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Flora Sánchez-Jiménez, Antonio Pérez-Pérez, Carmen González-Yanes, Souad Najib, Cecilia L. Varone, et al.. Leptin receptor activation increases Sam68 tyrosine phosphorylation and expression in human trophoblastic cells. *Molecular and Cellular Endocrinology*, 2010, 332 (1-2), pp.221. 10.1016/j.mce.2010.10.014 . hal-00654963

HAL Id: hal-00654963

<https://hal.science/hal-00654963>

Submitted on 24 Dec 2011

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Accepted Manuscript

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PII: S0303-7207(10)00502-2
DOI: doi:10.1016/j.mce.2010.10.014
Reference: MCE 7662

To appear in: *Molecular and Cellular Endocrinology*

Received date: 20-7-2010
Revised date: 27-9-2010
Accepted date: 19-10-2010

Please cite this article as: Sánchez-Jiménez, F., Pérez-Pérez, A., González-Yanes, C., Najib, S., Varone, C.L., Sánchez-Margalet, V., Leptin receptor activation increases Sam68 tyrosine phosphorylation and expression in human trophoblastic cells, *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.10.014

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Leptin receptor activation increases Sam68 tyrosine phosphorylation and expression in human trophoblastic cells

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ABSTRACT

Leptin is produced in placenta where it has been found to be an important autocrine signal for trophoblastic growth during pregnancy, promoting antiapoptotic and trophic effects. Leptin receptor is present in trophoblastic cells and leptin may fully activate signaling. We have previously implicated the RNA-binding protein Sam68 in leptin signal transduction in immune cells. In the present work we have studied the possible role of Sam68 in leptin receptor signaling in trophoblastic cells (JEG-3 cells). Leptin dose-dependently stimulated Sam68 phosphorylation in JEG-3 cells, as assessed by immunoprecipitation and immunoblot with anti-phosphotyrosine antibodies. As previously observed in other systems, tyrosine phosphorylation of Sam68 in response to leptin inhibits its RNA binding capacity. Besides, leptin stimulation dose-dependently increases Sam68 expression in JEG-3 cells, as assessed by quantitative PCR. Consistently, the amount of Sam68 protein is increased after 24 h of leptin stimulation of trophoblastic cells. In order to study the possible role of Sam68 on leptin receptor synthesis, we employed antisense strategy to knockdown the expression of Sam68. We have found that a decrease in Sam68 expression leads to a decrease in leptin receptor amount in JEG-3 cells, as assessed both by quantitative PCR and immunoblot. These results strongly suggest the participation of Sam68 in leptin receptor signaling in human trophoblastic cells, and therefore, Sam68 may mediate some of the leptin effects in placenta.

INTRODUCTION

Leptin hormone, the protein product of the *LEP* gene (Zhang et al 1994), is a multifunction hormone with a tertiary structure resembling that of members of the long-chain helical cytokine family (Flier 1995). The pleiotropic nature of leptin is supported by the universal distribution of leptin receptor (LEPR), which shows structural similarity to the class I cytokine receptor family (Lee et al 1996, Lollmann et al 1997, Myers 2004, Tartaglia et al 1995, Tartaglia 1997). Several alternatively spliced isoforms of leptin receptor have been identified, differing in the lengths of their cytoplasmic regions (Lee et al 1996, Myers 2004). Short isoforms (LEPR_S) are distributed in almost all peripheral tissues and show distinct signaling capabilities that include the activation of mitogen-activated protein kinase (MAPK) pathway (Yamashita et al 1998, Bjorbaek et al 1997). A long form (LEPR_L), which predominates in the hypothalamus, exhibits consensus aminoacid sequences to bind Janus tyrosine kinases (JAK/STAT) promoting the complete activation of this isoform (Ghilardi and Skoda 1997).

Similar to other receptors of the family, the JAK (Janus kinases)/STAT (signal transducers and activators of transcription) pathway is one of the main signaling cascades activated by leptin in LEPR_L (Ahima and Osei 2004, Sahu 2003, Sweeney 2002). In this context, we have found constitutive association of JAK2 and JAK3 with LEPR_L in human peripheral blood mononuclear cells, with the subsequent activation by tyrosine phosphorylation of STAT3 in both human peripheral blood mononuclear cells and trophoblastic cells (Sanchez-Margalet and Martin-Romero 2001, Perez-Perez et al 2008). Different pathways in addition to STATs are known to be involved in LEPR signaling. Thus, the mitogen-activated protein kinase (MAPK) family and the phosphatidylinositol 3-kinase (PI3K) signaling cascade become also activated by leptin,

as we have previously found in peripheral blood mononuclear cells (Santos-Alvarez et al 1999, Najib and Sanchez-Margalet 2002a) and trophoblastic cells (Perez-Perez et al 2008, Perez-Perez et al 2009, Perez-Perez et al 2010).

Leptin has been found to play a role in reproduction and pregnancy (Sagawa et al 2002), particularly in placenta, where it may act as an autocrine hormone (Hauguel-de Mouzon et al 2006). Expression of leptin and the different isoforms of leptin receptor are found in placenta (Li et al 2004, Smith and Waddell 2002). We have previously demonstrated that both human trophoblastic explants and human trophoblastic JEG-3 cells express the long isoform of leptin receptor, mediating the activation of signaling (Perez-Perez et al 2008). Possible physiological effects of placenta-derived leptin include angiogenesis, growth, and immune modulation. In addition, leptin is a trophic and mitogenic factor for trophoblastic cells by virtue of inhibiting apoptosis and promoting growth and proliferation (Santos-Alvarez et al 1999) via MAPK activation (Perez-Perez et al 2008, Najib and Sanchez-Margalet 2002a, Perez-Perez et al 2009, Perez-Perez et al 2010). In fact, we have reported that human chorionic gonadotropin (Maymo et al 2009) and estradiol (Gambino et al 2010) up-regulates leptin expression most likely by using the MAPK signal transduction pathway. Insulin has also growth promoting effects in JAr placental cells (Bifulco et al 2002, Taylor and Shalloway 1994) suggesting potential crosstalks between insulin and leptin signalling pathways.

Sam68 (Src-associated in mitosis) was originally identified as the only known substrate for Src family tyrosine kinases during mitosis (Taylor and Shalloway 1994, Fumagalli et al 1994) and is a member of the STAR (signal transduction and activation of RNA metabolism) family (Taylor and Shalloway 1994). Based on the domain organization of the protein, Sam68 could connect signal transduction pathways and RNA metabolism. In fact, there are many data demonstrating that signaling mediated phosphorylation of

Sam68 may alter its ability to bind RNA, thus providing a direct way to modify the RNA metabolism by the activation of signaling pathways (Coyle et al 2003, Derry et al 2000, Wang et al 1995).

It has been proposed that Sam68 may provide a link between the PI3K and Ras pathways via the interaction of phosphorylated tyrosine and proline-rich motifs with proteins containing SH2 and SH3 domains respectively (Najib et al 2005). Thus, Sam68 may be part of a multifunctional module required for the activation of overlapping pathways. In fact, Sam68 can be used by insulin and leptin receptors (Martin-Romero and Sanchez-Margalet 2001, Sanchez-Margalet and Najib 1999, Sanchez-Margalet and Najib 2001), in a similar way to other tyrosine kinase substrates that may function as adaptor proteins in different signaling pathways. In this sense, we have reported in human peripheral blood mononuclear cells, that human leptin time and dose dependently stimulates tyrosine phosphorylation of Sam68, promoting the association with STAT3, probably by interacting with the SH2 domains (Sanchez-Margalet and Martin-Romero 2001).

However, the precise biological role of Sam68 is unknown, specially in trophoblastic cells, where it has not been studied. Even though Sam68 is an ubiquitous protein, its expression levels vary from tissue to tissue, with testis and ovary expressing higher levels of the protein (Sette et al 2010). However there is limited information regarding expression of Sam68 in placenta and whether it may be recruited by the activated leptin receptor. The aim of this study was to investigate whether acute leptin administration affects phosphorylation (function) and expression of Sam68 in JEG-3 trophoblastic cell and, in addition, whether downregulation of Sam68 may affect the expression of *LEPR*, as it has been previously reported in C2C12 muscle cells, in which, the positive

regulation of *LEPR* mRNA by leptin is one of the early events triggered by the hormone, which depends on both Sam68 and ERK activity (Maroni et al 2009).

2. Materials and methods

2.1. Antibodies and reagents

The recombinant human leptin was provided by from Sigma (Sigma Chemical); Antibodies against leptin receptor (LEPR), the anti-Sam68 (C-20 and N-terminal), anti-tubulin from Santa Cruz Biotechnology; the anti-actin and anti-leptin antibodies from Sigma, the anti-phosphotyrosine (RC20) antibodies cross-linked to horseradish peroxidase from Transduction Laboratories. Agarose immobilized poly(U) and protein A-sepharose were purchased from Pharmacia. Oligonucleotide antisense of Sam68 sequence 5'cagTGGCaCCTCtGGTgatg 3' and standard control sequence 5'catcACCaGAGGtGCCActg 3' were purchased from Biomedal S.L.

2.2 Cell culture and treatments

The human choriocarcinoma cell line JEG-3 was generously provided by Susana Genti-Raimondi, (Universidad Nacional de Córdoba, Argentina). Cells were grown in DMEM-F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 g/mL streptomycin at 37°C in 5% CO₂. JEG-3 cells were treated at different times in the absence or presence of different concentrations of leptin (0-10 nM), corresponding to the physiological range of leptin response previously tested in both normal and JEG-3 trophoblastic cells (Pérez-Pérez 2008, Pérez-Pérez 2009). The lisates were washed with cold PBS and solubilized for 30 min at 4°C in lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl₂, 1 mM

CaCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate. Total protein levels were determined by the bicinchoninic acid method (Smith et al 1985) using bovine serum albumin as standard.

2.3 Immunoprecipitation and poly(U)-binding assay

Soluble cellular lysates (0.5 mg of protein) were precleared with 50 µl of protein A-sepharose for 2 h at 4°C by end-over-end rotation. The precleared cellular lysates were incubated with appropriate antibodies for 3 h at 4°C (Sanchez-Margalet and Martin-Romero 2001). Next, 50 µl of protein A-sepharose was added to immune complexes and incubation was continued for 2 h at 4°C. For the poly(U)-binding assay, 60 µL of a 50% solution of agarose-poly(U) beads (Pharmacia) were added to 0.5 mg of proteins of cellular lysates. The mixture was rotated for 2 h at 4°C (Martin-Romero and Sanchez-Margalet 2001).

2.4 Western Blotting Analysis

The immunoprecipitates and poly(U)-affinity precipitates were washed three times with lysis buffer. We added 40 µl of SDS-stop buffer containing 100 mM of DTT to the immunoprecipitates followed by boiling for 5 min. The soluble supernatants were then resolved by 8-16% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Martin-Romero and Sanchez-Margalet 2001, Sanchez-Margalet and Najib 1999). The membranes were blocked with buffered saline-0.05% Tween 20 (PBST) containing 5% nonfat dry milk for 1 h at 23°C. The blots were then incubated with primary antibody for 1 h, washed in PBST, and further incubated with secondary antibodies using horse radish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (Santa Cruz). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (SuperSignal from Pierce), (Sanchez-Margalet et al 2003).

The bands obtained in the blots were scanned and analyzed by the PCBAS2.0 program. Values are expressed as means \pm S.E.M. Student's t-test was used for comparisons, with differences being considered statistically significant at $p < 0.05$.

2.5 Transfection experiments

For transfection experiments, JEG-3 cells were plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 ml of DMEM-F12 plus 10% FCS. Cells were incubated for 24 h. Medium was replaced, and transfection of cells was performed according to the standard liposome-mediated method optimized for JEG-3 cells (Zhang et al 2007). Typically, 1.3 μ g of the Sam68 antisense or sense (control) was transfected using 5 μ l of LipofectAMINE (Life Technologies). The medium was replaced after 8 h with DMEM-F12 without FCS for 24 h. Transfection analysis was performed by duplicate in each of at least three independent experiments.

2.6 RNA extraction and Quantitative real-time -PCR (qRT-PCR) assay

Abundance of Sam68, and *LEPR* mRNA was determined by qRT-PCR. Total RNA was extracted from JEG-3 using TRISURE reagent (Chomczynski 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μ g of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). qRT-PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: *LEPR*: forward, 5' ATAGTTCAGTCACCAAGTGC 3'; reverse, 5' GTCCTGGAGAACTCTGATGTCC 3'; cyclophilin: forward, 5' CTTCCCCGATACTTCA 3'; reverse, 5' TCTTGGTGCTACCTC 3' and Sam68: forward, 5' TTTGTGGGGAAGATTCTTGG

3'; reverse, 5'GGGGGTCCAAAGACTTCAAT 3'. Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad). A typical reaction contained 10 μ M of forward and reverse primer, 3 μ l of cDNA, and the final reaction volume was 25 μ l. 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 sec at 95 °C and 1 min annealing and extension at 58 °C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. 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.

2.7 Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as mean \pm standard deviation (S.D.) in arbitrary units (A.U.). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad Software). Statistical significance was assessed by ANOVA followed by different post hoc tests, as indicated in each figure. A p value <0.05 was considered statistically significant.

3. Results

3.1 Effect of leptin treatment on Sam68 tyrosine-phosphorylation in JEG-3 trophoblastic cells.

We have previously implicated Sam68 as a signaling molecule in the LEPR system in human monocytes and lymphocytes (Sanchez-Margalet et al 2003). We have now tested this possible implication of Sam68 in leptin signaling in trophoblastic cells. Using JEG-3 trophoblastic cells, we studied the tyrosine phosphorylation of Sam68 in response to leptin stimulation. Cells were stimulated with increasing concentrations of leptin (0-10 nM) for 5 min, and lysed. Total extracts from control and leptin-treated trophoblastic cells were immunoprecipitated with N-terminal anti-Sam68 antibodies, and analysed by means of immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 1a, immunoprecipitated Sam68 was tyrosine phosphorylated when the cells were incubated with leptin for 5 min. Maximal phosphorylation of Sam68 was observed at 1 nM leptin, but 0.1 nM was enough to partially increase tyrosine-phosphorylation of Sam68, thus indicating that the leptin signaling involves this protein in trophoblastic cells. The phosphorylated band corresponded with an apparent molecular mass of about 68 kDa, consistent with previously reported data in different systems. The amount of protein immunoprecipitated in every lane was controlled by immunoblot with the same immunoprecipitating antibody.

3.2 Tyrosine phosphorylation of Sam68 by leptin inhibits the binding to poly(U)

Since leptin stimulation promotes the tyrosine phosphorylation of Sam68 we sought to check the possible regulation of Sam68 association with RNA by leptin. Thus, we used immobilized poly(U) polymer, since it has been previously shown that Sam68 binds this polymeric RNA specifically (Taylor and Shalloway 1994, Wang et al 1995, Martin-Romero and Sanchez-Margalet 2001).

As shown in Fig. 2, leptin stimulation increased the tyrosine phosphorylation level (A) and resulted in an inhibition of the binding efficiency of Sam68 to poly(U) in a dose-dependent manner (B). Thus, at 10 nM leptin almost no Sam68 is able to bind RNA.

3.2 Effect of leptin treatment on Sam68 mRNA expression in JEG-3 trophoblastic cells

Many different receptors positively modulate the expression of the signaling proteins. Thus, we have previously shown an increase of Sam68 expression in response to insulin in CHO-IR cells and adipocytes (Sanchez-Margalet et al 2003). In order to check the effect of leptin on Sam68 expression in trophoblastic cells, JEG-3 cells were incubated in the absence of serum with and without leptin (0-10 nM) for 16 h. We evaluated mRNA expression by means of qRT-PCR, using cyclophilin as an internal control for reaction efficiency. As shown in Fig. 3, leptin stimulation for 16 h increased Sam68 expression about two times the basal levels in trophoblastic cells. Similar experiments were performed using Western blot to further analyze the leptin effect on Sam68 expression by studying the amount of protein. As shown in Fig. 4, leptin dose-dependently stimulates Sam68 expression by using antibodies that specifically recognize Sam68. The amount of total protein in every sample was controlled using anti-actin antibodies.

3.3 Study of the effect of the downregulation of Sam68 on LEPR expression in trophoblastic cells

We next studied the relationship between Sam68 and LEPR in the opposite direction. Thus, we checked the effect of downregulation of Sam68 on *LEPR* expression. Thus, we reduced Sam68 levels by means of an antisense strategy, transfecting JEG-3 cells

with an antisense oligonucleotide of *Sam68* for 36 h, using the sense oligonucleotide as a control. Then, we evaluated *LEPR* expression in both conditions by using qRT-PCR. As shown in Fig. 5, *LEPR* mRNA was significantly reduced when *Sam68* was downregulated. The amount of *Sam68* mRNA was measured to check the down-regulation method. Next, the amount of LEPR was measured by Western blot, using antibodies that specifically recognize LEPR. As shown in Fig. 6, *Sam68* knock-down significantly diminished LEPR protein amount, thus providing consistent results to those obtained by qRT-PCR. The total amount of protein in every sample was controlled using anti-tubulin antibodies and the transfection control was checked by using anti-*Sam68* antibodies.

4. Discussion

Previously published results suggested that leptin has a role in reproduction, particularly in the fetoplacental physiology. Trophoblastic cells produce leptin locally, and the autocrine action of leptin may be important for trophoblastic cell survival, growth and proliferation. Insulin has also growth promoting effects in JAr placental cells (Bifulco et al 2002, Boileau et al 2001) suggesting potential crosstalks between insulin and leptin signaling pathways. However, the molecular mechanisms underlying the effects of leptin on trophoblastic cells are not completely understood (Sagawa et al 2002). To gain insight into the mechanisms of leptin action, we sought to identify cellular proteins that may be pertinent to its function. In this context, one possible candidate for integrating signal transduction pathways is *Sam68*, a member of the STAR protein family that not only contains KH domains, but also proline- and tyrosine-rich sequences that respectively interact with RNA and proteins (Lukong and Richard 2003). Previous data from other authors indicated that *Sam68* could play a role in proliferation and apoptosis

in an RNA-binding dependent manner (Taylor et al 2004). Sam68 may therefore play a role in RNA metabolism including alternative splicing (Gorla et al 2006, Rajan et al 2008). In addition, Sam68 has been found to function as an adaptor protein in signal transduction systems, including insulin receptor in hepatic and adipose cells (Najib et al 2005, Sanchez-Margalet and Najib 1999, Sanchez-Margalet and Najib 2001, Sanchez-Margalet et al 2003, Najib and Sanchez-Margalet 2002b) as well as leptin receptor itself in mononuclear cells (Martin-Romero and Sanchez-Margalet 2001, Martin-Romero et al 2000). Moreover, we have found that tyrosine phosphorylation of Sam68 in response to leptin in mononuclear cells inhibits Sam68 ability to bind RNA (Martin-Romero and Sanchez-Margalet 2001). In this context, a role for Sam68 linking signal transduction with RNA metabolism has been proposed (Najib et al 2005, Lukong and Richard 2003). However, there are no data regarding the role of Sam68 in leptin signaling in placenta, or the regulation of Sam68 expression in placenta in response to leptin or any other signal. That is why, in the present study, we have investigated the possible participation of Sam68 in leptin receptor signaling and the regulation of *Sam68* expression by leptin in JEG-3 human choriocarcinoma cells. JEG-3 cells express both leptin and its receptor (Magarinos et al 2007) and maintain many characteristics of human trophoblast cells. They have been widely used to study placental cellular signaling (Perez-Perez et al 2008, Standley and Standley 2002, Zygmunt et al 1998) showing comparable results.

In the present work, we have first reported that leptin indeed upregulates phosphorylation of Sam68 in placental cells. Moreover, this effect is dependent on the dose, in a similar way to that previously observed in other systems in response to insulin (Sanchez-Margalet and Najib 1999) or leptin (Martin-Romero and Sanchez-Margalet 2001). As a result of this tyrosine phosphorylation of Sam68 in response to leptin, we have found that the RNA binding capacity of Sam68 is diminished in

trophoblastic JEG-3 cells. This result is consistent with previous reported data demonstrating that tyrosine phosphorylation of Sam68 by kinases of the Src (Wang et al 1995) and Brk (Derry et al 2000, Haegebarth et al 2004) family negatively regulates its RNA binding function. More precisely, we have found that leptin stimulation of mononuclear cells promotes tyrosine phosphorylation of Sam68 mediating an inhibition on the Sam68 RNA binding activity, determined as binding to poly(U) (Martin-Romero and Sanchez-Margalet 2001). Thus, Sam68 may play a role in the transduction of the leptin signal from the plasma membrane to RNA metabolism via a rapid mechanism mediated by phosphorylation. Therefore, the leptin-stimulated tyrosine phosphorylation of Sam68 and its subsequent inhibition on RNA binding capacity might contribute to the leptin effects observed in trophoblastic cells. In fact, we have previously found that Sam68 participates in leptin and insulin signaling, showing dose-dependent phosphorylation and interaction with different signaling proteins. Thus, in different systems, Sam68 has been shown participate in different pathways including JAK-STAT (Sanchez-Margalet and Martin-Romero 2001), MAPK and PI3K (Martin-Romero and Sanchez-Margalet 2001, Sanchez-Margalet and Najib 1999, Sanchez-Margalet and Najib 2001, Najib and Sanchez-Margalet 2002b). Nevertheless, the possible participation of Sam68 in these signaling pathways in trophoblastic cells remains to be investigated .

Other receptor systems have been found to positively modulate the expression of kinases substrates and other molecules recruited to the receptor signaling upon stimulation with the specific ligand (Montezano et al 2010). In this context, we have also found that leptin stimulation of JEG-3 cells increases the expression of Sam68 in a similar way to that observed in response to insulin (Sanchez-Margalet et al 2003).

To further investigate the relationship between Sam68 and LEPR in trophoblastic cells, by using Sam68 antisense strategy, we studied the effect of Sam68 on *LEPR* mRNA expression levels, as well as the amount of LEPR protein. We have found that Sam68 does play a role in the *LEPR* expression in trophoblastic cells, as we clearly observed a significant inhibition of *LEPR* expression after 36 h of transfection with *Sam68* antisense oligonucleotide, as assessed both by qRT-PCR and Western blot analysis. Even though the role of Sam68 mediating the increase in *LEPR* expression has been recently reported in muscle cell line (Maroni et al 2009), this is the first time that Sam68 has been implicated in the expression of *LEPR* in JEG-3 cells, where it also functions as a leptin signaling protein. Therefore, Sam68 may positively regulate *LEPR* expression in a similar way to that observed in other signaling proteins and receptor systems (Edvinsson 2009, Rice and Garner 1999). Thus, we have provided some evidence for the possible role of Sam68 in leptin signaling produced by JEG-3 cells and that may be extensible for *in vivo* trophoblastic cells, describing its effect on the positive regulation of *LEPR* expression.

We have previously demonstrated the role of leptin as a trophic factor for placenta promoting growth and preventing apoptosis of human trophoblastic cells (Perez-Perez et al 2008, Magarinos et al 2007). Since Sam68 has been found to be involved in cell proliferation and survival (Barlat et al 1997, Li et al 2002, Busa et al 2007), the recruitment of Sam68 to LEPR signaling in JEG-3 cells may be useful to mediate these effects of leptin on human placenta. However, this hypothesis should be confirmed in future studies by using a more physiological system such as trophoblastic explants from healthy donors.

In conclusion, leptin dose-dependently stimulates expression and phosphorylation of Sam68 in JEG-3 trophoblastic cells, and we have found that down-regulation of Sam68,

by antisense strategy, reduces *LEPR* expression levels, suggesting a link between leptin signaling and Sam68, probably contributing to signal transduction complexes in JEG-3 trophoblastic cells.

Acknowledgements

This work was supported by a Grant from the Instituto de Salud Carlos III (ISCIII PS09/00119). Flora Sánchez-Jiménez and Antonio Pérez-Pérez are research fellow supported by the Instituto de Salud Carlos III (Contrato Post-formación especializada, Río Hortega), ISCIII CM07/00025, Spain. We are grateful to Beatriz Puche for providing language help.

FIGURE LEGENDS

Figure 1. Leptin stimulates Sam68 tyrosine-phosphorylation in JEG-3 trophoblastic cells

JEG-3 cells were incubated in the presence of increasing concentrations of leptin (0-10 nM) for 5 min. Cells were lysed and soluble clarified cell lysates were subjected to immunoprecipitation with anti-Sam68 antibodies. Immunoprecipitates were resolved by SDS-PAGE and Western blot with anti-phosphotyrosine antibodies (A). The same lysates were analyzed by immunoblot using the Sam68 antibodies to control the amount of protein in every lane (B). An experiment representative of three independent experiments is shown and densitograms with standard error are shown. * $p < 0.05$ *versus* control.

Figure 2. Tyrosine phosphorylation of Sam68 by leptin stimulation inhibits the Sam68 binding to poly(U).

JEG-3 cells were incubated in the presence of different increasing concentrations of leptin for 5 minutes and lysed. Precleared lysates were then incubated with Sepharose-coupled poly(U) beads for Sam68 affinity precipitation. Samples were then washed and analyzed by immunoblot with anti-phosphotyrosine (A) or anti-Sam68 (B) antibodies. A representative experiment of three is shown and densitograms are expressed as means with standard error * $p < 0.05$ *versus* control in Sam68 binded to poly(U).

Figure 3. Leptin up-regulates Sam68 RNA expression level in JEG-3 trophoblastic cells.

Cells were obtained and treated with increasing leptin dose (0-10 nM) during 16 h. Total RNA was extracted as previously described in Materials and Methods. Leptin mRNA was quantified with qRT-PCR. Cyclophilin was used as internal standard. Results shown are from a representative experiment and are expressed as means \pm SD for three independent experiments * $p < 0.05$ *versus* control.

Figure 4. Leptin increases Sam68 protein abundance in JEG-3 trophoblastic cells.

JEG-3 cells were treated with increasing leptin doses (0-10 nM) during 16 h. Cells were then lysed and proteins were separated on SDS PAGE gels. Sam68 expression was determined by Western blot analysis using specific anti-Sam68 antibodies. Loading controls were performed by immunoblotting the same membranes with anti- β -actin. Bands densitometry is shown in a representative experiment of three independent experiments with standard error, * $p < 0.05$ *versus* control.

Figure 5. Downregulation of Sam68 diminishes *LEPR* expression, determined by qRT-PCR in trophoblastic cells.

JEG-3 cells were transfected with antisense of *Sam68* for 24 h. After transfection, cells were treated with leptin 10 nM during 12 h. Total RNA was extracted as indicated in Materials and Methods and cyclophilin was used as internal standard and quantified with qRT-PCR. (A) *LEPR* expression in the absence (control) or presence of *Sam68* antisense. (B) The downregulation of *Sam68* expression with the antisense was also

checked by qRT-PCR. We show a representative experiment with the means \pm SD of three independent experiments. * $p < 0.05$ versus control.

Figure 6. Downregulation of Sam68 diminishes *LEPR* protein abundance, determined by Western blot, in trophoblastic cells.

After JEG-3 transfection with Sam68 antisense or Sam68 sense as control (0) for 24 h, cells were treated with leptin 10 nM during 12 h. They were lysed and proteins were separated on SDS-PAGE gels. *LEPR* protein amount was determined by Western blot analysis. Total amount of protein in each sample was controlled by immunoblotting the same membranes with anti-tubulin antibodies. Bands densitometry for *LEPR* in Sam68 antisense-treated cells and control cells is shown in a representative experiment of three independent ones. Densitograms with standard error are shown. * $p < 0.05$ versus control.

- Ahima, R.S., Osei, S.Y., 2004. Leptin signaling. *Physiol. Behav.* 81, 223-241.
- Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B., Schweighoffer, F., 1997. A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. *J. Biol. Chem.* 272, 3129-3132.
- Bifulco, G., Di Carlo, C., Caruso, M., Oriente, F., Di Spiezio Sardo, A., Formisano, P., Beguinot, F., Nappi, C., 2002. Glucose regulates insulin mitogenic effect by modulating SHP-2 activation and localization in JAr cells. *J. Biol. Chem.* 277, 24306-24314.
- Bjorbaek, C., Uotani, S., da Silva, B., Flier, J.S., 1997. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J. Biol. Chem.* 272, 32686-32695.
- Boileau, P., Cauzac, M., Pereira, M.A., Girard, J., Hauguel-De Mouzon, S., 2001. Dissociation between insulin-mediated signaling pathways and biological effects in placental cells: role of protein kinase B and MAPK phosphorylation. *Endocrinology* 142, 3974-3979.
- Busa, R., Paronetto, M.P., Farini, D., Pierantozzi, E., Botti, F., Angelini, D.F., Attisani, F., Vespasiani, G., Sette, C., 2007. The RNA-binding protein Sam68 contributes to proliferation and survival of human prostate cancer cells. *Oncogene* 26, 4372-4382.
- Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* 15, 532-4, 536-7.
- Coyle, J.H., Guzik, B.W., Bor, Y.C., Jin, L., Eisner-Smerage, L., Taylor, S.J., Rekosh, D., Hammariskjold, M.L., 2003. Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK. *Mol. Cell. Biol.* 23, 92-103.
- Derry, J.J., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A.W., Chen, T., Tyner, A.L., 2000. Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. *Mol. Cell. Biol.* 20, 6114-6126.
- Edvinsson, L., 2009. Cerebrovascular endothelin receptor upregulation in cerebral ischemia. *Curr. Vasc. Pharmacol.* 7, 26-33.
- Flier, J.S., 1995. The adipocyte: storage depot or node on the energy information superhighway? *Cell* 80, 15-18.
- Fumagalli, S., Totty, N.F., Hsuan, J.J., Courtneidge, S.A., 1994. A target for Src in mitosis. *Nature* 368, 871-874.

- Gambino, Y.P., Maymo, J.L., Perez-Perez, A., Duenas, J.L., Sanchez-Margalet, V., Calvo, J.C., Varone, C.L., 2010. 17Beta-estradiol enhances leptin expression in human placental cells through genomic and nongenomic actions. *Biol. Reprod.* 83, 42-51.
- Ghilardi, N., Skoda, R.C., 1997. The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol. Endocrinol.* 11, 393-399.
- Gorla, L., Cantu, M., Micciche, F., Patelli, C., Mondellini, P., Pierotti, M.A., Bongarzone, I., 2006. RET oncoproteins induce tyrosine phosphorylation changes of proteins involved in RNA metabolism. *Cell. Signal.* 18, 2272-2282.
- Haegebarth, A., Heap, D., Bie, W., Derry, J.J., Richard, S., Tyner, A.L., 2004. The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2. *J. Biol. Chem.* 279, 54398-54404.
- Hauguel-de Mouzon, S., Lepercq, J., Catalano, P., 2006. The known and unknown of leptin in pregnancy. *Am. J. Obstet. Gynecol.* 194, 1537-1545.
- Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., Friedman, J.M., 1996. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379, 632-635.
- Li, Q.H., Haga, I., Shimizu, T., Itoh, M., Kurosaki, T., Fujisawa, J., 2002. Retardation of the G2-M phase progression on gene disruption of RNA binding protein Sam68 in the DT40 cell line. *FEBS Lett.* 525, 145-150.
- Li, R.H., Yu, M.M., Cheung, A.N., Wong, Y.F., 2004. Expression of leptin and leptin receptors in gestational trophoblastic diseases. *Gynecol. Oncol.* 95, 299-306.
- Lollmann, B., Gruninger, S., Stricker-Krongrad, A., Chiesi, M., 1997. Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochem. Biophys. Res. Commun.* 238, 648-652.
- Lukong, K.E., Richard, S., 2003. Sam68, the KH domain-containing superSTAR. *Biochim. Biophys. Acta* 1653, 73-86.
- Magarinos, M.P., Sanchez-Margalet, V., Kotler, M., Calvo, J.C., Varone, C.L., 2007. Leptin promotes cell proliferation and survival of trophoblastic cells. *Biol. Reprod.* 76, 203-210.
- Maroni, P., Citterio, L., Piccoletti, R., Bendinelli, P., 2009. Sam68 and ERKs regulate leptin-induced expression of OB-Rb mRNA in C2C12 myotubes. *Mol. Cell. Endocrinol.* 309, 26-31.
- Martin-Romero, C., Sanchez-Margalet, V., 2001. Human leptin activates PI3K and MAPK pathways in human peripheral blood mononuclear cells: possible role of Sam68. *Cell. Immunol.* 212, 83-91.

- Martin-Romero, C., Santos-Alvarez, J., Goberna, R., Sanchez-Margalet, V., 2000. Human leptin enhances activation and proliferation of human circulating T lymphocytes. *Cell. Immunol.* 199, 15-24.
- Maymo, J.L., Perez Perez, A., Sanchez-Margalet, V., Duenas, J.L., Calvo, J.C., Varone, C.L., 2009. Up-regulation of placental leptin by human chorionic gonadotropin. *Endocrinology* 150, 304-313.
- Montezano, A.C., Burger, D., Paravicini, T.M., Chignalia, A.Z., Yusuf, H., Almasri, M., He, Y., Callera, G.E., He, G., Krause, K.H., Lambeth, D., Quinn, M.T., Touyz, R.M., 2010. Nicotinamide adenine dinucleotide phosphate reduced oxidase 5 (Nox5) regulation by angiotensin II and endothelin-1 is mediated via calcium/calmodulin-dependent, rac-1-independent pathways in human endothelial cells. *Circ. Res.* 106, 1363-1373.
- Myers, M.G., Jr, 2004. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog. Horm. Res.* 59, 287-304.
- Najib, S., Martin-Romero, C., Gonzalez-Yanes, C., Sanchez-Margalet, V., 2005. Role of Sam68 as an adaptor protein in signal transduction. *Cell Mol. Life Sci.* 62, 36-43.
- Najib, S., Sanchez-Margalet, V., 2002a. Human leptin promotes survival of human circulating blood monocytes prone to apoptosis by activation of p42/44 MAPK pathway. *Cell. Immunol.* 220, 143-149.
- Najib, S., Sanchez-Margalet, V., 2002b. Sam68 associates with the SH3 domains of Grb2 recruiting GAP to the Grb2-SOS complex in insulin receptor signaling. *J. Cell. Biochem.* 86, 99-106.
- Perez-Perez, A., Gambino, Y., Maymo, J., Goberna, R., Fabiani, F., Varone, C., Sanchez-Margalet, V., 2010. MAPK and PI3K activities are required for leptin stimulation of protein synthesis in human trophoblastic cells. *Biochem. Biophys. Res. Commun.* 396, 956-960.
- Perez-Perez, A., Maymo, J., Duenas, J.L., Goberna, R., Calvo, J.C., Varone, C., Sanchez-Margalet, V., 2008. Leptin prevents apoptosis of trophoblastic cells by activation of MAPK pathway. *Arch. Biochem. Biophys.* 477, 390-395.
- Perez-Perez, A., Maymo, J., Gambino, Y., Duenas, J.L., Goberna, R., Varone, C., Sanchez-Margalet, V., 2009. Leptin stimulates protein synthesis-activating translation machinery in human trophoblastic cells. *Biol. Reprod.* 81, 826-832.
- Rajan, P., Gaughan, L., Dalglish, C., El-Sherif, A., Robson, C.N., Leung, H.Y., Elliott, D.J., 2008. The RNA-binding and adaptor protein Sam68 modulates signal-dependent splicing and transcriptional activity of the androgen receptor. *J. Pathol.* 215, 67-77.
- Rice, K.M., Garner, C.W., 1999. IGF-I regulates IRS-1 expression in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 255, 614-617.

Sagawa, N., Yura, S., Itoh, H., Mise, H., Kakui, K., Korita, D., Takemura, M., Nuamah, M.A., Ogawa, Y., Masuzaki, H., Nakao, K., Fujii, S., 2002. Role of leptin in pregnancy-a review. *Placenta* 23 Suppl A, S80-6.

Sahu, A., 2003. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Front. Neuroendocrinol.* 24, 225-253.

Sanchez-Margalet, V., Gonzalez-Yanes, C., Najib, S., Fernandez-Santos, J.M., Martin-Lacave, I., 2003. The expression of Sam68, a protein involved in insulin signal transduction, is enhanced by insulin stimulation. *Cell Mol. Life Sci.* 60, 751-758.

Sanchez-Margalet, V., Martin-Romero, C., 2001. Human leptin signaling in human peripheral blood mononuclear cells: activation of the JAK-STAT pathway. *Cell. Immunol.* 211, 30-36.

Sanchez-Margalet, V., Martin-Romero, C., Santos-Alvarez, J., Goberna, R., Najib, S., Gonzalez-Yanes, C., 2003. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. *Clin. Exp. Immunol.* 133, 11-19.

Sanchez-Margalet, V., Najib, S., 2001. Sam68 is a docking protein linking GAP and PI3K in insulin receptor signaling. *Mol. Cell. Endocrinol.* 183, 113-121.

Sanchez-Margalet, V., Najib, S., 1999. p68 Sam is a substrate of the insulin receptor and associates with the SH2 domains of p85 PI3K. *FEBS Lett.* 455, 307-310.

Santos-Alvarez, J., Goberna, R., Sanchez-Margalet, V., 1999. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell. Immunol.* 194, 6-11.

Sette, C., Messina, V., Paronetto, M.P., 2010. Sam68: a new STAR in the male fertility firmament. *J. Androl.* 31, 66-74.

Smith, J.T., Waddell, B.J., 2002. Leptin receptor expression in the rat placenta: changes in ob-ra, ob-rb, and ob-re with gestational age and suppression by glucocorticoids. *Biol. Reprod.* 67, 1204-1210.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.

Standley, P.R., Standley, C.A., 2002. Identification of a functional Na⁺/Mg²⁺ exchanger in human trophoblast cells. *Am. J. Hypertens.* 15, 565-570.

Sweeney, G., 2002. Leptin signalling. *Cell. Signal.* 14, 655-663.

Tartaglia, L.A., 1997. The leptin receptor. *J. Biol. Chem.* 272, 6093-6096.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Wool, E.A., Monroe, C.A., Tepper, R.I., 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83, 1263-1271.

Taylor, S.J., Resnick, R.J., Shalloway, D., 2004. Sam68 exerts separable effects on cell cycle progression and apoptosis. *BMC Cell Biol.* 5, 5.

Taylor, S.J., Shalloway, D., 1994. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. *Nature* 368, 867-871.

Wang, L.L., Richard, S., Shaw, A.S., 1995. P62 association with RNA is regulated by tyrosine phosphorylation. *J. Biol. Chem.* 270, 2010-2013.

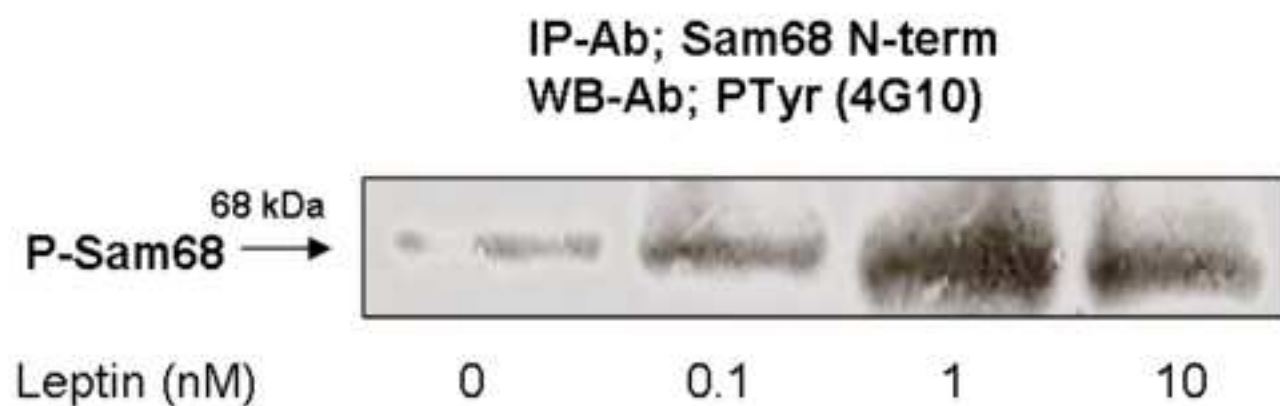
Yamashita, T., Murakami, T., Otani, S., Kuwajima, M., Shima, K., 1998. Leptin receptor signal transduction: OBRa and OBRb of fa type. *Biochem. Biophys. Res. Commun.* 246, 752-759.

Zhang, M., Guller, S., Huang, Y., 2007. Method to enhance transfection efficiency of cell lines and placental fibroblasts. *Placenta* 28, 779-782.

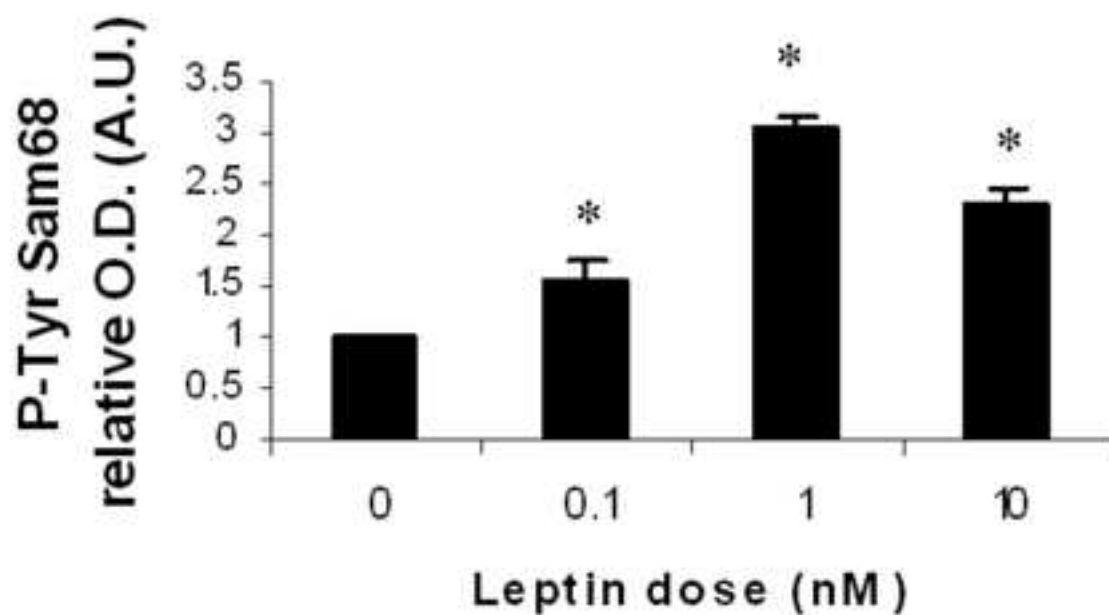
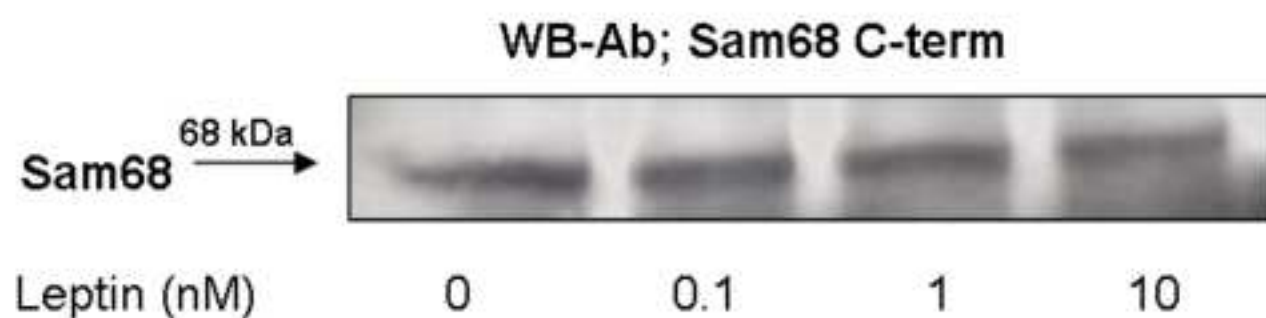
Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature* 372, 425-432.

Zygmunt, M., Hahn, D., Kiesenbauer, N., Munstedt, K., Lang, U., 1998. Invasion of cytotrophoblastic (JEG-3) cells is up-regulated by interleukin-15 in vitro. *Am. J. Reprod. Immunol.* 40, 326-331.

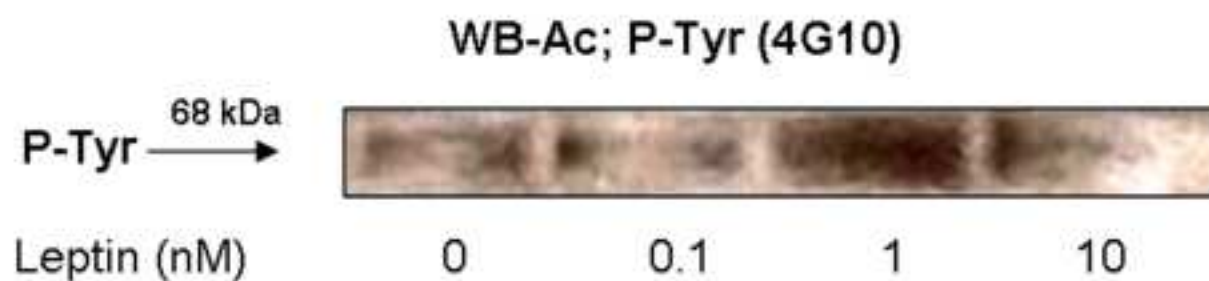
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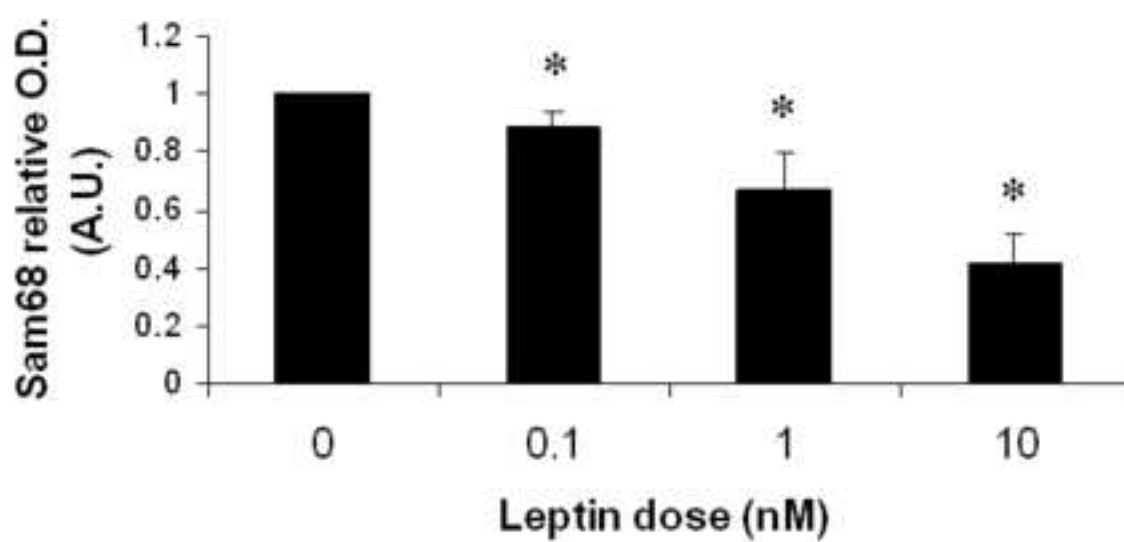
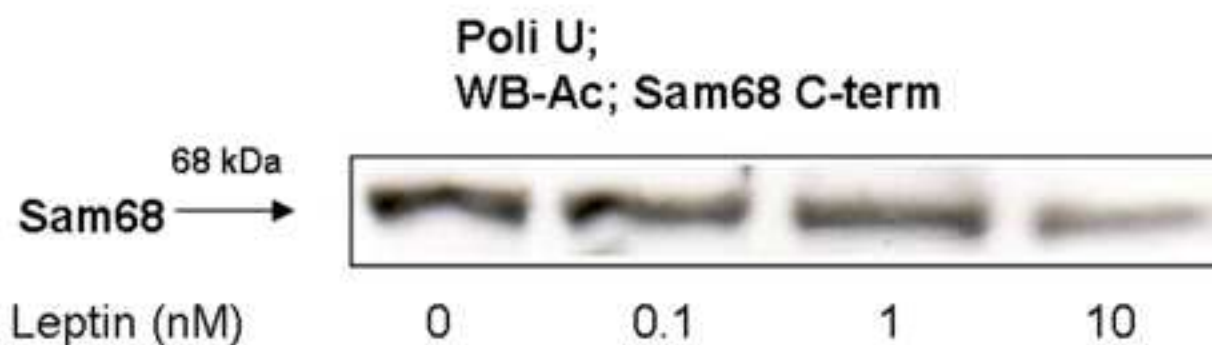
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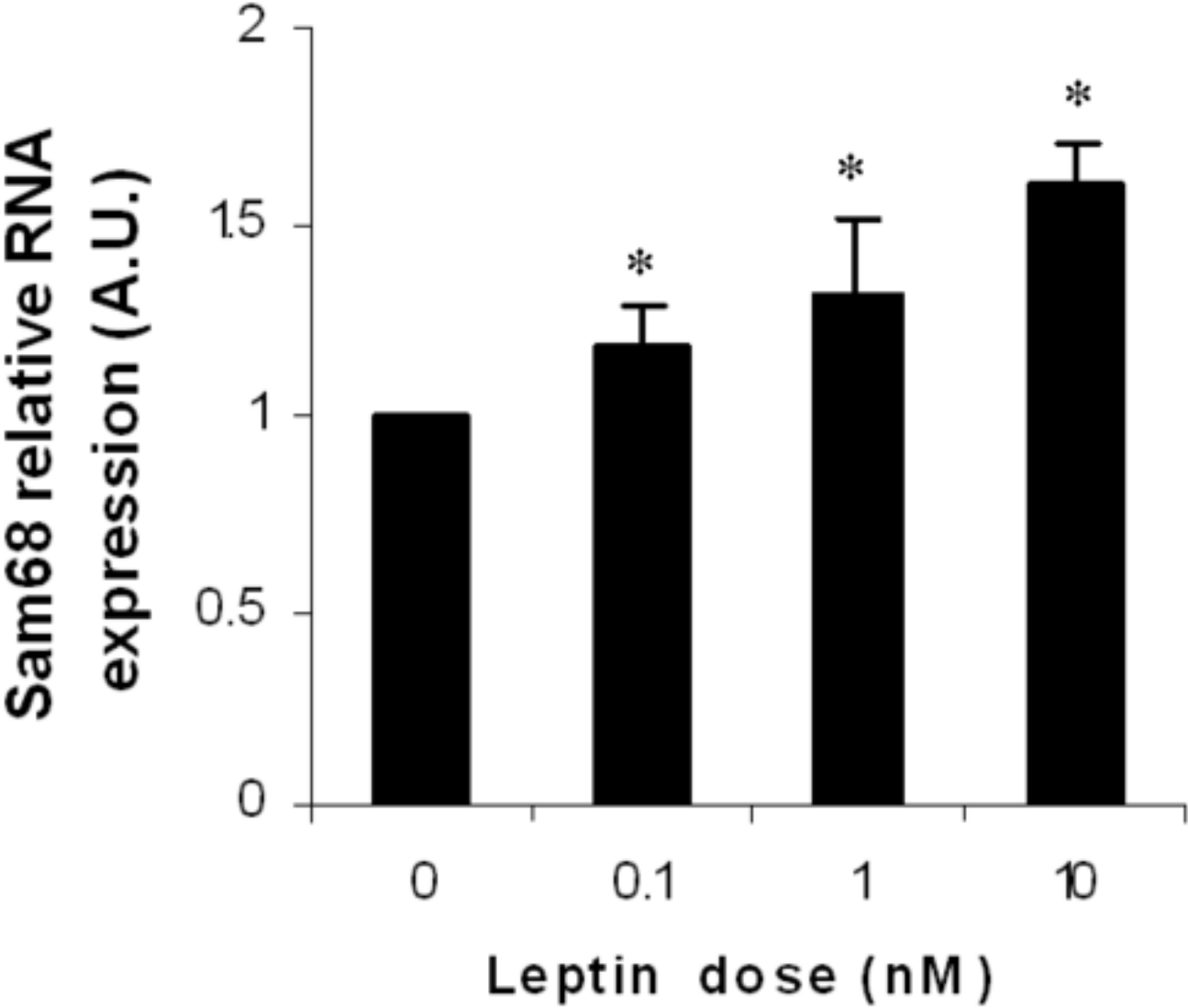


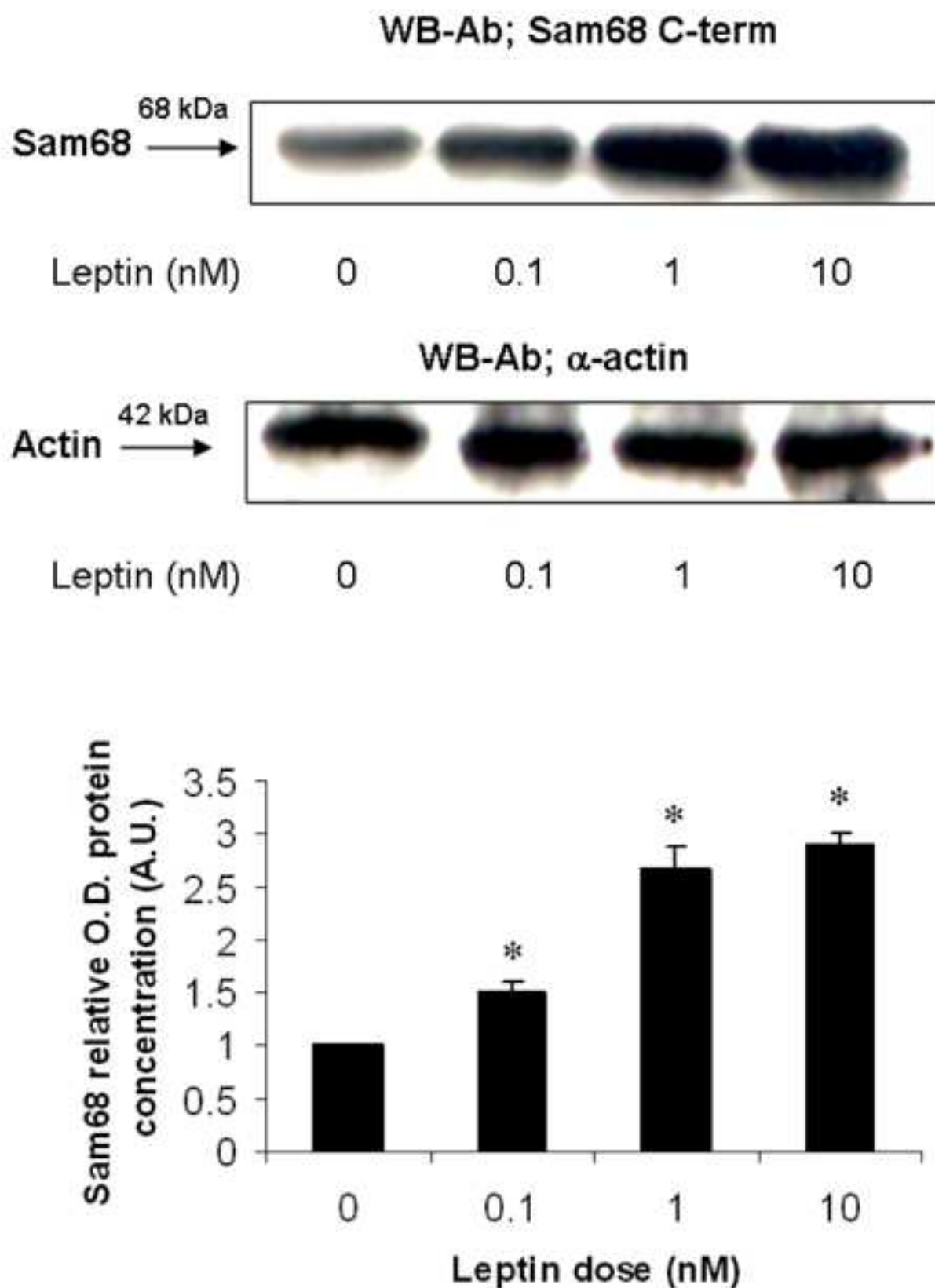
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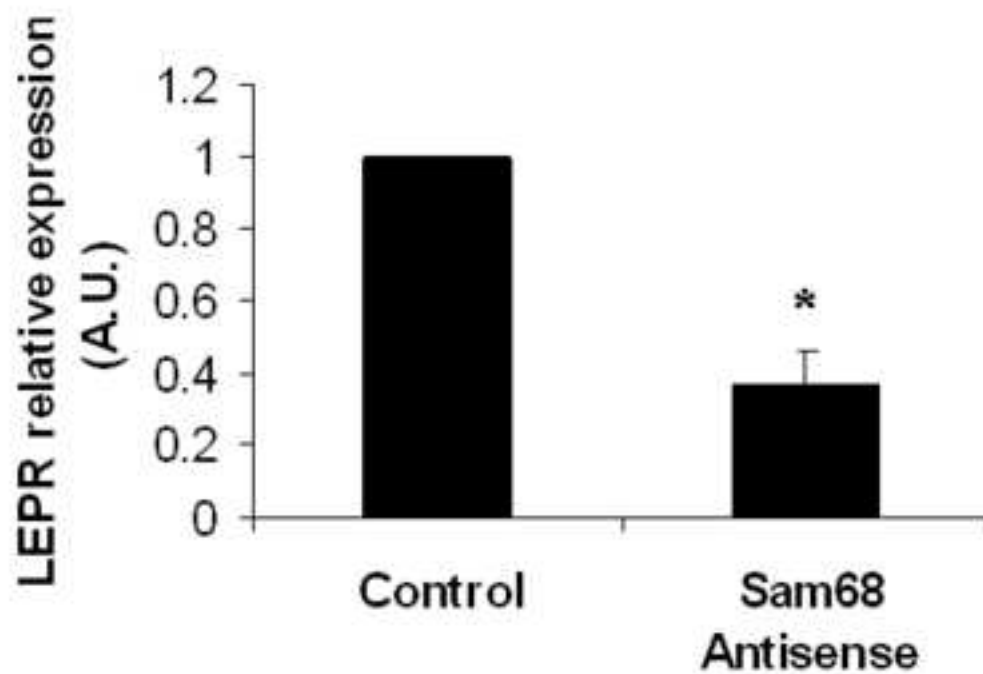
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