation [12]. We also confirmed that result studying the appearance M30 cytokeratin 18 fragment that is only revealed after caspase cleavage of the protein in human placental trophoblast cells [21]. These findings suggest that leptin may be protective to the deleterious effects of maternal fever during pregnancy preventing the high temperature-mediated apoptosis of the trophoblastic cells. This effect may be of physiological relevance since trophoblastic cells are an important source of leptin production during pregnancy [30,31] and even more leptin is produced under stressful condition [30,32].

Trophoblastic apoptosis, as other types of cell apoptosis, includes the extrinsic and intrinsic pathways culminating in the activation of caspases. It has been reported that p53, a key component of cellular mechanisms that are activated by cellular stress, is involved in spontaneous abortion [33]. However, it remains incompletely understood how p53 is stabilized in placenta and in response to different stress signals. For that reason in this work we investigated whether this key cell cycle-signaling protein was involved in the apoptosis mediated by high temperature in human placental explants. We found a significant increase in p53 expression and phosphorylation in placental explants under high temperature conditions, but p53 protein level did not change accordingly. This may be due to increased degradation of p53 that may counterbalance the increased expression. Moreover, a decrease in p53 mRNA level and p53 phosphorylation was observed in response to 10 nM leptin, demonstrating that leptin regulates p53 pathway under high temperature conditions in human placental explants.

Under normal conditions, p53 is a short-lived protein that is highly regulated and maintained at low or undetectable levels [34,35]. After stress, such as serum deprivation [17], p53 is activated mostly at the post-translational level by a complex series of modifications that include the phosphorylation of specific residues. It was reported that p53 is serine-phosphorylated after DNA damage and other types of stress [36–38]. Therefore, it might be speculated that activation (phosphorylation) of p53 by high temperature promotes apoptosis and placental leptin may play an important role controlling this process. In this sense, we also demonstrated an increased serine-phosphorylation of p53 triggered by high temperature, as well as a significant reduced serine-phosphorylation of p53 in response to 10 nM leptin. In addition, our results suggest that phosphorylation of Ser-46 regulates the transcriptional activation of the p53AIP1 (apoptosis-inducing gene) as previously was demonstrated by Oda et al [39].

It is known that Mdm-2 is induced by activated p53, which is negatively regulated, at least in part by mediating its ubiquitination and subsequent degradation in the proteasome [34,40]. This mechanism may explain the lack of differences in p53 protein level in spite of the changes in p53 gene expression. Although little is known about leptin effect on Mdm2 levels in placenta, we demonstrated an increased Mdm-2 expression in human placental explants incubated at high temperature. In addition, leptin 10 nM

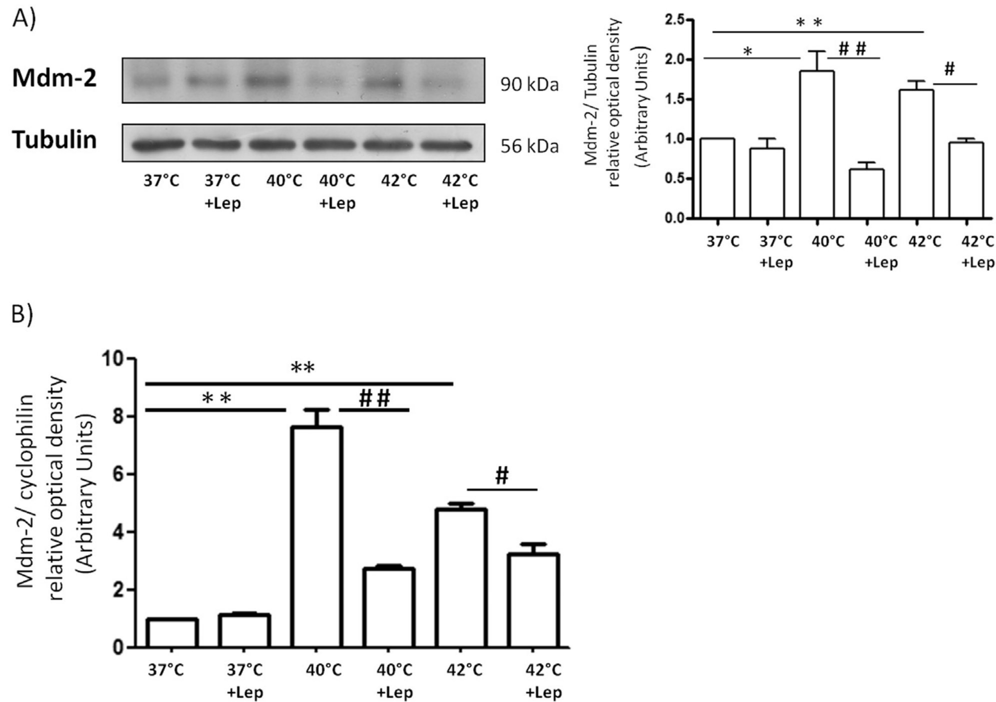


Fig. 4. Leptin diminishes Mdm-2 expression in human placental explants incubated at 40 °C and 42 °C. Mdm-2 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 3 h. A) Mdm-2 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. 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 40 °C and 42 °C without leptin (#) or 37 °C (*).#p < 0.01, ##p < 0.001, *p < 0.01, **p < 0.001. B) Mdm-2 expression in human placental explants was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

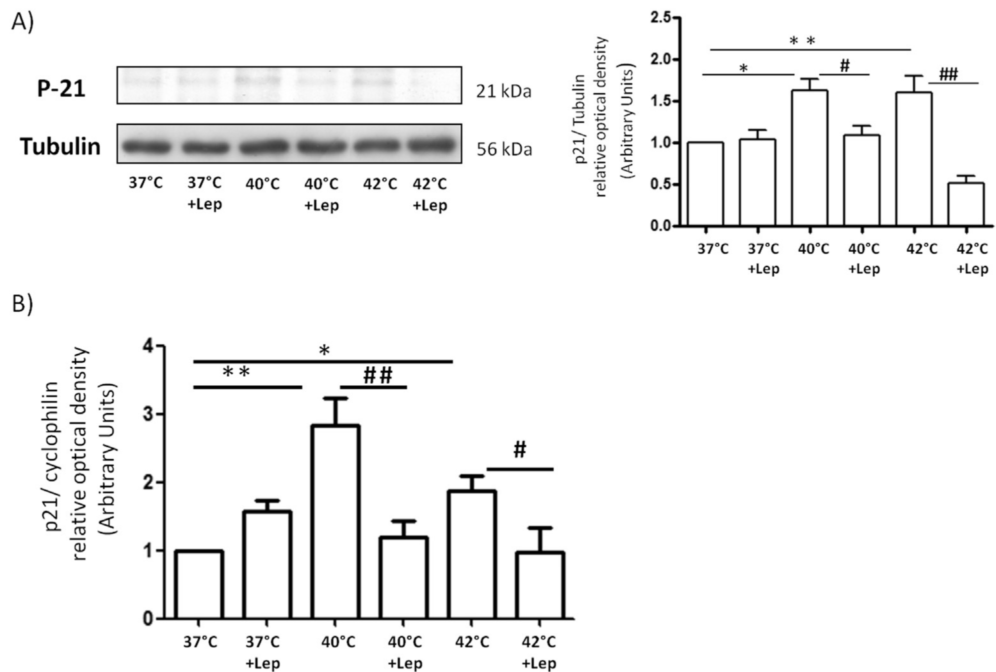


Fig. 5. Leptin diminishes p21 expression in human placental explants incubated at 40 °C and 42 °C. P21 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 3 h. A) p21 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. 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 40 °C and 42 °C without leptin (#) or 37 °C (*).#p < 0.01, ##p < 0.001, *p < 0.01, **p < 0.001. B) p21 expression in human placental explants was determined by *q*RT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

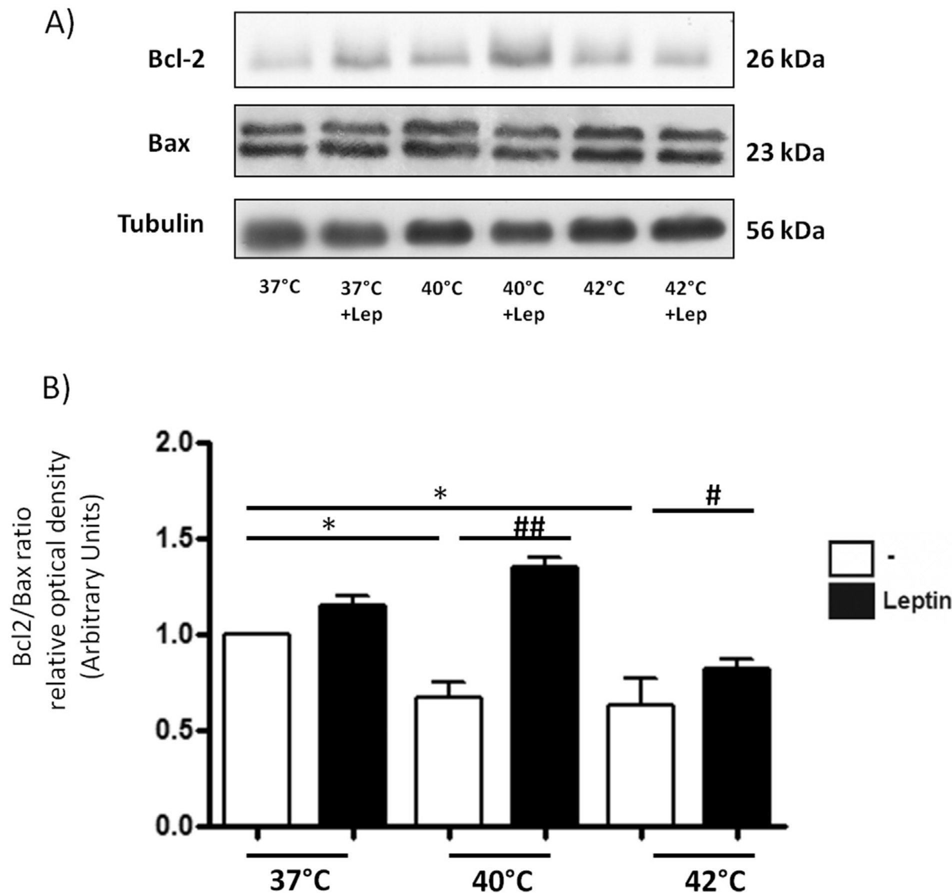


Fig. 6. Leptin enhances BCl-2/Bax ratio in human placental explants incubated at 40 °C and 42 °C. Placental explants were processed as described in Materials and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 3 h (black box). Placental extracts were prepared and proteins were separated on SDS-PAGE gels. A) BCL-2 and BAX expression were determined by Western blot analysis as indicated in the Figure. 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-tubulin. B) Bcl-2/Bax Ratio. Leptin increased BCL-2/BAX ratio. 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 40 °C and 42 °C without leptin (#) or 37 °C (*). #$p < 0.01$, ##$p < 0.05$, *$p < 0.01$.

not only decreases p53 expression but also Mdm-2 expression, suggesting that the leptin effect on Mdm-2 may be mediated by p53 phosphorylation level.

The p21 gene is induced by p53 in almost all cell types by a variety of stress agents, suggesting that multiple pathways can promote its activation [36,45]. Here we have found an increase in p21 expression in human placental explants incubated at high temperature as well as a reduced p21 expression in response to leptin 10 nM. Thus, our data demonstrate that maternal fever could be an important cause for p53-mediated activation of p21 and subsequent placental growth arrest, which may be counterbalanced by leptin.

Finally, it is well known that BCL-2-family proteins are central regulators of cell life and death. The first pro-apoptotic member of the family, BAX (BCL-2 Antagonist X) was identified as a BCL-2-interacting protein that opposed BCL-2 (anti-apoptotic protein) and promoted apoptotic cell death [41]. Moreover, p53 also interacts with BCL-2 at the outer mitochondrial membrane to promote the oligomerization of Bax. This in turn drive the formation of pores in the mitochondrial membrane, resulting in the release of cytochrome c and other apoptotic activators from the mitochondria [42,43]. Moreover, mitochondria function is the main operations center in which the intrinsic apoptotic pathway [44]. We observed a reduced BCL-2/BAX ratio in human placental explants incubated at high temperature, which was increased in response to 10 nM leptin. The modulation of this ratio by leptin was due principally to an increase in BCL-2 protein. Therefore, in this study we have determined an activated intrinsic apoptotic pathway when placental explants were incubated at high temperature, which may be prevented by leptin incubation. Whether extrinsic pathway is severely impaired in placental explants incubated at high temperature would require further investigation.

Taken together, our findings provide evidence for an inhibitory leptin effect on the cell apoptosis program triggered by high temperature, suggesting a trophic role of leptin in the physiology of placenta. In addition, we have provided some evidence for the possible anti-apoptotic mechanisms exerted by leptin in placenta. However, further additional studies are needed to fully explain the effect of leptin on the regulation of p53 expression and BCL-2-family proteins in response to hyperthermia. This temperature-induced apoptosis may provide a model to further understand certain placental pathologies associated with fever, and may unravel new therapeutic targets. Finally, leptin seems to reduce the high temperature promoted apoptosis in trophoblast cells from human placental villi, by preventing p53 signaling.

Funding

The work funded by the Spanish Grant from ISCIII (PI09/00119 and PI12/01172), co-funded in part by FEDER.

Conflict of interest

Authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2016.03.009.

References

[1] M.J. Edwards, Review: Hyperthermia and fever during pregnancy, Birth Defects Res. A Clin. Mol. Teratol. 76 (2006) 507–516.
[2] J.M. Graham Jr., M.J. Edwards, M.J. Edwards, Teratogen update: gestational effects of maternal hyperthermia due to febrile illnesses and resultant patterns of defects in humans, Teratology 58 (1998) 209–221.
[3] A.N. Andersen, V. Goossens, L. Gianaroli, R. Felberbaum, M.J. de, K.G. Nygren, Assisted Reproductive Technology in Europe, Results generated from European registers by ESHRE, Hum. Reprod. 2007 (22) (2003) 1513–1525.
[4] S.A. Collier, S.A. Rasmussen, M.L. Feldkamp, M.A. Honein, Prevalence of self-reported infection during pregnancy among control mothers in the National Birth Defects Prevention Study, Birth Defects Res. A Clin. Mol. Teratol. 85 (2009) 193–201.
[5] N.H. Morken, N. Gunnes, P. Magnus, B. Jacobsson, Risk of spontaneous preterm delivery in a low-risk population: the impact of maternal febrile episodes, urinary tract infection, pneumonia and ear-nose-throat infections, Eur. J. Obstet. Gynecol. Reprod. Biol. 159 (2011) 310–314.
[6] M.J. Edwards, R.D. Saunders, K. Shiota, Effects of heat on embryos and foetuses, Int. J. Hyperth. 19 (2003) 295–324.
[7] M.C. Ziskin, J. Morrissey, Thermal thresholds for teratogenicity, reproduction, and development, Int. J. Hyperth. 27 (2011) 374–387.
[8] B. Huppertz, D. Hemmings, S.J. Renaud, J.N. Bulmer, P. Dash, L.W. Chamley, Extravillous trophoblast apoptosis—a workshop report, Placenta 26 (Suppl A) (2005) S46–S48. S46–8.
[9] A.N. Sharp, A.E. Heazell, I.P. Crocker, G. Mor, Placental apoptosis in health and disease, Am. J. Reprod. Immunol. 64 (2010) 159–169.
[10] B. Toth, M. Bastug, C. Scholz, P. Arck, S. Schulze, S. Kunze, K. Friese, U. Jeschke, Leptin and peroxisome proliferator-activated receptors: impact on normal and disturbed first trimester human pregnancy, Histol. Histopathol. 23 (2008) 1465–1475.
[11] P. Fernandez-Riejos, S. Najib, J. Santos-Alvarez, C. Martin-Romero, A. Perez-Perez, C. Gonzalez-Yanes, V. Sanchez-Margalet, Role of leptin in the activation of immune cells, Mediat. Inflamm. 2010 (2010) 568343, http://dx.doi.org/10.1155/2010/568343. Epub;%2010 Mar 23.: 568343.
[12] A. Perez-Perez, J. Maymo, J.L. Duenas, R. Goberna, J.C. Calvo, C. Varone, V. Sanchez-Margalet, Leptin prevents apoptosis of trophoblastic cells by activation of MAPK pathway, Arch. Biochem. Biophys. 477 (2008) 390–395.
[13] A. Perez-Perez, J. Maymo, Y. Gambino, J.L. Duenas, R. Goberna, C. Varone, V. Sanchez-Margalet, Leptin stimulates protein synthesis-activating translation machinery in human trophoblastic cells, Biol. Reprod. 81 (2009) 826–832.
[14] A. Perez-Perez, Y. Gambino, J. Maymo, R. Goberna, F. Fabiani, C. Varone, V. Sanchez-Margalet, MAPK and PI3K activities are required for leptin stimulation of protein synthesis in human trophoblastic cells, Biochem. Biophys. Res. Commun. 396 (2010) 956–960.
[15] A. Tzschoppe, E. Struwe, W. Rascher, H.G. Dorr, R.L. Schild, T.W. Goecke, M.W. Beckmann, B. Hofner, J. Kratzsch, J. Dotsch, Intrauterine growth restriction (IUGR) is associated with increased leptin synthesis and binding capability in neonates, Clin. Endocrinol. (Oxf) 74 (2011) 459–466.
[16] A.E. Heazell, A.N. Sharp, P.N. Baker, I.P. Crocker, Intra-uterine growth restriction is associated with increased apoptosis and altered expression of proteins in the p53 pathway in villous trophoblast, Apoptosis 16 (2011) 135–144.
[17] A.R. Toro, J.L. Maymo, F.M. Ibarbalz, A. Perez-Perez, B. Maskin, A.G. Faletti, V. Sanchez-Margalet, C.L. Varone, Leptin is an anti-apoptotic effector in placental cells involving p53 downregulation, PLoS One 9 (2014) e99187.
[18] H.Y. Chang, X. Yang, Proteases for cell suicide: functions and regulation of caspases, Microbiol. Mol. Biol. Rev. 64 (2000) 821–846.
[19] D.W. Nicholson, Caspase structure, proteolytic substrates, and function during apoptotic cell death, Cell Death Differ. 6 (1999) 1028–1042.
[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[21] M. Kadyrov, P. Kaufmann, B. Huppertz, Expression of a cytokeratin 18 neo-epitope is a specific marker for trophoblast apoptosis in human placenta, Placenta 22 (2001) 44–48.
[22] D.R. Green, G. Kroemer, Cytoplasmic functions of the tumour suppressor p53, Nature 458 (2009) 1127–1130.
[23] X. Li, P. Dumont, P.A. Della, C. Shetler, M.E. Murphy, The codon 47 polymorphism in p53 is functionally significant, J. Biol. Chem. 280 (2005) 24245–24251.
[24] A.R. Toro, A. Perez-Perez, G.I. Corrales, V. Sanchez-Margalet, C.L. Varone, Mechanisms involved in p53 downregulation by leptin in trophoblastic cells, Placenta 36 (2015) 1266–1275.
[25] T. Miyashita, J.C. Reed, Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, Cell 80 (1995) 293–299.
[26] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.
[27] J.A. Arroyo, V.D. Winn, Vasculogenesis and angiogenesis in the IUGR placenta, Semin. Perinatol. 32 (2008) 172–177.
[28] R. Levy, D.M. Nelson, To be, or not to be, that is the question. Apoptosis in human trophoblast, Placenta 21 (2000) 1–13.
[29] M.P. Magarinos, V. Sanchez-Margalet, M. Kotler, J.C. Calvo, C.L. Varone, Leptin promotes cell proliferation and survival of trophoblastic cells, Biol. Reprod. 76 (2007) 203–210.
[30] R. Bajoria, S.R. Sooranna, B.S. Ward, R. Chatterjee, Prospective function of placental leptin at maternal-fetal interface, 23 ed, 2002, pp. 103–115.
[31] H. Masuzaki, Y. Ogawa, N. Sagawa, K. Hosoda, T. Matsumoto, H. Mise, H. Nishimura, Y. Yoshimasa, I. Tanaka, T. Mori, K. Nakao, Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans, Nat. Med. 3 (1997) 1029–1033.
[32] S. Iwagaki, Y. Yokoyama, L. Tang, Y. Takahashi, Y. Nakagawa, T. Tamaya, Augmentation of leptin and hypoxia-inducible factor 1alpha mRNAs in the pre-eclamptic placenta, Gynecol. Endocrinol. 18 (2004) 263–268.
[33] D. Pietrowski, H. Bettendorf, E.K. Riener, C. Keck, L.A. Hefler, J.C. Huber, C. Tempfer, Recurrent pregnancy failure is associated with a polymorphism in the p53 tumour suppressor gene, Hum. Reprod. 20 (2005) 848–851.
[34] C.L. Brooks, W. Gu, Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation, Curr. Opin. Cell Biol. 15 (2003) 164–171.
[35] O. Laptenko, C. Prives, Transcriptional regulation by p53: one protein, many possibilities, Cell Death Differ. 13 (2006) 951–961.
[36] E. Appella, C.W. Anderson, Post-translational modifications and activation of p53 by genotoxic stresses, Eur. J. Biochem. 268 (2001) 2764–2772.
[37] S.Y. Shieh, M. Ikeda, Y. Taya, C. Prives, DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2, Cell 91 (1997) 325–334.
[38] S.Y. Shieh, J. Ahn, K. Tamai, Y. Taya, C. Prives, The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites, Genes Dev. 14 (2000) 289–300.
[39] K. Oda, H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura, Y. Taya, p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53, Cell 102 (2000) 849–862.
[40] C.L. Brooks, W. Gu, p53 ubiquitination: Mdm2 and beyond, Mol. Cell 21 (2006) 307–315.
[41] Z.N. Oltvai, C.L. Milliman, S.J. Korsmeyer, Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death, Cell 74 (1993) 609–619.
[42] J.E. Chipuk, T. Kuwana, L. Bouchier-Hayes, N.M. Droin, D.D. Newmeyer, M. Schuler, D.R. Green, Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis, Science 303 (2004) 1010–1014.
[43] Y. Tomita, N. Marchenko, S. Erster, A. Nemajerova, A. Dehner, C. Klein, H. Pan, H. Kessler, P. Pancoska, U.M. Moll, WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization, J. Biol. Chem. 281 (2006) 8600–8606.
[44] K.F. Ferri, G. Kroemer, Organelle-specific initiation of cell death pathways, Nat. Cell Biol. 3 (2001) E255–E263.