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Leptin activates cyclin D1 promoter gene in Ishikawa endometrial cancer cells: role of STAT and cAMP response element.

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Summary

Leptin, a cytokine mainly produced by adipocytes, in addition to the control weight homeostasis by regulating food intake and energy expenditure, is implicated in multiple biological actions. Epidemiological studies demonstrate a positive association between obesity and an increased risk of developing different cancers include breast, prostate, colon and endometrial cancer in both pre- and postmenopausal women. It has been shown that leptin receptors ObR (short and long isoforms) are expressed in both cancer and non-cancer endometrium and a recent study demonstrated that leptin promotes endometrial cancer growth and invasiveness through STAT/MAPK and Akt pathways. However, the involvement of leptin in endometrial carcinogenesis still needs to be elucidated.

In this study we evaluated the molecular mechanism underlying the proliferative role of leptin in Ishikawa human endometrial cancer cells analyzing cell-cycle profile with flow cytometric analysis and the expression of cell-cycle regulators with RT-PCR and Western blotting analysis. Leptin treatment significantly reduced the numbers of G0/G1-phase cells associated with the increase of cell population in S and G2/M phases.

Upon leptin exposure we evidenced an up-regulation of cyclin D1 expression together with a down-regulation of cyclin-dependent kinase inhibitor p21 WAF1/CIP1.

Mutagenesis studies, eletrophoretic mobility and chromatin immunoprecipitation assay revealed that cyclic AMP-responsive element binding

(CREB) protein and activation of signal transducers and activators of transcription 3 (STAT3) binding protein motifs, present on cyclin D1 promoter, were important for the up-regulatory effects induced by leptin on cyclin D1 expression in endometrial cancer Ishikawa cells.

In conclusion, our findings for the first time demonstrated that the increased proliferation by leptin in human endometrial cancer cells is due, at last in part, to the up-regulation of the cell-cycle positive regulator cyclin D1. This gives a great emphasis to the role of leptin in promoting endometrial cancer establishing a direct association between obesity and endometrial carcinogenesis.

Introduction

Leptin, a product of the obese (*ob*) gene, mainly secreted by adipocytes, is involved in the control of body weight and results strongly correlated to the body fat mass *(Zhang et al, 1994; Bray 2002)*. In addition to its regulatory role in energy metabolism, it is implicated in the modulation of many other processes such as reproduction *(Brann et al, 2002)*, lactation *(Neville et al, 2002)*, hematopoiesis *(Bennett et al, 1996)*, immune responses *(Lord et al, 1998)*, cell differentiation (*Motta et al, 2007*) and proliferation (*Chen et al, 2007*).

Leptin circulates as a 16 kDa protein particularly bound to plasma protein and exerts its action through the trans-membrane leptin receptor (ObR) which belongs to a family of class I cytokine receptors (*Tartaglia 1997*). Several isoforms of the leptin receptor, generated by mRNA alternative splicing have been discovered but ObRs, the short form, and ObRl, the long form, are the two major isoforms prevalently expressed in mammalian cells (Figure 1).

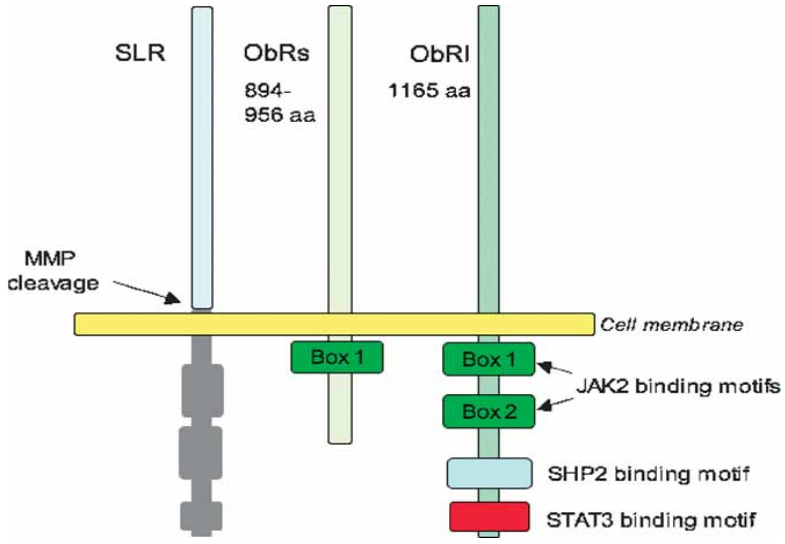


Figure 1. Schematic representation of leptin receptors, ObRs (short form) ObRl (long form). ObRl and ObRs share a common extracellular leptin-binding domain, but contain intracellular domains of variable lengths.

ObRl is highly expressed in the hypothalamus (*Bjorbaek et al, 1997*), but lower levels of this receptor have been identified in many peripheral organs, such as pancreas (*Morton et al, 1999*), prostate (*Stattin et al, 2001*), keratinocytes (*Frank et al, 2000*), vagal afferent neurons (*Buyse et al, 2001*), stomach mucosa cells (*Goiot et al, 2001*), placenta (*Ebenbichler et al, 2002*), bone (*Lee et al, 2002*) and endometrial cells (*Yuan et al, 2004*).

The short forms of ObRs are ubiquitously expressed *(Fei et al, 1997)*. Their functions are not clear, but there is evidence that ObRs can be involved in intra- and trans-cellular leptin transport (*Hileman et al, 2000*).

Only the long form, has the intracellular motifs necessary for the activation of signaling pathways (*Tartaglia et al, 1997*). As with other class I cytokine receptors, the leptin signaling is thought to be transmitted mainly by the JAK/STAT (Janus Kinase /Signal Transducers and Activators of Transcription) pathway (*Bahrenberg et al, 2002; Ahima & Osci et al, 2004*). JAKs associate constitutively with conserved Box 1 and 2 motifs in the intracellular domain of ObRl. Binding of leptin to ObRl results in autophosphorilation of JAK 1 and JAK 2 as well as phosphorilation of the cytoplasmatic domain of ObRl and the downstream transcription factors STATs (*Ahima & Osci et al, 2004*). The leptin signal is terminated by induction of SOCS3 (suppressor of cytokine signaling), a member of a family of proteins which inhibits the JAK/STAT signaling cascade (*Bjorback et al, 1999, Emilsson et al, 1999*). SOCS proteins have a variable amino-terminal domain, a central SH2 domain and a carboxy-terminal domain termed the SOCS-box motif. They are induced by cytokines

and act in a negative feedback loop to inhibit the receptor. It has been shown that overexpression of SOCS-3 inhibits leptin mediated tyrosine phosphorilation of JAK 2 (*Bjorback et al, 1999, Emilsson et al, 1999*).

In addition to STAT3 activation, leptin regulates other key signaling pathways: Ras/ERK1/2 (Ras/Extracellular signal-regulated kinase 1/2) cascade and PI-3K/Akt/GSK3 (phosphoinositide 3 kinase/ protein kinase B/glycogen synthase kinase 3) growth/antiapoptotic pathway. Besides, leptin has been found to induce phospholipase C (PLC)-gamma, protein kinase C (PKC), nitric oxide (NO) and p38 kinase (*Bjorbaek et al, 1997; Sweeney et al, 2002; Zabeau et al, 2003*). Ultimately, induction of ObRl can activate several genes involved in cell proliferation, including c-fos, c-jun, junB, and egr-1 (*Sweeney et al; 2002; Zabeau et al, 2003; Frankenberry et al, 2004*). (Figure 2)

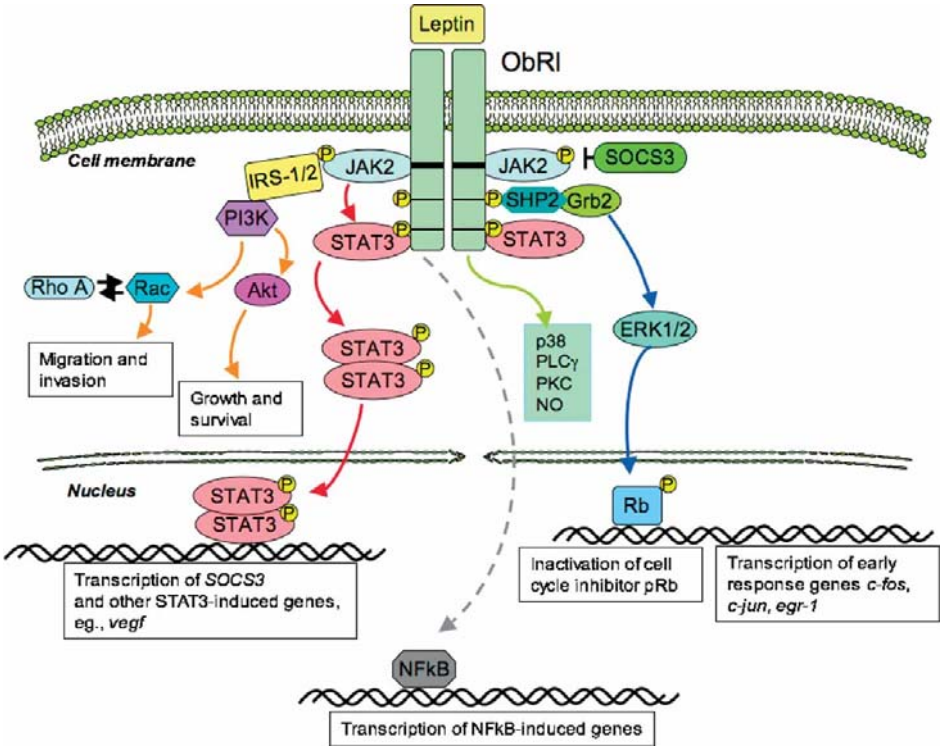


Figure 2. ObRl stimulates a broad spectrum of intracellular signaling pathways.

Recently, leptin is considered as a new growth factor. In fact, many studies have demonstrated that this cytokine is able to stimulate the proliferation of various cell types and it plays an important role in the development and progression of several cancer cells such as breast (*Okumura et al, 2002*), prostate (*Somasundar et al, 2004*), ovarian (*Choi et al, 2004*), colorectal (*Hardwick et al, 2001*), pancreatic (*Morioka et al, 2007*), and lung cancers (*Tsuchiya et al, 1999*).

Previous studies suggested that leptin signaling can crosstalk with both polypeptide growth factor signaling and with steroid receptor function. For instance, insulin is known to increase leptin expression (*Cusin et al, 1995; Saladin et al, 1995; Hardie et al, 1996; Leroy et al, 1996*), but it can also induce leptin resistance by the inhibition of leptin signaling through JAK2 (*Kellerer et al, 2001*).

Of particular interest is the link between leptin activity and ERα. Recent reports demonstrated that ERα and ObR are coexpressed in malignant mammary tissue and breast cancer cell line (*Dieudonne et al, 2002; Hu et al, 2002; Laud et al, 2002*). Notably, mitogenic effects of leptin and leptin-dipendent activation of STAT3 require SRC-1, a member of the p160 family of steroid receptor modulators (*Yin et al, 2004*), which might represent crosstalk between steroid receptor and leptin-induced transcriptional mechanisms. Furthermore, leptin has been found to modulate both estrogen syntesis and ERα activity. Our previous works demonstrated in breast cancer epithelial cells that leptin is an amplifier of E_2 signaling through a double mechanism: an enhanced aromatase gene expression (*Catalano et al, 2003*) and a direct trans-activation of ERα (*Catalano et al, 2004*). In addition, leptin and E_2 enhance

primary tumor mass either *in vivo* in MCF-7 cell tumor xenograft and *in vitro* in MCF-7 three dimensional cultures through an up-regulation of E-cadherin expression (*Mauro et al, 2007*).

The level of serum leptin is strongly correlated to body fat content, thus hyperleptinemia is a common feature of obese patients. Epidemiological studies have suggested a positive correlation between obesity and an increased risk of developing different cancers, include breast, prostate, colon and endometrial. There is convincing and consistent evidence from both case-control and cohort studies that obesity is tightly related to endometrial cancer in both pre- and postmenopausal women *(Calle and Thun et al, 2004).*

Endometrial cancer is the most common gynaecological malignancy and the fourth most common malignancy in women in the developed world after breast, colorectal and lung cancer. The incidence is estimated at 15-20 per 100,000 women per year (*Ryan et al, 2005).* The majority of cases can be divided into two broad categories based on clinic-pathological and molecular characteristics: type I oestrogen-dependent with endometriod morphology and Type II non-oestrogen-dependent with serous papillary or clear cell morphology.

Increased endometrial cancer risk has been associated with early menarche and late menopause, suggesting a relationship of risk with greater lifetime exposure to estrogens at pre-menopausal levels. Other hormone-related factors associated with risk are parity and use of exogenous estrogens for oral contraception or postmenopausal replacement therapy (*Emons et al, 2000*). Furthermore, risk has been

related to plasma concentrations of estrogens, progesterone, androgens, SHBG, and insulin (*Potischman et al, 1996; Troisi et al, 1997*). Although, it is generally thought that excess weight influences endometrial cancer risk through changes in endogenous hormone metabolism, an additional candidate that may play a crucial role in the same scenario could be leptin (*Kaaks et al, 2001; Key et al, 2001*).

Indeed, several studies demonstrated that serum leptin levels among cases with endometrial cancer were significantly higher compared to controls (*Petridou et al, 2002; Yuan et al, 2004*).

Expression of leptin and its functional receptors include short and long isoforms (ObRl and ObRs) has been shown in both cancer and non-cancer endometrium (*Gonzales et al, 2000; Yuan et al, 2004; Sharma et al, 2006; Koda et al, 2007*). The levers of ObRl was similar in cancer and normal tissue, but the short isoforms were significantly decreased in malignant cells. Moreover, induction of the expression of this receptor resulted in inhibited proliferation of cancer cells due to delayed start of the mitotic S phase suggesting that loss of ObRs in endometrial cancer might contribute to malignant progression (*Yuan et al, 2004*).

A recent report demonstrated that leptin promotes endometrial cancer growth and invasiveness through STAT/MAPK and Akt pathways. Particularly, treatment with leptin resulted in increased proliferation and induces invasion of ECC1 and Ishikawa cells (*Sharma et al, 2006*).

However, the molecular mechanism involved in leptin induced endometrial cancer cell proliferation still needs to be elucidated.

In recent years, a large body of evidence has shown that disruption of cell cycle control mechanism is a common pathway in human cancer and over-expression of cyclin D1 is one of the most commonly observed alterations. Cyclin D1, an important cell cycle regulator is required for completion of the G1/S transition in normal mammalian cells (*Fu et al, 2004*). (Figure 3)

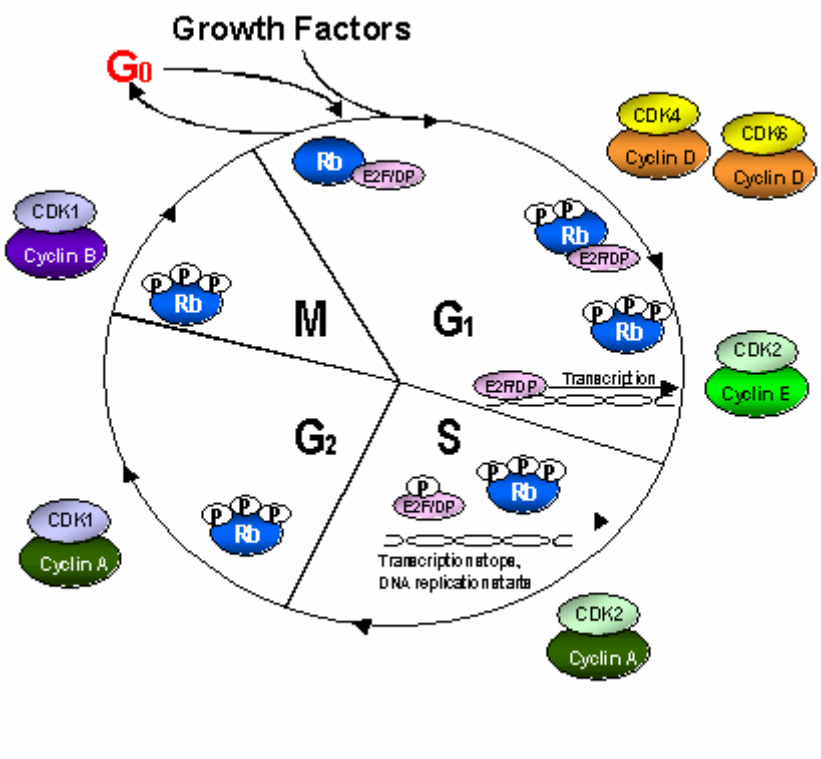


Figure 3. The mammalian cell cycle.

This cycle modulator increases from normal endometrium to hyperplasia and carcinoma, suggesting that it may play a role in endometrial carcinogenesis.

In this study, we explored the molecular mechanism eliciting the biological effect of leptin in endometrial carcinoma cells' growth. By performing a panel of different assays, we have demonstrated that leptin enhances cyclin D1 expression through STAT3 binding site (GAS) and cyclic AMP-response element (CRE) located

within the cyclin D1 promoter. Our findings have provided evidence for better understanding of the association between obesity and endometrial cancer progression.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin, streptomycin, fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate-buffered saline were purchased from Eurobio (Les Ullis Cedex, France).

Triazol reagent by Invitrogen (Carlsbad, CA).

FuGENE 6 by Roche Applied Science (Indianapolis, IN).

TaqDNA polymerase, RETROscript kit, TnT® T7/T3 coupled rabbit reticulocyte lysate system, 100-bp DNA ladder, Dual Luciferase kit, and TK Renilla luciferase plasmid were provided by Promega (Madison, WI).

Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, H89 and recombinant human leptin were purchased by Sigma (Milan, Italy).

Antibodies against phospho-CREB (ser133) (1B6), CREB (48H2), phospho p44/42 MAPK (Thr 202/Tyr 204) (#9101S), p44/42 MAPKinase (#9102) and U0126 (inhibitor of MAPK) were provided by Cell Signaling.

AG 490 were provided by Biomol (Milan, Italy).

Antibodies against Cyclin D1 (M-20), GAPDH (FL-335), P21 (H-164), pSTAT3 (B-7), and STAT3 (F-2) were provided by Santa Cruz Biotechnology.

An ECL system, [γ^{32}P]ATP, and Sephadex G-50 spin columns were purchased from Amersham Biosciences (Buckinghamshire, UK).

QuickChange kit (Stratagene, La Jolla, CA)

RNase A (Calbiochem, La Jolla, CA).

Plasmids

The plasmids containing the human cyclin D1 promoter or its deletions (p-2966/+142, p-944/+142, p-848/+142, p-136/+142) were kindly provided by Prof A. Weisz (University of Naples, Italy). These fragments were inserted into the luciferase vector pXP2.

Site-directed mutagenesis

The cyclin D1 promoter plasmids bearing STAT3 binding recognition site (GAS) mutated site (pGAS mut) and cyclic AMP-responsive element (CRE) mutated site (pCRE mut) were created by site-directed mutagenesis using QuickChange kit.

Briefly, this was based on a PCR reaction with two complementary oligonucleotide primers containing the mutation. The PCR was performed with the *Pfu* DNA polymerase during 16 cycles (30sec at 95°C, 30sec at 55°C and 8min at 68°C), using as template the human cyclin D1 promoter P-136/+142 and the following mutagenic primers:5'-CGGACTACAGGGGAGTAGCGTTGAAGTTGCAAAGTCCTGGAG-3' and 5'-CTCCAGGACTTTGCAACTTCAACGCTACTCCCCTGTAGTCCG-3' (GAS MUT); 5'-GATCTTTGCTTAACAACAGTAACTCTACACGGACTACAGGGGAG -3'and 5'-CTCCCCTGTAGTCCGTGTAGAGTTACTGTTGTTAAGCAAAGATC-3' (CRE MUT). To create the plasmid mutated in both responsive elements (pGAS/CRE mut), we used as template pCRE mut and the primers for GAS site above mentioned. The PCR products were then incubated with *Dpn*I which only digests the parental methylated cDNA and the constructed mutated expression vectors were confirmed by DNA sequencing.

Cell Culture

Ishikawa human endometrial cancer cells were obtained from D. Picard (University of Geneva, Geneva, Switzerland). Ishikawa cells were maintained in DMEM without phenol red supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. Cells were switched to medium without serum 48h before each experiment.

DNA Flow Cytometry

Ishikawa cells at 50-60% confluence were shifted to serum-free medium (SFM) for 48 h and then untreated or treated with 1000 ng/mL leptin in SFM for 24h. Thereafter, cells were trypsinized, centrifuged at 1500 rpm for 3 min, washed with PBS, and then treated with 20 μg/ml RNase A. DNA was stained with 100μg/ml propidium iodide for 30 min at 4 °C protected from light, and cells were analyzed with the FAC-Scan (Becton Dickingson and Co., Franklin Lakes, NJ).

Total RNA extraction and reverse transcription-PCR assay

Total cellular RNA was extracted from Ishikawa cells using Triazol reagent as suggested by the manufacturer. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of genes expression was performed by the reverse transcription-PCR method. cDNA was synthesized by oligo (dT) using a RETROscript kit as suggested by the manufacturer. The cDNAs obtained were further amplified by a PCR using the following primers: 5'-TCTAAGATGAAGGAGACC ATC-3' and 5'-GCGGTAGT AGGACAGGAAGTTGTT-3' (cyclin D1); 5'-GCTTC ATGCCAGCTACTTCC-3' and 5'-CTGTGCTCACTTCAGGGTCA-3' (p21); 5'-CTC AACATCTCCCCCTTCTC-3' and 5'-CAAATCCCATATCCTCGTCC-3' (36B4).

The PCR was performed for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min), 30 cycles for p21 (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) and 15 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) to amplify 36B4 in the presence of 1μl of first strand cDNA, 1 μM each of the primers

mentioned above, 0.5 mM dNTP, *Taq* DNA polymerase (2 units/tube), and 2.2 mM magnesium chloride in a final volume of 25 µl. To check for the presence of DNA contamination, a reverse transcription-PCR was performed on 1 µg of total RNA without Moloney murine leukemia virus reverse transcriptase (the negative control). The PCR products were analyzed on 2% agarose gel and stained with ethidium bromide. DNA quantity in each lane was analyzed by scanning densitometry. Standard DNA (100-bp DNA ladder) was run to provide the appropriate size marker.

Immunoblotting

Ishikawa cells were grown in 10 cm dishes to 50–60% confluence and lysed in 500 µl of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (aprotinin, PMSF, and sodium ortho-vanadate). Equal amounts of total protein were resolved on an 11% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, probed with rabbit polyclonal antiserum directed against the human cyclin D1 (1:1000) and p21(1:1000). The antigen-antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-rabbit IgG and revealed using the ECL System. The blots were then exposed to film, and the bands of interest were quantified by densitometer (model 620; Bio-Rad). The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%.

Transient transfection assay

Ishikawa cells were starved with serum free medium for 24 h and then transfected using the FuGENE 6 reagent with the mixture containing 0.25 μg of human cyclin D1 promoter constructs. Twenty-four hours after transfection, the cells were untreated or treated with 1000 ng/mL leptin for 6, 12 and 24h. TK Renilla luciferase plasmid (10 ng per each well) was used. Firefly and Renilla luciferase activities were measured by Dual Luciferase kit. The firefly luciferase data for each sample were normalized based on the transfection efficiency measured by Renilla luciferase activity.

Electrophoretic mobility shift assay

Nuclear extracts from Ishikawa cells were prepared as previously described (Anderws et al, 1991). Briefly, Ishikawa cells plated into 60 mm dishes were scraped into 1.5 ml of cold phosphate-buffered saline (PBS). Cells were pelleted for 10 sec and resuspended in 400 μl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 sec. Samples were then centrifuged for 2 min and the supernatant fraction was discarded. The pellet was resuspended in 50 μl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min

at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at –70°C. The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ^{32}P] ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are as follows: 5'-AGGGGAGTTTTGTTGAAGTTGCAAA-3' and 5'-TTTGCAACTTCAACAAAACTCCCCT-3' (GAS); 5'-TTAACAACAGTAACGT CACACGGACTA-3' and 5'-TAGTCCGTGTGACGTTACTGTTGTTAA-3' (CRE); 5'-AGGGGAGT***AGC***GTTGAAGTTGCAAA-3' and 5'-TTTGCAACTTCAAC***GCT*** ACTCCCCT-3' (GAS MUT); 5'-CTTAACAACAGTAA***TTG***CACACGGACTA-3' and 5'-TAGTCCGTGTG***CAA***TTACTGTTGTTAAG-3' (CRE MUT).

In vitro transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system. The protein-binding reactions were carried out in 20 mL of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/mL BSA, 50 μg/mL poly(dI/dC)] with 50,000 cpm of labeled probe, 20 μg of Ishikawa nuclear protein or an appropriate amount of CREB protein and 5 μg of poly (dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. For experiments involving STAT3 and CREB antibodies, the reaction mixture was incubated with these antibodies at 4°C for 12h. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25x Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at -70°C.

Chromatin immunoprecipitation assay

According to the ChIP assay procedure previously described (*Shang et al, 2000*), Ishikawa cells were grown in 100 mm dishes to 50-60% confluence, shifted to serum free medium (SFM) for 24 hours and then untreated or treated with 1000 ng/mL leptin for 10 min, 30 min and 1 h. Thereafter, cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 min. Then, cells were sonicated four times for 10 sec at 30% of maximal power (Sonics, Vibra Cell 500W) and collected by centrifugation at 4°C for 10 min at 14 000 rpm. The supernatants were diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl) and immunocleared with 80 µl of sonicated salmon sperm DNA/protein A agarose for 1 hour at 4°C. The precleared chromatin was immunoprecipitated with a specific anti-STAT3, anti-CREB antibodies and with a normal mouse serum IgG (Nms) as negative control. At this point, 60 µl salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 hours at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The

immunocomplexes were eluated with elution buffer (1% SDS, 0.1 M $NaHCO_3$), reverse crosslinked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for 1 hour. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. 2 µl of 10 mg/ml yeast tRNA were added to each sample and DNA was precipitated with 95% EtOH for 24 hours at –20°C, and then washed with 70% EtOH and resuspended in 20 µl of TE buffer. 3 µl of each sample were used for PCR amplification with the following primers flanking GAS/CRE sequence present in the cyclin D1 promoter region: 5'-TGCGCCCGCCCCCGCCCCCCTC-3' and 5'-TGTTCCATGGCTGGGGCTCTT-3'. The PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The amplification products obtained in 35 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Each datum point represents the mean ± S.E. of three different experiments. Data were analyzed by ANOVA test using the STATPAC computer program.

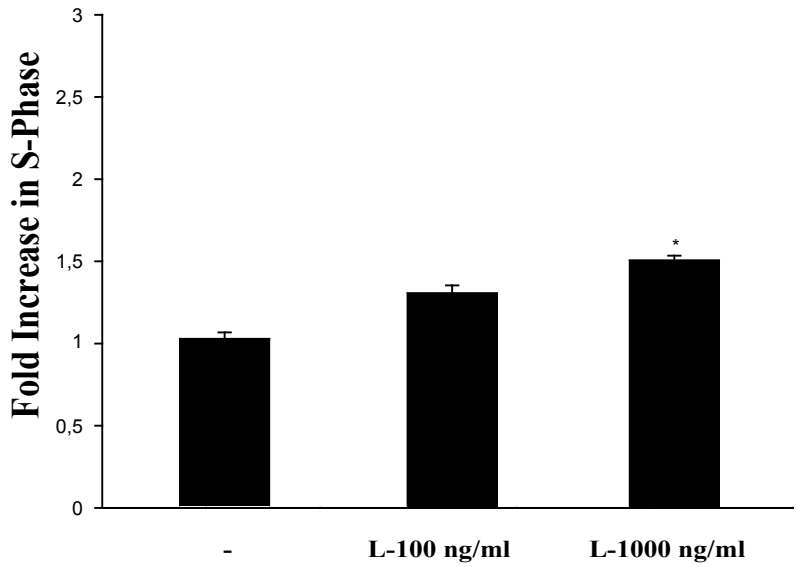
Results

Leptin modulates cell cycle progression in endometrial cancer cells

On the basis of previous studies demonstrating the expression of the Ob and ObR in both cancer and non-cancer endometrium (*Gonzalez et al, 2000; Kitawaki et al, 2000; Koshiba et al, 2001*), and the stimulatory effects of leptin on proliferation of Ishikawa and ECC1 cells in a time- and dose-dependent manner (*Sharma et al, 2006*), we first investigated the role of leptin on Ishikawa cell cycle progression. Cells were synchronized with serum starvation for 48h and then induced to re-enter the cell cycle by treatment with hormone. Flow cytometric analysis revealed that 24h leptin 1000 ng/ml treatment significantly reduced the numbers of G0/G1-phase cells accompanied with the increase of cell population in S phase, compared with control group (Fig. 4).

Leptin enhances Cyclin D1 and down-regulates $p21^{WAF1/Cip1}$ expression in Ishikawa cells

Considering that the cyclin D1 is a critical modulator in the cell cycle G1/S transition and its over-expression is one of the most commonly observed alterations in human endometrial cancers (*Horn et al, 2006*), we aimed to examine the potential ability of leptin to modulate cyclin D1 mRNA and protein content in Ishikawa human endometrial cancer cells. Results of RT-PCR showed an increased cyclin D1 mRNA after the treatment with leptin 1000 ng/ml for 6, 12 and 24 h. mRNA expression of the cyclin D1 gene was normalized using the human housekeeping gene 36B4 (Fig. 5A). The leptin-induced expression of cyclin D1 was confirmed at protein level, at all



	S-Phase	G2/M	G0/G1
-	28 ± 1	20 ± 1,2	52 ± 0,76
L-100 ng/ml	35 ± 1,2	23 ± 0,89	42 ± 2
L-1000 ng/ml	41 ± 0,73	24 ± 1,17	35 ± 1,76

Figure 4 Leptin increases the fraction of Ishikawa cells in the S phase of the cell cycle. Ishikawa cells were synchronized in serum-free medium for 48 h and were exposed to 100 ng/ml and 1000 ng/ml leptin for 24 h or left untreated (-). The distribution of Ishikawa cells in the cycle was determined by flow cytometry using propidium-iodide stained nuclei. The results indicate the fold increase of Ishikawa cells in S phase after serum starvation or leptin treatment. The histograms represent the means S.E. of three separate experiments done in triplicate. * P <0,01, compared with serum-starved condition. The table shows the distribution of Ishikawa cells in the various phases of cell cycle.

times investigated, by Western blotting analysis (Fig. 5B).

To further study the involvement of leptin in cell cycle progression, the expression of $p21^{WAF1/Cip1}$, which plays as a major negative regulator in the G1 checkpoint, was analyzed. $p21^{WAF1/Cip1}$ mRNA and protein levels were decreased in leptin treated samples than untreated cells (Figs. 5A and 5B).

These observations indicate that leptin by stimulating expression of the positive regulator, cyclin D1, and inhibiting the expression of the negative regulator $p21^{WAF1/Cip1}$**,** promotes the entry of G1 into S phase in cell cycle progression.

Leptin-induced cyclin D1 expression is STAT, MAPK and PKA dependent in proliferating Ishikawa cells.

Leptin exerts its biological functions through binding to its receptors that mediate a downstream signal by activating multiple signaling pathways (*Garofalo et al, 2006*). To gain insight into the mechanism underlying the modulatory role of leptin on cyclin D1 expression in endometrial cancer cells, we examined the changes in signal transductional pathways involved in mediating leptin action. Cellular proteins were extracted from Ishikawa cells treated with 1000 ng/ml leptin for various time periods, and by western blotting analysis we determined the status of STAT3 and ERK1/2 phosphorylation. As shown in Figure 6A, leptin significantly induced phosphorylation of STAT3 within 15 minutes of treatment while an increased phosphorylation of ERK was observed after 5 minutes of leptin stimulation followed by a decline (Fig. 6B). Besides, we also examined effect of leptin on phosphorylated

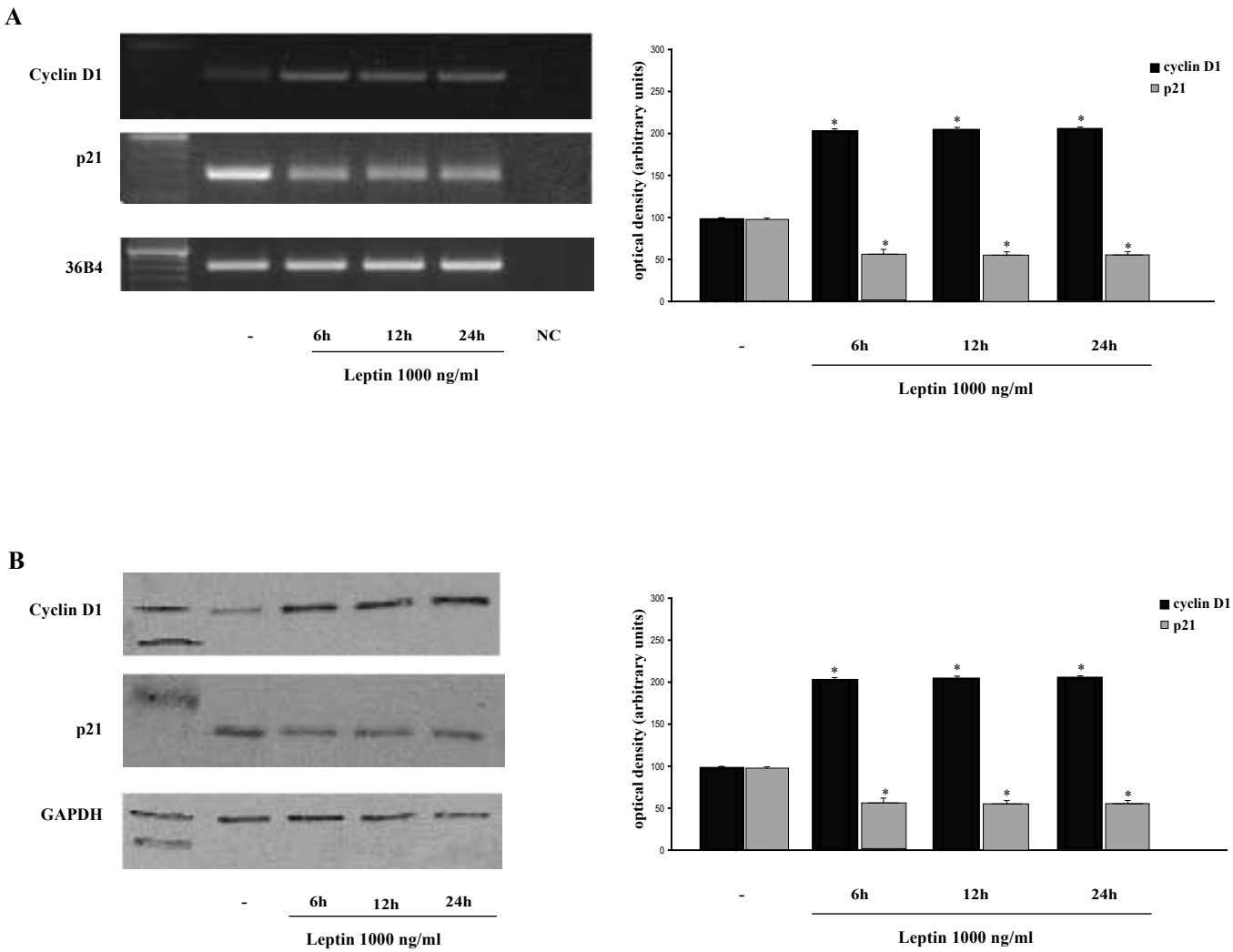


Figure 5 Effects of Leptin on cyclin D1 and p21WAF1/Cip1 expression in Ishikawa cells.
Ishikawa cells were serum-starved for 48 h followed by treatment with 1000 ng/ml leptin for 6, 12 and 24 h or left untreated (-). A, total RNA was isolated from Ishikawa cells and reverse transcribed. cDNA was subjected to PCR using specific primers for cyclin D1 (30 cycles), p21 (30 cycles) or 36B4 (15 cycles). NC: negative control, RNA sample without the addition of reverse transcriptase. B, Protein extracts obtained from Ishikawa cells were immunoblotted with rabbit polyclonal antiserum against human cyclin D1 and p21. GAPDH served as loading control. The histograms represent the means S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.01$ compared to vehicle.

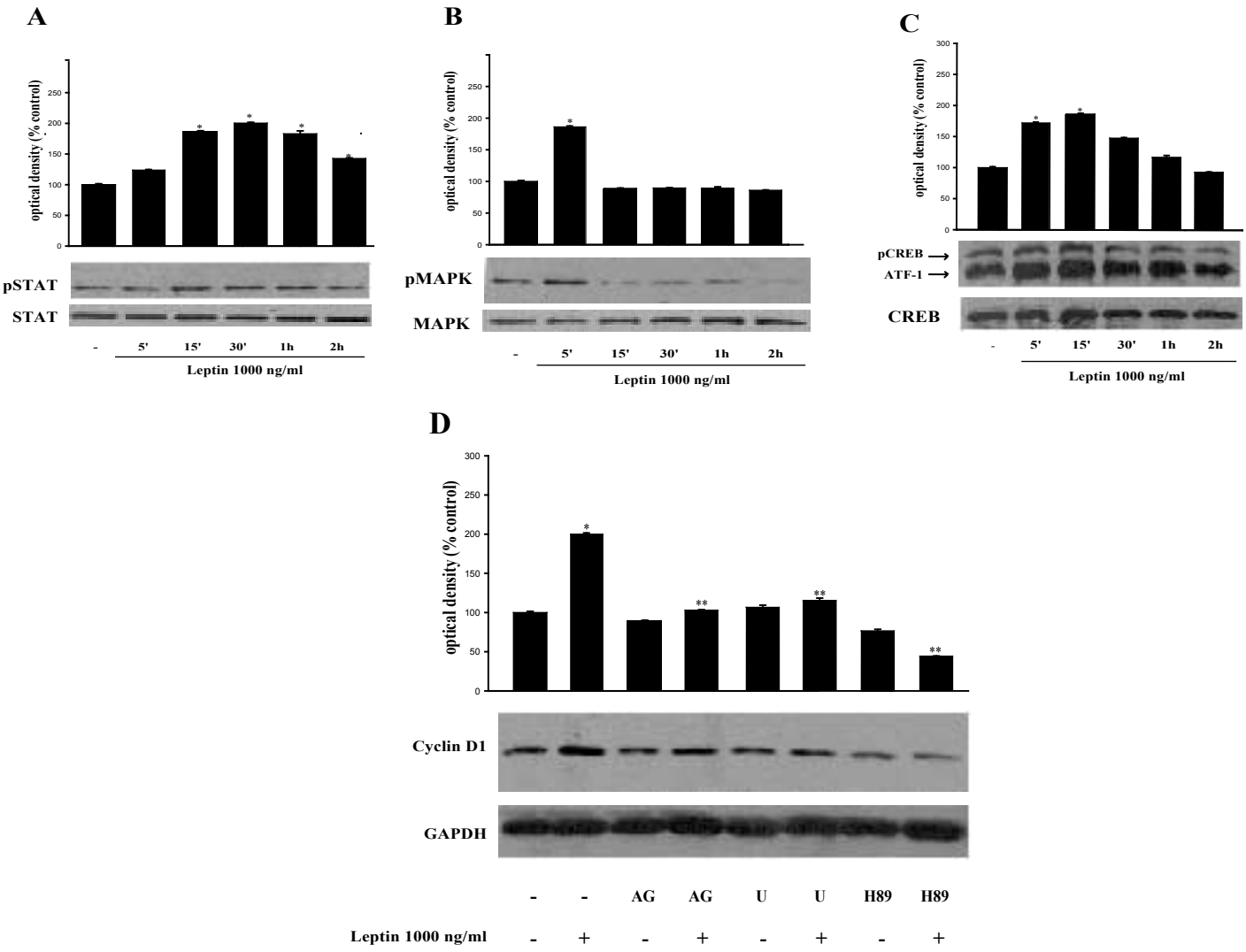


Figure 6 Activation of leptin signaling in up-regulation of cyclin D1 expression.
Ishikawa cells were serum-starved for 48 h and treated with 1000 ng/ml leptin for various time intervals or left untreated (-). Protein extracts obtained from Ishikawa cells were immunoblotted with a specific antibodies against total or phosphorylated (p) forms of STAT3 (A), MAPK (B) and CREB/ATF-1 (C). The histograms represent the means S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.05$ compared to vehicle. (D) Ishikawa cells were serum-starved for 48 h and treated with 1000 ng/ml leptin for 24 h or left untreated (-). For combined treatment, cells were pretreated with AG490 (20 uM), U0126 (10 uM) and H89 (10 uM) for 30 min followed leptin treatment. The histograms represent the means S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.01$ compared to vehicle; ** $p<0.01$ compared to leptin treatment.

CREB/ATF-1, a downstream substrate of MAPK, but also an effector of PKA (*Delghandi et al, 2005*). The CREB Ab produces two bands and recognizes both CREB (upper band) and ATF-1 (lower band). Notably, leptin exposure for 15 minutes significantly induced phosphorylation of CREB/ATF1 (Fig. 6C).

Leptin had no effect on total STAT3, ERK and CREB protein expression levels.

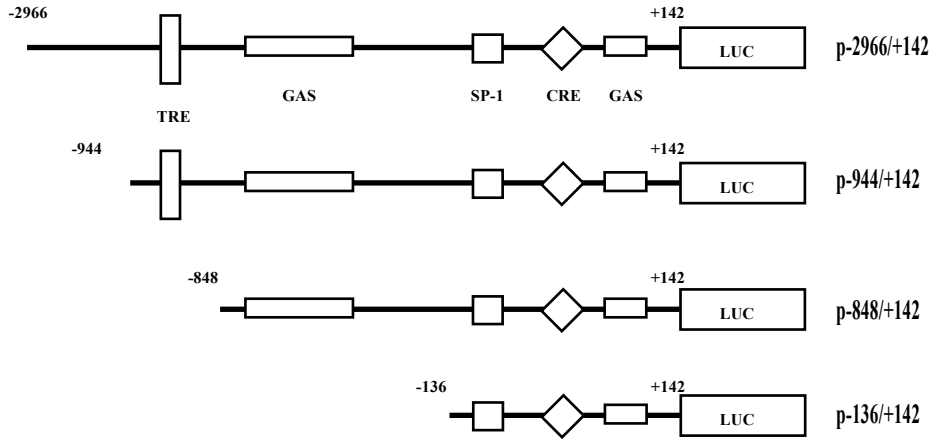
Next, to investigate the signal transduction pathways involved in leptin-induced cyclin D1 expression, chemical inhibitor of JAK/STAT (AG490), ERK1/2 (U0126) and PKA (H89) were added to serum starved Ishikawa cells before the treatment with 1000 ng/ml of leptin. Our results revealed that, AG490, U0126 and H89 effectively prevent leptin induction of cyclin D1 expression level (Fig. 6D).

Effects of leptin on activity of human cyclin D1 promoter/luciferase reporter gene constructs in Ishikawa cells.

To evaluate whether leptin is able to activate cyclin D1 promoter, we transiently transfected Ishikawa cells with a luciferase reporter construct containing the upstream region of the cyclin D1 gene spanning from -2966 to + 142. As shown in Figure 7, a significant increase in promoter activity was observed in the transfected cells exposed to leptin 1000 ng/ml for 6, 12 and 24 h.

Cyclin D1 promoter contains multiple regulatory elements, including binding sites for AP-1, STATs, NF-kB, Oct-1, Sp1, CRE and TCF/LEF (*Saxena et al, 2007; Bartusel et al, 2005; Brockman et al, 2005; Natsume et al, 2003; Allan AL et al, 2001*).

A



B

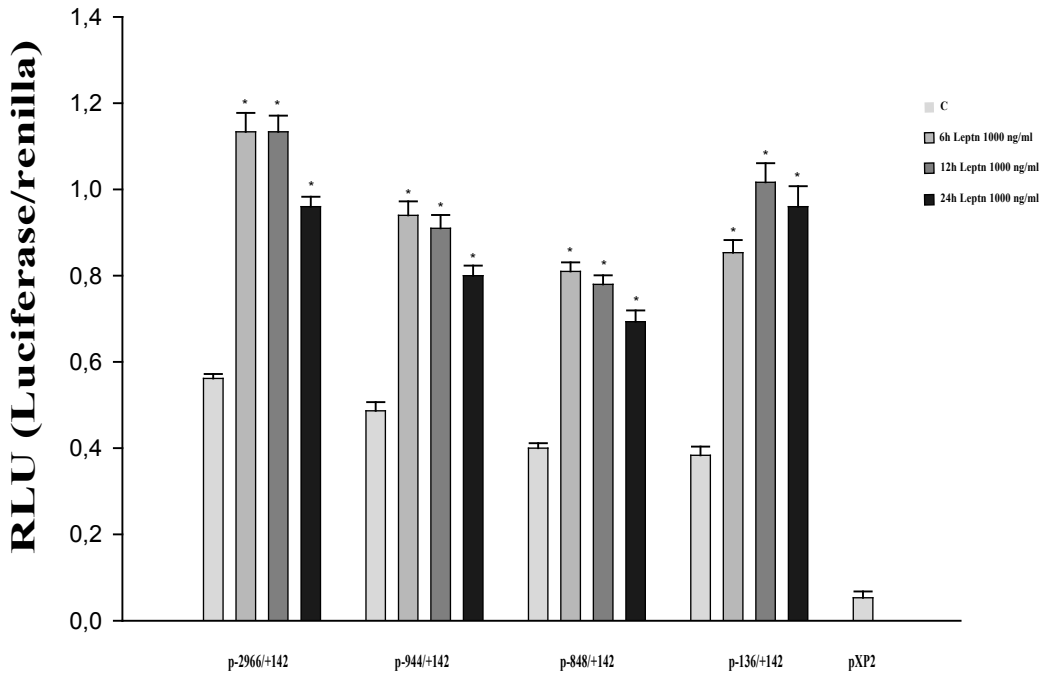


Figure 7 Leptin enhances human cyclin D1 promoter in Ishikawa cells.
A. Schematic representation of human cyclin D1 promoter fragments used in this study. All of the promoter constructs contain the same 3' boundary (+142). The 5' boundaries of the promoter fragments varied from -2966 to -136. Each fragment was subcloned into the pXP2 vector. B. Transcriptional activity of Ishikawa cells with promoter constructs is shown. Ishikawa cells were serum-starved for 24 h, transfected for 24 h and left untreated (-) or treated with 1000 ng/ml leptin for 6, 12 and 24 h. The values represent the means S.E. of three separate experiments. In each experiment, the activities of the transfected plasmid was assayed in triplicate transfections. pXP2: basal activity measured in cells transfected with pXP2 basal vector.
* $p<0.01$ compared to vehicle.

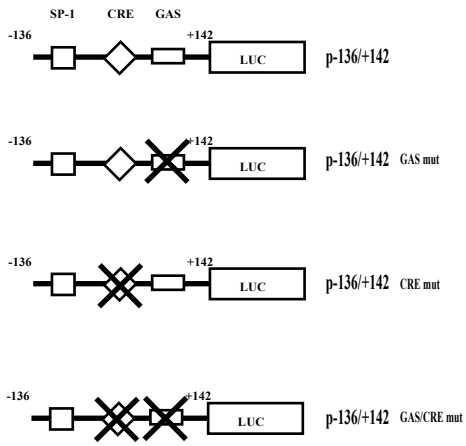
To delimit the *cis*-element involved in cyclin D1 transcriptional activation by leptin, we transiently transfected Ishikawa cells with plasmids containing a series of 5’ deleted segments of human cyclin D1 promoter. Schematic representation of constructs is shown in Figure 7A.

In transfection experiments performed using p-944/+142, p-848/+142 and p-136/+142 the responsiveness to leptin was still observed at all time investigated (Fig. 7B), suggesting that the region from -136 to +142 was required for the trans-activation of cyclin D1 by leptin.

The nucleotide sequence analysis of this region evidenced STAT3 binding motif (GAS) and a cyclic AMP-responsive element (CRE) located at position -52 and -27 respectively, putative effectors of leptin signaling as previously demonstrated in other systems (*Mauro et al, 2007, Saxena et al, 2007*). Thus, mutation analysis of the GAS and CRE sites on cyclin D1 promoter was carried out to define further their role for functional interaction with leptin.

The effects of leptin on different GAS and CRE mutant constructs showed that mutation of the GAS site and CRE alone moderately affected the regulation of cyclin D1 promoter activity by leptin (Fig. 8B). In contrast, mutation of both GAS and CRE completely abolished leptin responsiveness of cyclin D1 promoter in Ishikawa cells (Fig. 8B). These results suggest that leptin signaling pathways stimulate cyclin D1 transcription through both GAS and CRE motifs. Whereas either of these two sites may be sufficient alone to induce cyclin D1 promoter activity, both of them are necessary for optimal leptin action.

A



B

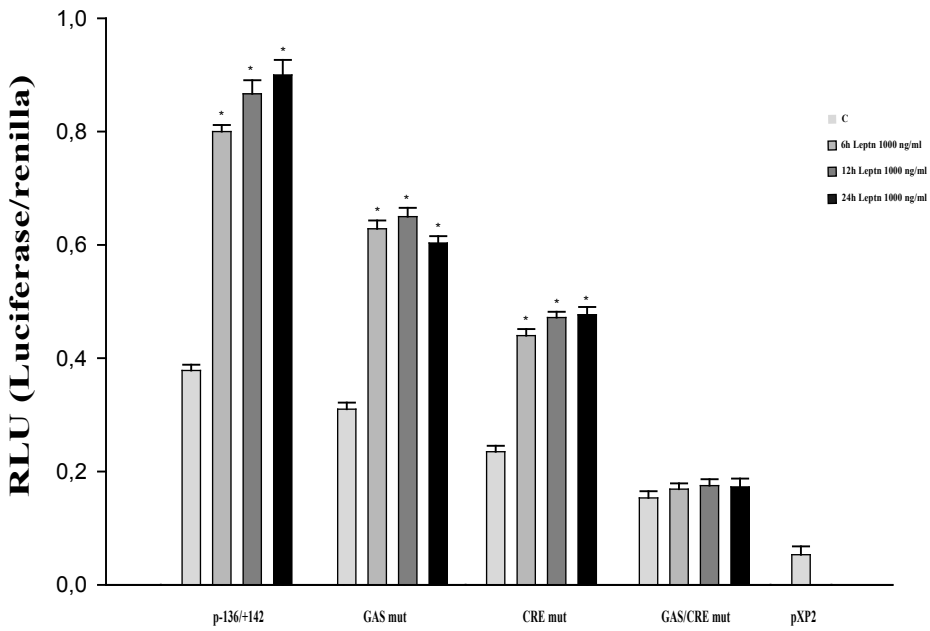


Figure 8 Leptin transactivates cyclin D1 gene promoter.
A. Schematic representation of the mutated plasmids used in this study. B.Transcriptional activity of Ishikawa cells with promoter constructs is shown. Ishikawa cells were serum-starved for 24 h, transfected for 24 h and left untreated (-) or treated with 1000 ng/ml leptin for 6, 12 and 24 h. The values represent the means S.E. of three separate experiments. In each experiment, the activities of the transfected plasmid was assayed in triplicate transfections. pXP2: basal activity measured in cells transfected with pXP2 basal vector. * $p<0.01$ compared to vehicle.

Leptin increases STAT3-DNA and CREB-DNA binding activity to cyclin D1 promoter.

To further define whether GAS and CRE are responsible for the transcriptional activation of cyclin D1 by leptin, we performed EMSA experiments.

Using synthetic oligodeoxyribonucleotides corresponding to the GAS and CRE motifs, we observed the formation of a complex in nuclear extract Ishikawa cells (Figs. 9 and 10, *lane 1*), which was abrogated by 100 fold molar excess of unlabeled probe (Figs. 9 and 10, *lane 2*) demonstrating the specificity of the DNA binding complex. This inhibition was not observed when a mutated oligodeoxyribonucleotides were used as competitor (Figs. 9 and 10, *lane 3*). Leptin induced both GAS and CRE activation compared with untreated at the same time-point (Figs. 9 and 10, *lane 4, 5, 6*). Incubation of anti-STAT3 with the nuclear extracts resulted in a greatly reduced band, indicating the presence of STAT3 protein in the complex (Fig. 9, *lane 7*). Similarly, incubation of anti-CREB with the nuclear extracts resulted in reduced and supershifted bands (Fig. 10, *lane 7*). IgG did not affect either GAS or CRE complex formation (Figs. 9 and 10, *lane 8*). Using transcribed and translated *in vitro* CREB protein, we obtained a complex migrating at the same level as that of Ishikawa nuclear extracts (Fig. 10, *lane 9*).

Leptin enhances recruitment of STAT3 and CREB to the promoter region of cyclin D1.

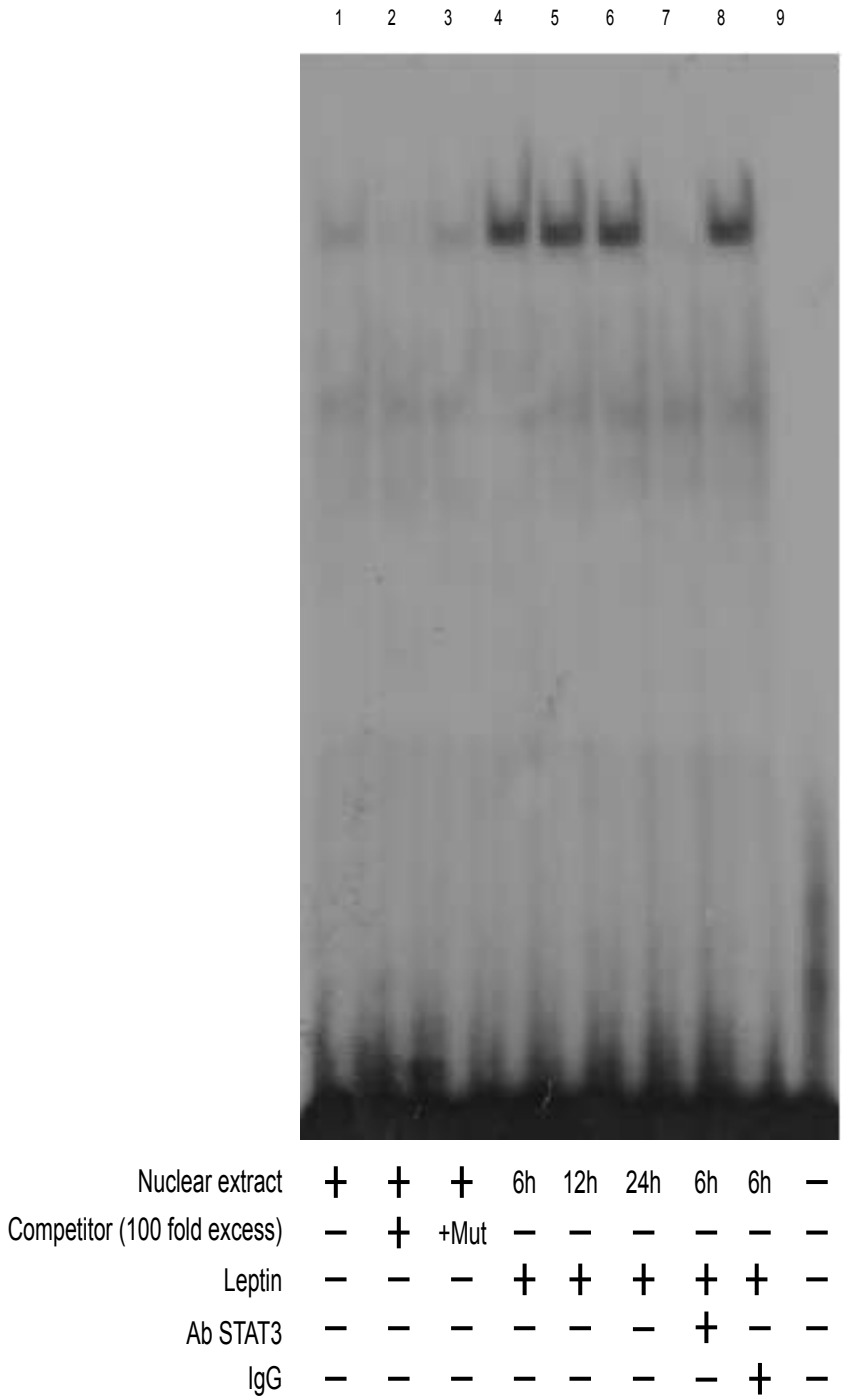


Figure 9 Effects of *in vitro* leptin treatment on STAT3-DNA binding activity in Ishikawa cells.
Nuclear extracts from Ishikawa cells were incubated with a double-stranded STAT3-specific consensus sequence probe labeled with [32P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lane 2) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated GAS (lane 3). Ishikawa nuclear extracts treated with 1000 ng/ml leptin for 6, 12 and 24 h incubated with probe (lane 4, 5 and 6). The specificity of the binding was tested by adding to the reaction mixture a STAT3 antibody (lane 7). IgG did not affect either GAS complex formation (lane 8). Lane 9 contains probe alone.

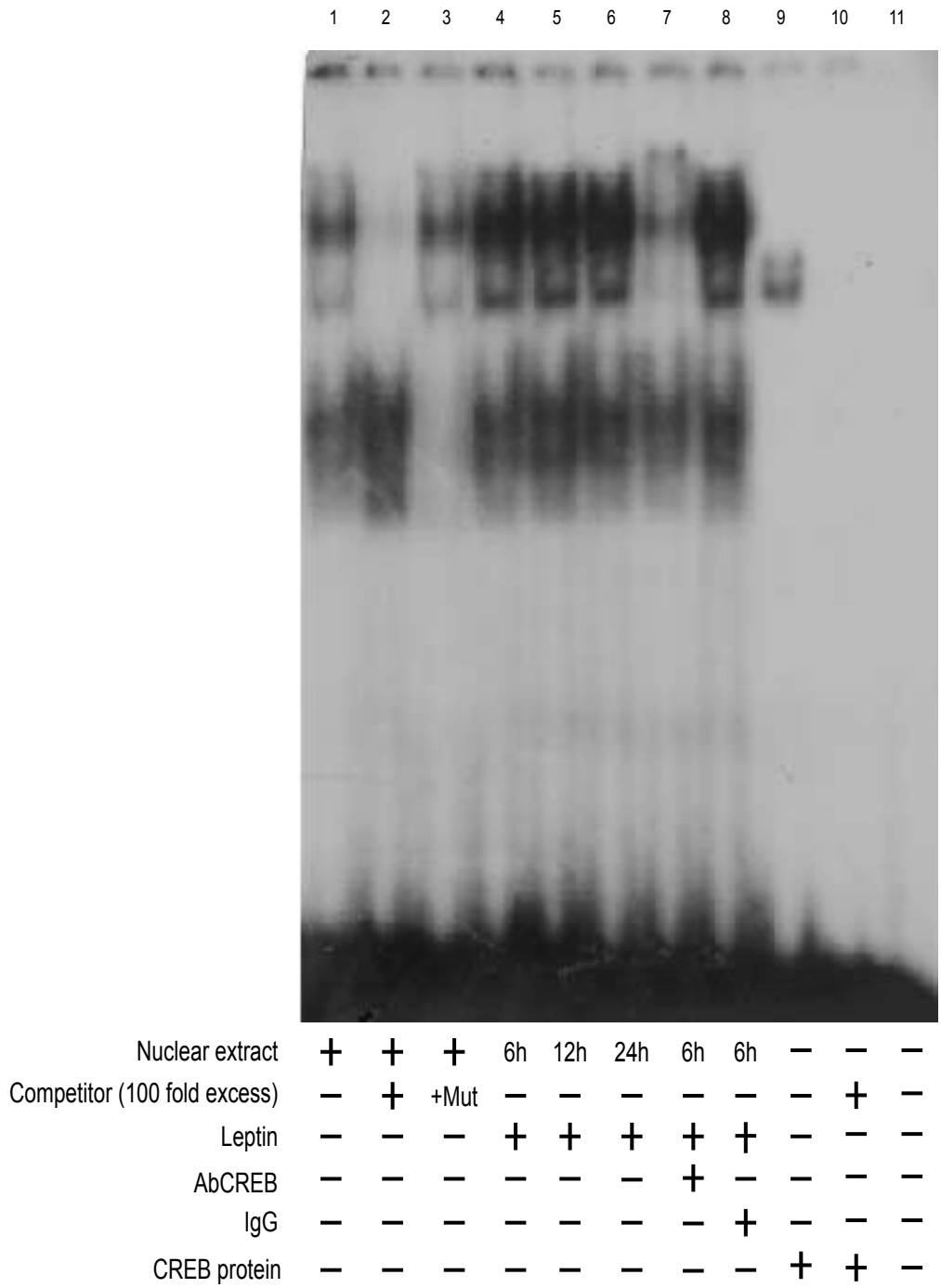


Figure 10 Effects of in vitro leptin treatment on CREB-DNA binding activity in Ishikawa cells.
Nuclear extracts from Ishikawa cells were incubated with a double-stranded CREB-specific consensus sequence probe labeled with [32P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lane 2) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated CRE (lane 3). Ishikawa nuclear extracts treated with 1000 ng/ml leptinfor 6, 12 and 24 h incubated with probe (lane 4, 5 and 6). The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (lane 7). IgG did not affect either CRE complex formation (lane 8). We used as positive control a transcribed and translated *in vitro* CREB protein (lane 9) or in combination with 100-fold molar exess of unlabeled probe (lane 10). Lane 11 contains probe alone.

Although our findings clearly demonstrated the role of STAT3 and CREB in leptin mediated regulation of cyclin D1 promoter, we further sought to determine that STAT3 and CREB directly participate in leptin mediated cyclin D1 gene regulation using chromatin immunoprecipitation assay. Using specific antibodies against STAT3, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from Ishikawa cells cultured with or without leptin 1000 ng/ml for various periods. The resulting precipitated genomic DNA was then analyzed by PCR using primers spanning the STAT3 binding elements in the promoter region of the cyclin D1. As shown in Figure 11A, ChIP analysis with anti-STAT3 antibodies revealed that treatment with leptin for 1h increased STAT3 recruitment to cyclin D1 promoter. Interestingly, we also observed upon leptin stimulation a significant increase in CREB recruitment to the cyclin D1 promoter as evidenced by ChIP analysis using anti-CREB antibody (Fig. 11B). Our data suggest that cyclin D1 may be a target for leptin mediated growth stimulation of Ishikawa cells and molecular mechanisms involve recruitment of STAT3 and CREB transcription factors.

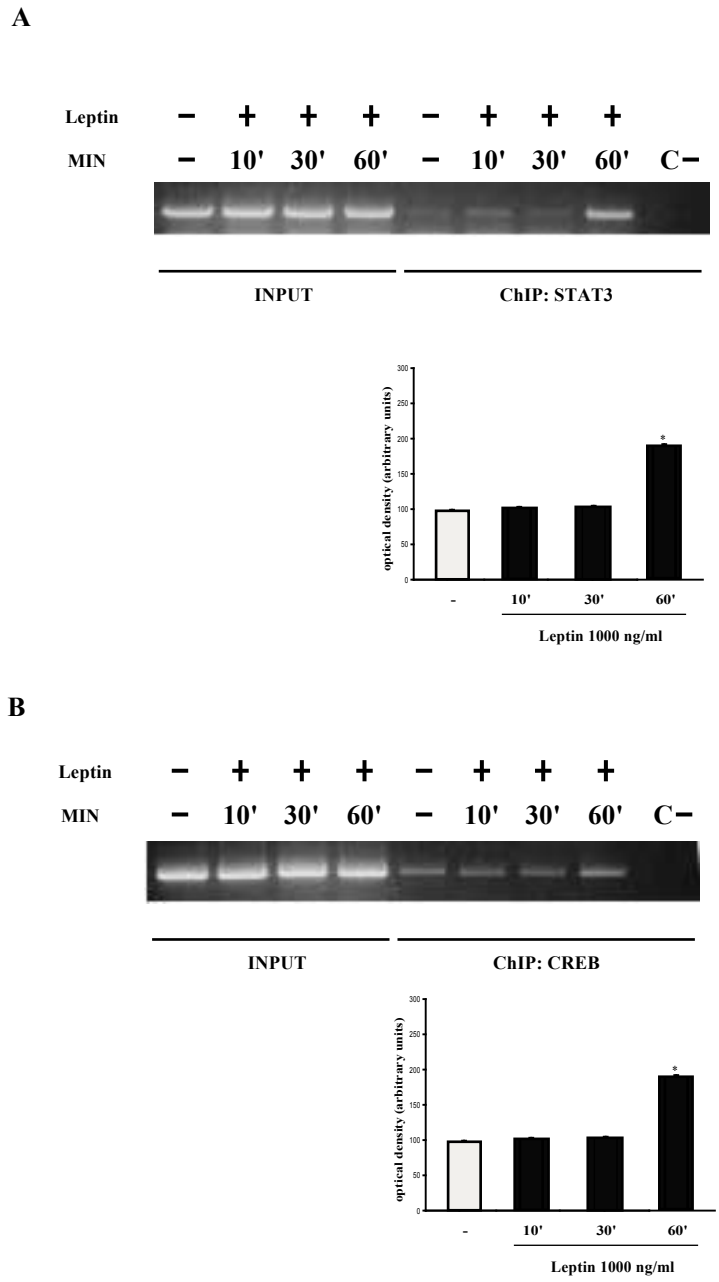


Figure 11 Recruitment of STAT3 and CREB to the cyclin D1 promoter in Ishikawa cells. The cells were serum-starved for 48 h and left untreated (-) or treated with 1000 ng/ml leptin for various time intervals. The preacleared chromatin was immunoprecipitated with specific antibody anti-STAT3 and anti-CREB. Cyclin D1 promoter sequences contain GAS and CRE sites was detected by PCR with specific primers, as detailed in Materials and Methods. To determine input DNA, the cyclin D1 promoter fragment was amplified from 3 l, purified soluble chromatin before immunoprecipitation. PCR products obtained at 35 cycles. ChIP with non-immune IgG was used as negative control (C-). The histograms represent the means S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.01$ compared to vehicle.

Discussion

Increasing epidemiologic data in humans and many *in vitro* investigative reports have linked obesity with various disease states and suggested a strong link between leptin and tumor progression (*Garofalo et al, 2006; Somasundar et al, 2003*). Indeed, several reports have described a mitogenic effect of leptin on gastric (*Goiot et al, 2001*), breast (*Okumura et al, 2002*), ovarian (*Choi et al, 2004*) and prostate cancer cells (*Somasundar et al, 2004*).

Leptin levels have been shown to have a positive correlation with endometrial cancer (*Petridou et al, 2002; Yuan et al, 2004*). For instance, both short and long isoforms of leptin receptor mRNA and proteins, were expressed in endometrial cancer (*Gonzales et al, 2000; Yuan et al, 2004; Sharma et al, 2006; Koda et al, 2007*). However, even thought, a growth stimulatory effect of leptin in human endometrial cancer cells was recently proposed (*Sharma et al, 2006*), the molecular mechanisms remains to be fully elucidated.

Thus, in the present study, we focused, in Ishikawa endometrial cancer cells, leptin signaling on cell cycle progression. Our experimental results showed that leptin treatment is able to speed up the progression reducing G0/G1 arrest with the increase of cell population in S phase, in a dose-dependent manner.

The cell cycle is regulated by the coordinate action of cyclin-dependent kinases (cdk), specific cyclin proteins and cdk inhibitors (*Hilakivi-Clarke et al, 2004*). Cyclin D1 and cyclin-dependent kinases are required for completion of the G1/S transition in normal mammalian cells (*Fu et al, 2004*).

In recent years, a large body of evidences have shown that overexpression of cyclin D1 is one of the most commonly observed alterations in human cancer, bringing cell cycle as a critical interface between hormonal signaling and tumorgenesis (*Chen et al, 2007*). Particularly, cyclin D1 overexpression in endometrial glands increases progressively in intensity and extent from normal endometrium to complex hyperplasia and carcinoma (*Ruhul et al, 2001*).

Of interest, we found that leptin exposure up-regulates both cyclin D1 mRNA and protein levels at all time investigated with a concomitant decrease of $p21^{WAF1/Cip1}$ expression.

In addition, in our study, we demonstrated that leptin stimulated cyclin D1 expression requires JAK/ STAT, MAPK and PKA activation, as it emerges by the observation that the chemical inhibitors of the above mentioned pathways completely reversed the increase of cyclin D1 protein levels.

It is worth noting that our findings recall previous reports indicating the involvement of JAK/STAT and MAPK signaling pathways in leptin mediated cell growth in diverse cellular contexts (*Dieudonne et al, 2002*; *Hardwick et al, 2001; Choi et al*, *2004*). For instance, recently, in Ishikawa endometrial cancer cells, leptin through ERK1/2 has been linked to cell proliferation (*Sharma et al, 2006*; *Gong et al, 2007*), whereas in MCF-7 cells, we evidenced that leptin signaling through ERK1/2 is able to potentiate estrogen action and aromatase activity promoting breast cancer cell growth (*Catalano et al, 2003; Catalano et al, 2004*).

It is well documented how RAS-MAPK pathway induces activation of CREB

kinase, a member of the $p90^{RSK}$ family that corresponds to RSK2 and thereby phosphorylates CREB Ser^{133} (*Xing et al, 1996; Dalby et al, 1998; Bannister et al, 1995*).

Although CREB is a major downstream substrate of ERK1/2, it is also classically known as a PKA effector. The interrelationship of PKA and JAK/STAT-dependent intracellular mechanism of leptin action was previously suggested (*Matsuoka et al, 1999*) as well as the involvement of PKA in leptin induced human ovarian proliferation (*Sirotkin et al, 2007*). These observations well fit with our results demonstrating a significant increases of CREB/ATF-1 phosphorylation upon leptin exposure.

Therefore, investigating the potential ability of leptin to modulate cyclin D1 promoter gene, we performed transient transfection experiments in Ishikawa cells using diverse deletion constructs of cyclin D1 promoter gene. The results indicated that leptin signaling up-regulates the full-length promoter activity of cyclin D1. Moreover, we documented that the region spanning from -136 to +142, which contains GAS and CRE sites as potential target of leptin, is required for the responsiveness to leptin. Our mutation analyses of the GAS and CRE sites on cyclin D1 promoter showed that both motifs are the mediators of cyclin D1 regulation by leptin. The results of mutants suggest that, whereas loss of either GAS or CRE alone leads to a partial reduction of cyclin D1 promoter activity, loss of both completely abolished leptin-induced promoter activation.

Under the present experimental conditions, our data imply that leptin can act on

the cyclin D1 promoter through two different responsive elements and must have at least one of these sites to augment cyclin D1 promoter activity.

Previously, in breast cancer cells, Leslie et al reported that cyclin D1 is transcriptionally regulated by STAT3 (*Leslie et al, 2006*) and activation of cyclin D1 through CRE by estrogens has been suggested (*Liu et al, 2002; Castro-Rivera et al, 2001; Sabbah et al, 1999*). Moreover, GAS and CRE has been shown to be a potential target of leptin signaling. Indeed, a recent work, showed that leptin-activated STAT3 binds to its cognate sites in cyclin D1 promoter leading to hyperacetylation and overexpression of cyclin D1 gene through a recruitment of distinct coactivator complexes (*Saxena et al, 2007*). On the other hand, our previous findings reported, in MCF-7 breast cancer cell line, activation of E-cadherin gene promoter by leptin through CRE site (*Mauro et al, 2007*).

Our EMSA experiments extended the aforementioned observations because nuclear extracts from Ishikawa cells treated with leptin showed an increased binding to the GAS and CRE sequence located in the cyclin D1 promoter region. These findings were supported by ChIP assay demonstrating the ability of leptin to enhance the recruitment of STAT3 and CREB to the promoter of cyclin D1.

Overall, these results indicate that the leptin mediated growth in Ishikawa cells involves, at least in part, the direct stimulation of cyclin transcription and sustain the molecular basis of a direct association between obesity and endometrial carcinogenesis.

In summary, these data, for the first time, elucidated the molecular mechanisms

by which leptin activates cyclin D1 expression in Ishikawa cells. The transcriptional pathways engaged by leptin receptor signaling occur through two distinct transcription factor binding sites in the cyclin D1 promoter region which may be considered as potential target of novel pharmacological tools for endometrial cancer treatment particularly in obese women.

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Fas Ligand Expression in TM4 Sertoli Cells is Enhanced by Estradiol "In situ" Production

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The testis is an immunologically privileged site of the body where Sertoli cells work on to favor local immune tolerance by testicular autoantigens segregation and immunosuppressive factors secretion. Fas/Fas Ligand (FasL) system, expressed prevalently in Sertoli cells, has been considered to be one of the central mechanisms in testis immunological homeostasis. In different cell lines it has been reported that the proapoptotic protein FasL is regulated by 17-β estradiol (E2). Thus, using as experimental model mouse Sertoli cells TM4, which conserve a large spectrum of functional features present in native Sertoli cells, like aromatase activity, we investigated if estradiol "in situ" production may influence FasL expression. Our results demonstrate that an aromatizable androgen like androst-4-ene-3,17-dione (Δ4) enhanced FasL mRNA, protein content and promoter activity in TM4 cells. The treatment with N^6,2′-*O*-dibutyryladenosine-3′-5′-cyclic monophosphate [$(Bu)_2$cAMP] (simulating FSH action), that is well known to stimulate aromatase activity in Sertoli cells, amplified Δ4 induced FasL expression. Functional studies of mutagenesis, electrophoretic mobility shift (EMSA) and chromatin immunoprecipitation (ChIP) assays revealed that the Sp-1 motif on FasL promoter was required for E2 enhanced FasL expression in TM4 cells. These data let us to recruit FasL among those genes whose expression is up-regulated by E2 through a direct interaction of ERα with Sp-1 protein. Finally, evidence that an aromatizable androgen is able to increase FasL expression suggests that E2 production by aromatase activity may contribute to maintain the immunoprivilege status of Sertoli cells.

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The immunoprivilege of male gonad lies on blood-testis barrier, prevalently maintained by Sertoli cell functions. This physical barrier between the general circulation and testicular tissue probably conceals antigens from the immune system and prevents effector cell access (Filippini et al., 2001; Bart et al., 2002; Ferguson et al., 2002). This immune protective function together with the secretion of hormonal and nutritive factors produced by Sertoli cells, under FSH control, substain germ cells functional maturation along all spermatogenesis process (Griswold et al., 1988; De Cesaris et al., 1992).

The Fas/FasL system was first identified in T cells (Suda et al., 1993; Lynch et al., 1995) where it plays a key role in eliminating T cell populations following antigenic stimulation and clonal proliferation. This system is also functional in the testis (Bellgrau et al., 1995; Sanberg et al., 1996) and in a variety of other tissues in which these proteins are constitutively expressed to maintain their immunoprivilege, such as eyes (Griffith et al., 1995), placenta (Guller, 1997; Uckman et al., 1997) and brain (Saas et al., 1997).

FasL is a type II trans-membrane protein that belongs to the tumor necrosis factor (TNF) family of cytokines and induces apoptosis in cells expressing Fas receptors (Suda et al., 1993). Fas (CD95, APO-1) is a transmembrane receptor protein, sharing a high degree of homology with the tumor necrosis factor/nerve growth factor receptor family (TNF/NGF-Rs) (Watanabe-Fukunaga et al., 1992; Nagata and Goldstein, 1995). It is characterized by an intracellular domain called "death domain" responsible for the activation of the intracellular signaling pathway following Fas-FasL interaction (Nagata and Goldstein, 1995).

The Fas/FasL expression during testicular development and its cell specific localization within the testis is still a matter of debate, but it is generally assumed that FasL is predominantly expressed in Sertoli cells (Suda et al., 1993; Bellgrau et al., 1995; French et al., 1996; Lee et al., 1997; Francavilla et al., 2000; D'Abrizio et al., 2004).

Among the different factors influencing FasL, it has been reported that 17-β estradiol (E2) is able to regulate the expression of this proapoptotic protein in human endometrial cells (Selam et al., 2001) and human ovarian tissue (Sapi et al., 2002). Moreover, estrogen treatment increases FasL expression in monocytes through the interaction of estrogen receptor with FasL promoter (Mor et al., 2003).

It has been well established that the estrogens biosynthesis, in the testis, is catalyzed by the enzyme complex referred to as aromatase cytochrome P450, which aromatizes the A ring of C19 androgens to the phenolic A ring of C18 estrogens (Armstrong and Dorrington, 1977; Van der Molen et al., 1981). The enzyme aromatase is composed of two polypeptides: an ubiquitous non-specific flavoprotein NADPH-cytochrome P450 reductase and a specific form of cytochrome P450 (P450arom encoded by the CYP 19 gene) (Simpson et al., 1994). In the testis an age-related change has been observed in the cellular localization of the aromatization event, primarily in Sertoli cells in immature animals, but located in Leydig and germ cells in adults (Levallet et al., 1998; Andò et al., 2001). Besides, the synthesis of estrogens is regulated at the level of the

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aromatizing enzyme system by Follicole-Stimulating Hormone (FSH) and cyclic AMP (Dorrington and Armstrong, 1975).
In the mouse Sertoli cell line TM4 we previously demonstrated P450arom immunocytochemical localization together with its enzymatic activity (Catalano et al., 2003).
In the present study, we investigated if an aromatizable androgen like androst-4-ene-3,17-dione (Δ4), after its conversion to E2, can modulate FasL expression in TM4 cells. Our results demonstrate that estradiol "in situ" production enhanced FasL mRNA, protein content and promoter activity. Many transcription factors have been reported to regulate FasL promoter by DNA-protein interaction upon diverse biological signals in different cells and tissues (Latinis et al., 1997; Kasihatla et al., 1998; Matsui et al., 1998; Mittelstadt and Ashwell, 1998; Kavurma et al., 2001; Kirschhoff et al., 2002; Kavurma and Khachigian, 2003).
Functional studies of mutagenesis, electrophoretic mobility shift analysis and ChIP assay lead us to demonstrate that the up-regulatory effects induced by E2 on FasL expression are mediated by a direct interaction of Estrogen Receptor alpha (ERα) with Sp-1 protein.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), Triazol Reagent and 100 bp DNA ladder by Invitrogen (Carlsbad, CA), L-Glutamine, penicillin, horse serum, Eagle's non-essential amino acids, calf serum (CS), streptomycin, bovine serum albumine (BSA), phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ullis Cedex, France). FuGENE 6, Sephadex G50 spin columns and poly (dI-dC) by Roche (Indianapolis, IN). GoTaq DNA polymerase, T4 polynucleotide Kinase, TNT master mix, Dual luciferase kit, Sp-1 human recombinant protein and TK renilla luciferase plasmid were provided by Promega (Madison, WI). The RETROscript kit and DNase I were purchased from Ambion (Austin, TX). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, androst-4-ene-3,17-dione (Δ4), 7α,19α-dimethyl-19-nortestosterone (mibolerone), formaldehyde, NP-40, proteinase K, tRNA, Tamoxifen (Tam), N^6,2′-*O*-dibutyryladenosine-3′-5′-cyclic monophosphate [$(Bu)_2$cAMP] and 1,3,5-Tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) by Sigma (Milan, Italy). Antibodies against ERα, ERβ, β-actin, Sp-1, and polymerase II (N20) were provided by Santa Cruz Biotechnology (Santa Cruz, CA) whereas anti-FasL antibody by BD biosciences (San Josè, CA). ECL System and [γ^{32}P]ATP were purchased by Amersham Pharmacia (Buckinghamshire, UK). Letrozole was provided by Novartis Pharma AG (Basel, Switzerland), Mithramycin by ICN Biomedicals, (Shelton, CT). Salmon sperm DNA/protein A agarose by UBI (Chicago, IL). Diarylpropionitrile (DPN) and ICI 182,780 were purchased from Tocris chemical (Bristol, UK). ABI Prism 7000 Sequence Detection System, TaqMan Ribosomal RNA Reagent kit, TaqMan Ribosomal RNA Control Reagent kit and SYBR Green Universal PCR Master Mix by Biosystems (Forster City, CA).

Cell cultures

The TM4 cell line, derived from the testis of immature BALB/c mice, was originally characterized based on its morphology, hormone responsiveness, and metabolism of steroids (Mather, 1980). This cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEM-F12 containing 2.5% fetal CS, 5% horse serum, 1 mg/ml penicillin–streptomycin. Human uterin cervix adenocarcinoma (HeLa) cells were obtained from the ATCC. HeLa cells were cultured in DMEM/F12 containing 5% CS, 1% L-Glutamine, 1% Eagle's non essential amino acids and 1 mg/ml penicillin–streptomycin.

Western blot analysis

TM4 cells were grown in 10 cm dishes to 70–80% confluence and lysed in 500 μl of 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Equal amounts of total proteins were resolved on a 11% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with: (1) mouse monoclonal ERα antibody, (2) rabbit polyclonal ERβ ??antibody, (3) mouse monoclonal FasL antibody, (4) mouse monoclonal β-actin antibody. The antigen-antibody complex was detected by incubation of membranes 1 h at room temperature with peroxidase-coupled goat anti-rabbit IgG or goat anti-mouse IgG and revealed using the ECL System. Blots were then exposed to film and bands of interest were quantified by densitometer (Mod 620 BioRad, USA). The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%.

Real-time RTPCR

Total cellular RNA was extracted from TM4 cells using "TRIAZOL Reagent" as suggested by the manufacturer. All RNA was treated with DNase I and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis prior to use. Two micrograms of total RNA was reverse transcribed in a final volume of 50 μl using a RETROscript kit as suggested by the manufacturer. cDNA was diluted 1:5 in nuclease free water, aliquoted and stored at −20°C. The cDNAs obtained were further amplified for FasL gene using the following primers: forward 5′-CGAGGAGTGTGGCCCATTT-3′ and reverse 5′-GGTTCCATATGTGTCTTCCCATTC-3′.
PCR reactions were performed in the ABI Prism 7000 Sequence Detection System, using 0.1 μM of each primer, in a total volume of 30 μL reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix for the dissociation protocol was used for FasL and 18S. Negative control contained water instead of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content. The 18S quantification was performed using a TaqMan Ribosomal RNA Reagent kit following the method provided in the TaqMan Ribosomal RNA Control Reagent kit. The relative FasL gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in FasL gene expression relative to 18S rRNA and calibrator, calculated following the ΔΔCt method, as follows:

$$\text{n-fold} = 2^{-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}})}$$

where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the 18S rRNA reference gene from the average Ct value of the different genes analyzed.

Transfection assay

Transient transfection experiments were performed using pGL_2 vectors containing different deleted segments of human FasL gene promoter (p-2365: −2365/−2; p-318: −318/−2; p-237: −237/−2) ligated to a luciferase reporter gene (kindly provided by Dr. Paya, Department of Immunology, Mayo Clinic Rochester, Minnesota, USA). Deletion of Sp-1 sequence in FasL gene promoter was generated by PCR using as template p-318 construct. The resulting plasmid encoding the human Fas-L gene promoter containing the desired deletion was designed p-280 Sp-1 and the sequence was confirmed by nucleotide sequence analysis.
FuGENE 6 was used as recommended by the manufacturer to transfect TM4 cells plated in 3.5 cm^2 wells with pGL_2 FasL promoter constructs (0.5 μg/well).
Another set of experiments was performed in HeLa cells cotransfecting p-318 FasL promoter (−318/−2) (0.5 μg/well) and the wild-type human ERα expression vector (HEGO) (0.5 μg/well) (Tora et al., 1989) or pCMV5-hERβ, containing human ERβ gene (0.5 μg/well) (a gift from JA Gustafsson).
Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK renilla luciferase plasmid (25 ng/well) was used to normalize the efficiency of the transfection. Twenty-four hours after transfection, the medium was changed and TM4 cells were treated in serum free medium (SFM) in the presence of Δ4, $(Bu)_2$cAMP, mibolerone, letrozole, PPT and DPN. HeLa cells, 24 h after transfection, were treated in the presence or absence of E2 for 24 h. The firefly and renilla luciferase activities were measured using Dual Luciferase Kit. The firefly luciferase data for each sample were

normalized on the basis of transfection efficiency measured by renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from TM4 as previously described (Andrews and Faller, 1991). Briefly, TM4 cells plated into 60 mm dishes were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 sec and resuspended in 400 μl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 sec. Samples were then centrifuged for 10 sec and the supernatant fraction discarded. The pellet was resuspended in 50 μl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at −70°C. The yield was determined by Bradford method (Bradford, 1976). The probe was generated by annealing single stranded oligonucleotides and labeled with [γ^{32}P] ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitor are the following (the nucleotide motifs of interest are underlined and mutations are shown as lowercase letters): Sp1 5′-AAATTGTGGGCGGAAACTTCCAGGGG-3′, mutated Sp-1 5′-AAATTGTGttCGGAAACTTCCAGGGG-3′. Oligonucleotides were synthesized by Sigma Genosys. The protein binding reactions were carried out in 20 μl of buffer (20 mM HEPES pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glicerol, 1 mg/ml BSA, 50 μg/ml poli dI/dC) with 50,000 cpm of labeled probe, 10 μg of TM4 nuclear protein and 5 μg of poly (dI-dC). The above-mentioned mixture was incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotide. For experiments involving Sp-1, ERα and ERβ antibodies, the reaction mixture was incubated with these antibodies at 4°C for 12 h. For in vitro mithramycin treatment, mithramycin (100 nM) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extract. As positive controls we used Sp-1 human recombinant protein (1 μl) and in vitro transcribed and translated ERα protein (1 μl) synthesized using T7 polymerase in the rabbit reticulocyte lysate system as direct by the manufacturer. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 X Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at −70°C.

Chromatin immunoprecipitation (ChIP)

According to the ChIP assay procedure previously described (Shang et al., 2000), TM4 cells were grown in 60 mm dishes to 50–60% confluence, shifted to SFM for 24 h and then treated with E2 (100 nM), ICI 182,780 (10 μM), E2 + ICI for 1 h. Thereafter, the cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 min. Then, cells were sonicated four times for 10 sec at 30% of maximal power (Sonics, Vibra Cell 500W) and collected by centrifugation at 4°C for 10 min at 14,000 rpm. The supernatants were diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl) and immunocleared with 80 μl of sonicated salmon sperm DNA/protein A agarose for 1 h at 4°C. The precleared chromatin was immunoprecipitated with a specific anti-Sp-1, anti ERα and anti polymerase II antibodies and with a normal mouse serum IgG (Nms) as negative control. At this point, 60 μl of salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluated with elution buffer (1% SDS, 0.1 M $NaHCO_3$), reverse crosslinked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. Two microliters of 10 mg/ml yeast tRNA were added to each sample and DNA was precipitated with 70% EtOH for 24 h at −20°C, and then washed with 95% EtOH and resuspended in 20 μl of TE buffer. One microlitre of each sample was used for PCR amplification with the following primers flanking Sp-1 sequence present in the Fas-L promoter region: 5′-GCAACTGAGGCCTTGAAGGC-3′ (forward) and 5′-GCAGCTGGTGAGTCAGGCCAG-3′ (reverse). The PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The amplification products obtained in 25 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Each datum point represents the mean ± SE of three different experiments. Data were analyzed by ANOVA test using the STATPAC computer program.

Results

Estradiol "in situ" production, by aromatase activity, enhances FasL expression in TM4 cell line

In TM4 cells, which exibit a spectrum of features in common with native Sertoli cells, like the presence of aromatase activity, we investigated if an aromatizable androgen Δ4, through its conversion into E2, may influence FasL mRNA and protein content by Real-time RT-PCR and Western blot analysis. Since aromatase expression and activity, in Sertoli cells, is under FSH control (Dorrington and Armstrong, 1975) we also evaluated the treatment with $(Bu)_2$cAMP (simulating FSH action) on FasL expression.

As shown in Figure 1A the treatment with Δ4 (100 nM) for 24 h resulted in an increase of FasL mRNA expression more than 1.9-fold. The simultaneous treatment with $(Bu)_2$cAMP (1 mM) and Δ4, further enhanced FasL mRNA expression compared with Δ4 treatment alone (2.4-fold), suggesting that $(Bu)_2$cAMP stimulates E2 "in situ" production by its action on aromatase activity. These up-regulatory effects were reversed by addition of the aromatase inhibitor letrozole (1 μM) (90%), while no significant difference was observed in the presence of a non-aromatizable androgen mibolerone (100 nM) with or without $(Bu)_2$cAMP.

Next, we performed Western blot analysis using a monoclonal antibody anti FasL. We detected a band of 37 kDa which intensity was increased upon Δ4 treatment. Exposure to $(Bu)_2$cAMP combined with Δ4 enhanced the effect induced by Δ4 alone. The addition of letrozole reversed these up-regulatory effects (Fig. 1B,C).

To evaluate whether E2 "in situ" production was able to activate FasL promoter we transiently transfected TM4 cells with vector containing human FasL promoter fused to the luciferase reporter gene. The treatment for 24 h with Δ4 or Δ4 + $(Bu)_2$cAMP displayed a significant increase of the basal promoter activity that was reversed by letrozole (Fig. 1D).

Effects of Δ4 on expression of human FasL promoter/luciferase reporter gene constructs in TM4 cells

To delimit the *cis*-elements involved in FasL transcriptional activation by Δ4, we transiently transfected TM4 cells with plasmids containing different deleted segments of human FasL promoter. Schematic representation of constructs is shown in Figure 2A. Transfected cells were untreated (C) or treated with 100 nM of Δ4 and 1 μM of letrozole.

p-318 plasmid showed a higher basal activity when compared with the other plasmids (p-2365, p-237) (Fig. 2B) suggesting the presence of a DNA sequences upstream from −318 to which transcription factors with repressor activity bind. These data well fit with previous results demonstrating that FasL gene promoter region, located between −318 and −237, plays a major role in promoting basal transcription in TM4 Sertoli cells (McClure et al., 1999).

In TM4 cells transfected with p-2365 and p-318 plasmids the treatment with Δ4 induced a significant increase of the basal

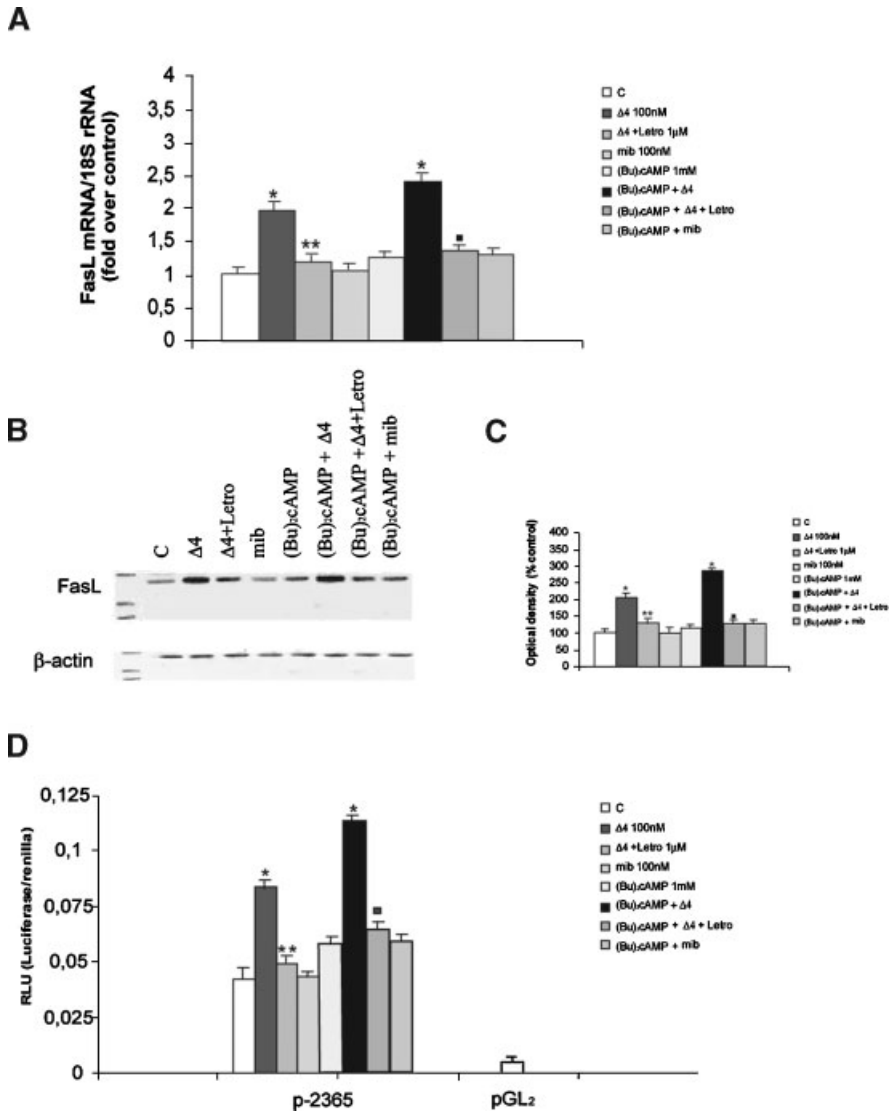

Fig. 1. Effects of Δ4 on FasL expression. A: Total RNA was obtained from TM4 cells untreated (control, C) or treated for 24 h with Δ4 (100 nM) mibolerone (mib 100 nM), $(Bu)_2$cAMP (1 mM), $(Bu)_2$cAMP + Δ4 and $(Bu)_2$cAMP + mib. One micromolar of aromatase inhibitor letrozole (Letro) was used. Real time RT-PCR was performed to analyze mRNA levels of FasL. Data represent the mean ± SE of values from three separate RNA samples. Each sample was normalized to its 18S ribosomal RNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator (control) calculated with the ΔΔCt method as indicated in the "Material and Methods" section. *$P < 0.01$ compared to control. **$P < 0.01$ compared to Δ_4 treated samples; ■ $P < 0.01$ compared to $(Bu)_2$cAMP + Δ4 treated samples. B: Immunoblot of FasL from TM4 cells treated in the absence (C) or in the presence of the above-mentioned treatments. C: The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. *$P < 0.01$ compared to control; **$P < 0.01$ compared to Δ_4 treated samples; ■ $P < 0.01$ compared to $(Bu)_2$cAMP + Δ4 treated samples. D: Transcriptional activity of TM4 cells transfected with p-2365 construct is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), mibolerone (mib 100 nM), $(Bu)_2$cAMP (1 mM), $(Bu)_2$cAMP + Δ4 and $(Bu)_2$cAMP + mib. One micromolar of aromatase inhibitor letrozole was used. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL_2: basal activity measured in cells transfected with pGL_2 basal vector. *$P < 0.01$ compared to control. **$P < 0.01$ compared to Δ4 treated samples; ■ $P < 0.01$ compared to $(Bu)_2$cAMP + Δ4 treated samples.

promoter activity that was completely reversed by letrozole. In contrast, Δ4 was unable to activate p-237 construct eliciting, in the region from −318 to −237, the presence of *cis*-element involved in estrogen responsiveness. In fact, this region contains Sp-1 site, a potential target of ER. In order to explore the role of the Sp-1 binding site in the regulation of FasL expression by Δ4, functional experiments were performed using the Sp-1 deleted plasmid (p-280 Sp-1). Luciferase assay revealed that the inducibility by Δ4 on FasL promoter was totally lost (Fig. 2D). These results suggest that the up-regulatory effects of estradiol production by aromatase activity require Sp-1 sequence motif.

ERβ is not involved in E2-modulating FasL expression

Before exploring more closely the possible interaction between E2/ER complex to Sp-1 and the role of this binding in modulating FasL expression, we set out to determine which functional ER(s) isoform was present in TM4 cells. By Western blotting

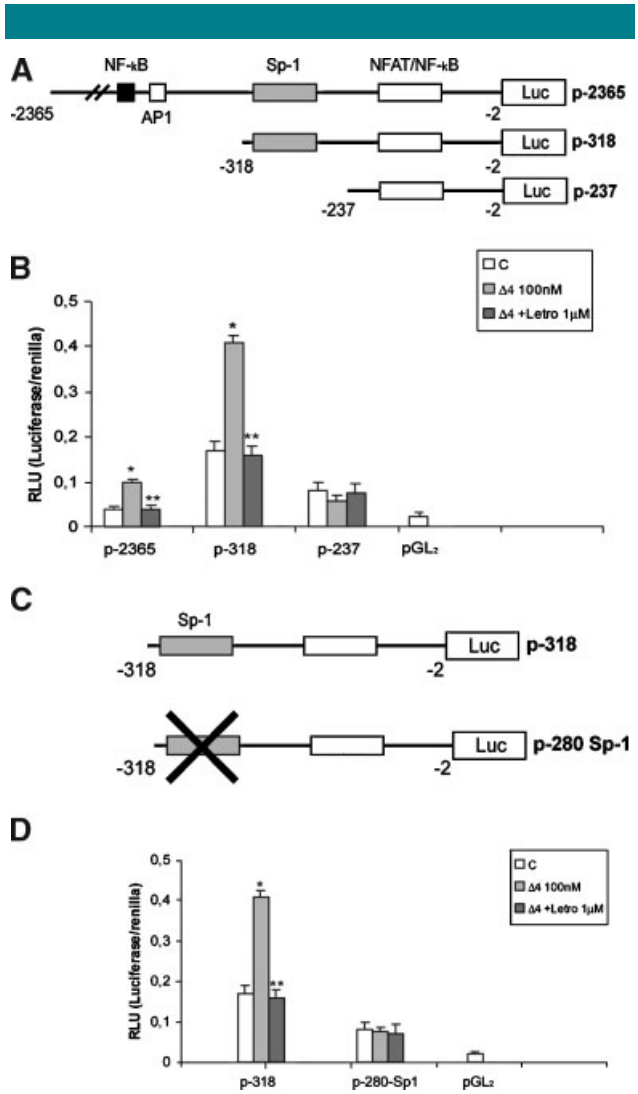

Fig. 2. Effects of estradiol "in situ" production on expression of human FasLpromoter/luciferase reporter gene construts in TM4 cells. A: Schematic map of the FasL promoter fragments used in this study. All of the promoter constructs contain the same 3′ boundary (−2). The 5′ boundaries of the promoter fragments varied from −237 to −2365. Each fragment was subcloned into the pGL_2 vector. B: Transcriptional activity of TM4 cells with promoter constructs is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), and Δ4 + letrozole (1 μM) for 24 h. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL_2: basal activity measured in cells transfected with pGL_2 basal vector. *$P < 0.01$ compared to control; **$P < 0.01$ compared to Δ4 treated samples. C: Schematic representation of the p-318 and p-280 Sp-1 constructs. The deletion of Sp-1 sequence is present in p-280 Sp-1 construct containing the region from −318 to −2 of FasL promoter gene. Each fragment was subcloned into the pGL_2 vector. D: Transcriptional activity of TM4 cells with p-280 Sp-1 construct is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), and Δ4 + letrozole (1 μM) for 24 h. The values represent the mean ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *$P < 0.01$ compared to control; **$P < 0.01$ compared to Δ4-treated samples.

analysis, we demonstrated in TM4 protein extracts the presence of both ER(s) (Fig. 3A, lane 2), As positive control, the breast cancer cell line MCF-7 (ERα positive) and human prostate cancer cell line LNCaP (ERβ positive) were used (Fig. 3A, lane 1).
In the presence of the two different ER antagonists ICI 182,780 (10 μM) and tamoxifen (10 μM) (Tam) the up-regulation of E2 on FasL expression was abrogated demonstrating that this effect was specifically dependent by ER (Fig. 3B,C).
To specify which isoforms of ER were mainly involved in FasL transactivation, we cotransfected HeLa cells (ER negative) with p-318 FasL promoter and the wild type human ERα or ERβ expression vector. The treatment with E2 (100 nM) for 24 h showed an increased transcriptional activation of FasL promoter only in cells cotransfected with ERα (Fig. 3D).
Finally, to demonstrate further the direct involvement of ERα in FasL transactivation we used 100 nM of the selective agonists of ERα [1,3,5-Tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT)] and ERβ [diarylpropionitrile (DPN)] in TM4 cells transiently transfected with p-318 FasL promoter. The treatment with PPT showed an increase of FasL promoter activity while no change was observed in the presence of DPN (Fig. 3E).

Effects of 17-β estradiol treatment on Sp1 DNA binding activity in TM4 cells

On the basis of the evidences that the up-regulatory effects of E2 on FasL require the crucial presence of Sp-1-RE, EMSA was performed using synthetic oligodeoxyribonucleotides corresponding to the putative Sp-1 binding site. In the presence of TM4 nuclear extracts (10 μg) we observed the formation of a specific complex (Fig. 4A, lane 1), which was abrogated by a 100-fold molar excess of unlabeled probe (Fig. 4A, lane 2). This inhibition was not observed when a mutated Sp-1 oligonucleotide was used as competitor (Fig. 4A, lane 3). E2-treatment induced a strong increase in Sp-1 DNA binding activity (Fig. 4A, lane 4) compared with basal levels. In the presence of ICI 182,780 the Sp-1 DNA binding activity was drastically reduced (Fig. 4A, lane 5). The addition of mithramycin (100 nM), that binds to GC boxes and prevents sequential Sp-1 binding, decreased the binding of E2 treated TM4 nuclear extracts on Sp-1 DNA sequence (Fig. 4A, lane 6). In a cell free system we observed in the presence of Sp-1 reconbinant protein a single band that causes the same shift respect to the complex revealed in TM4 nuclear extracts (Fig. 4A, lane 7) which was abrogated by 100-fold molar excess of unlabeled probe (Fig. 4A, lane 8). Transcribed and translated in vitro ERα protein did not bind directly to Sp-1 probe (Fig. 4A, lane 9). When the nuclear extracts from TM4 cells treated with E2 were incubated with either anti-Sp-1 or anti-ERα antibody, the original band DNA-protein complex was immunodepleted (Fig. 4B, lanes 3 and 4), whereas anti-ERβ antibody gave no effects (lane 5).
Taken together these results suggest that ERα is recruited by Sp-1 in our DNA binding complex.

17-β Estradiol enhances recruitment of Sp-1/ERα to the promoter region of FasL gene in TM4 cells

Interaction of ERα and Sp-1 with the FasL gene promoter was also investigated using a ChIP assay. After sonication and immunoprecipitation by anti ERα or anti Sp-1 antibodies, PCR was used to determine binding of ERα/Sp-1 protein to the −318 to −2 DNA region of the FasL gene promoter. Our results indicated that treatment with E2 induced an increased recruitment of Sp-1/ERα complex to the FasL promoter. The latter event was reduced in the presence of E2 + ICI. The enhanced recruitment of Sp-1/ERα was correlated with greater association of polymerase II to the FasL regulatory region

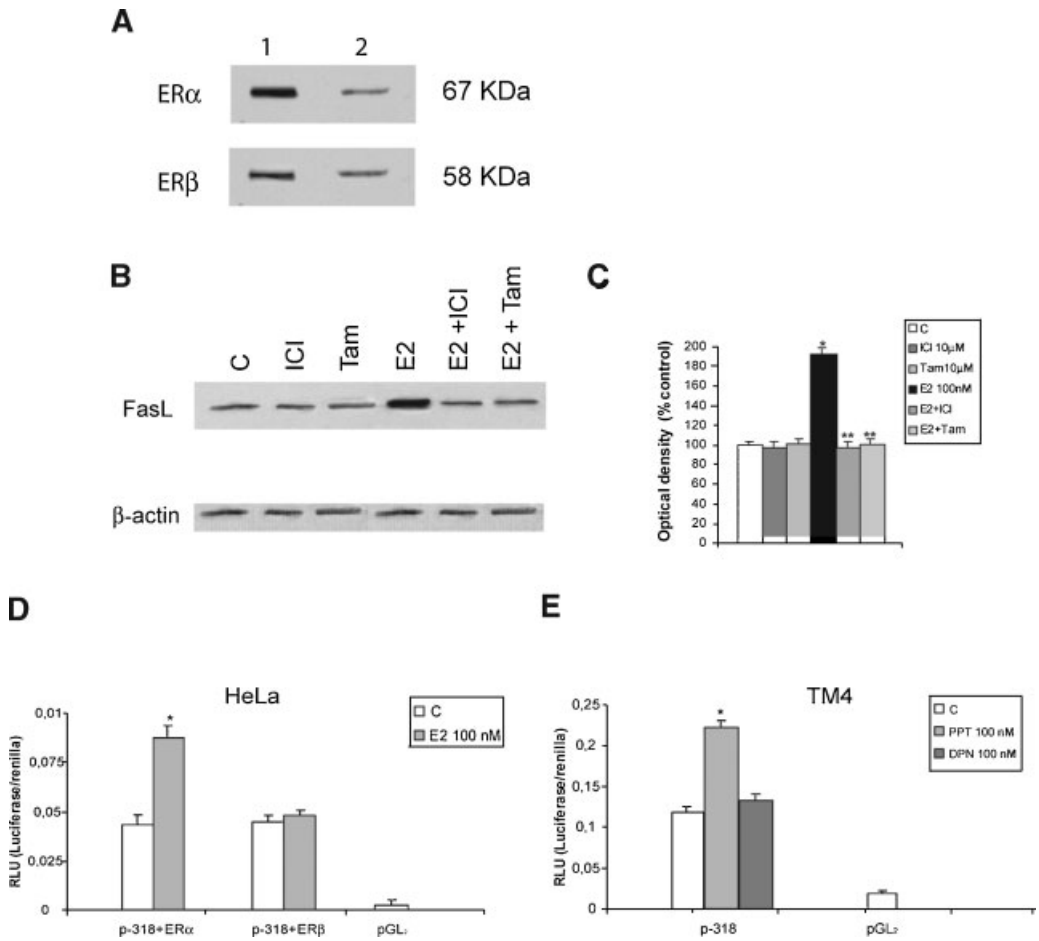

Fig. 3. 17β-Estradiol enhances FasL transcriptional activity through ERα. A: Lysates from TM4 cells were used to evaluate by Western blot analysis the expression of ERα and ERβ (lane 2). The human breast cancer cell line MCF-7 and human prostate cancer cell line LNCaP were used as positive control for ERα and ERβ respectively (lane 1). B,C: Immunoblot of FasL from TM4 cells treated in the absence (C) or in the presence of E2 (100 nM) for 24 h. The pure anti-estrogen ICI 182,780 (10 μM) and tamoxifen (Tam 10 μM) were used. The histograms represent the means ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. *$P < 0.01$ compared to control; **$P < 0.01$ compared to E2 treated samples. D: HeLa cells were transiently cotransfected with p-318 FasL promoter construct (−318/−2) and ERα or ERβ plasmids. The cells were untreated (C) or treated with E2 (100 nM) for 24 h. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *$P < 0.01$ compared to control. E: TM4 cells transfected with p-318 FasL promoter construct were untreated (C) or treated with PPT (100 nM) and DPN (100 nM) for 24 h. *$P < 0.01$ compared to control.

(Fig. 5A). No PCR product was observed using DNA immunoprecipitated with normal mouse serum IgG.

Discussion

In testis, Fas/FasL interaction has been thought to play an important role in the establishment of immunoprivilege. Several reports have demonstrated that Sertoli cells through FasL may trigger apoptotic cell death of sensitive lymphoid cells, which express on their cell surface Fas receptor. This has provided new insights into the concepts of tolerance and immunoprivilege (Bellgrau et al., 1995; Sanberg et al., 1996; Ferguson and Griffith, 1997). For instance, testis grafts from mice expressing FasL survived when transplanted into allogeneic animals. On the contrary, grafts derived from "gld" mice, which lack functional FasL, were rejected (Bellgrau et al., 1995).

In the present report, for the first time, we have provided evidences that, in TM4 cell line, an aromatizable androgen Δ4 induces a strong increase in FasL mRNA, protein content and promoter activity. These effects are reversed by addition of letrozole, an aromatase inhibitor, addressing how E2 "in situ" production by aromatase activity plays a crucial role in modulating the immunoprivileged status of these somatic cells. A further support to the specificity of the above described results raises from the evidence that no noticeable effect was produced by mibolerone, a non-aromatizable steroid.

It is well known that postnatal development and function of testicular Sertoli cells is regulated primarily by FSH, a glycoprotein hormone secreted by the pituitary gland (Dorrington and Armstrong, 1975). In the prepubertal testis, FSH is required for Sertoli cells proliferation to achieve the adult number of these cells (Griswold, 1998). This proliferative stage of Sertoli cells development is also characterized by the presence of FSH-dependent cytochrome P450 aromatase activity (Carreau et al., 2003; Sharpe et al., 2003). In our recent work (Catalano et al., 2003) we have documented in TM4 cell line a strong dose-dependent stimulation of aromatase activity

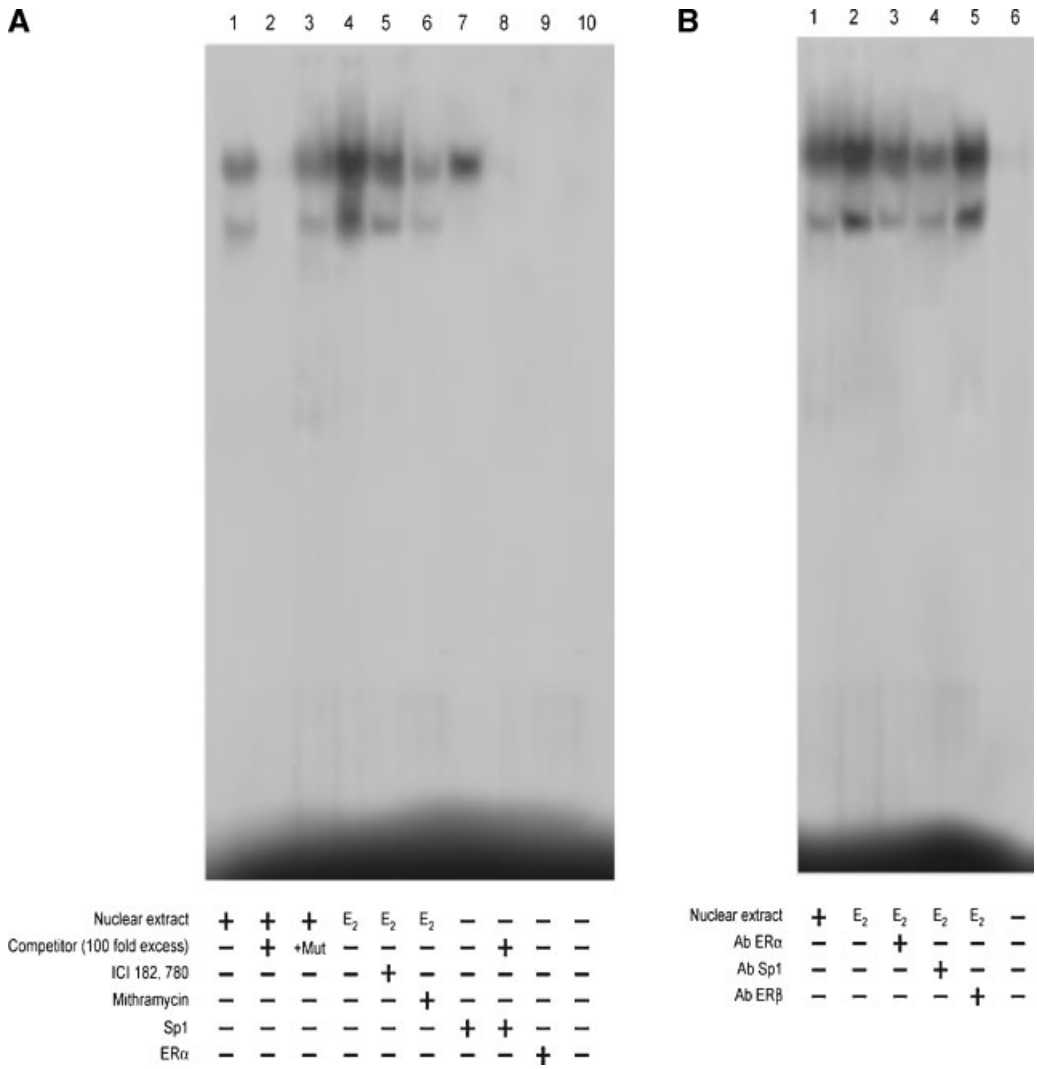

Fig. 4. Electrophoretic mobility shift assay of the Sp-1 binding site in the FasL promoter region. A: Nuclear extracts from TM4 cells were incubated with a double-stranded Sp1-specific consensus sequence probe labeled with [γ-^{32}P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). Competition experiments were perfomed adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 8) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated Sp-1 (lane 3). Nuclear extracts were obtained from TM4 cells treated with 100 nM of E2 (lane 4), E2 + ICI 182,780 (10 μM) (lane 5), E2 + mithramycin (100 nM) (lane 6) for 24 h. As control we used human Sp-1 recombinant protein and transcribed and translated in vitro ERα protein (lane 7 and 9). Lane 10 contains probe alone. B: Anti-ERα, anti Sp-1 and anti-ERβ antibodies (lanes 3–5) were incubated with E2-treated TM4 nuclear extracts. Lane 6 contains probe alone.

induced by $(Bu)_2$cAMP similar to that described previously in immature Sertoli cells (Andò et al., 2001). In the present study it is worth to emphasize that FSH induced an increased FasL expression through the enhancement of aromatase activity.

To elucidate the molecular mechanism involved in Δ4 enhanced FasL expression, we transiently transfected TM4 cells with different constructs containing deleted segments of the human FasL promoter.

A maximal constitutive reporter gene activity was observed with p-318 construct, containing the region between −318 and −2 bp from the transcriptional start site of the human FasL promoter. This is in agreement with previous results demonstrating that FasL gene promoter region from 318 to −237 bp plays a major role in promoting basal transcription in TM4 cells (McClure et al., 1999). Moreover, the induced activation by Δ4 was not observed in cells transfected with p-237 construct (−237 to −2) suggesting that the region between −318 and −237 bp contains elements that mediate the potentiating effects of estrogen on FasL expression.

A broadening number of transactivating factors has been identified as regulators of FasL gene expression (Kavurma and Khachigian, 2003), as nuclear factor in activated T cells (NF-AT) (Latinis et al., 1997), nuclear factor-kappa B (NF-KB) (Matsui et al., 1998), activator protein-1 (AP-1) (Kasihatla et al., 1998), interferon regulatory factor-1 (IFN-1) (Kirschhoff et al., 2002), early growth response factor (Egr) (Mittelstadt and Ashwell, 1998) and specificity protein-1 (Sp-1) (Kavurma et al., 2001). Sp-1 is involved in the transcriptional regulation of many genes and has also been identified to be important in the regulation of FasL gene expression and apoptosis. Indeed, this transcription factor is able to activate FasL promoter via a distinct recognition element, and inducible FasL promoter activation is abrogated by expression of the dominant-negative mutant form of Sp-1 (Kavurma et al., 2001). In addition, it has been recently

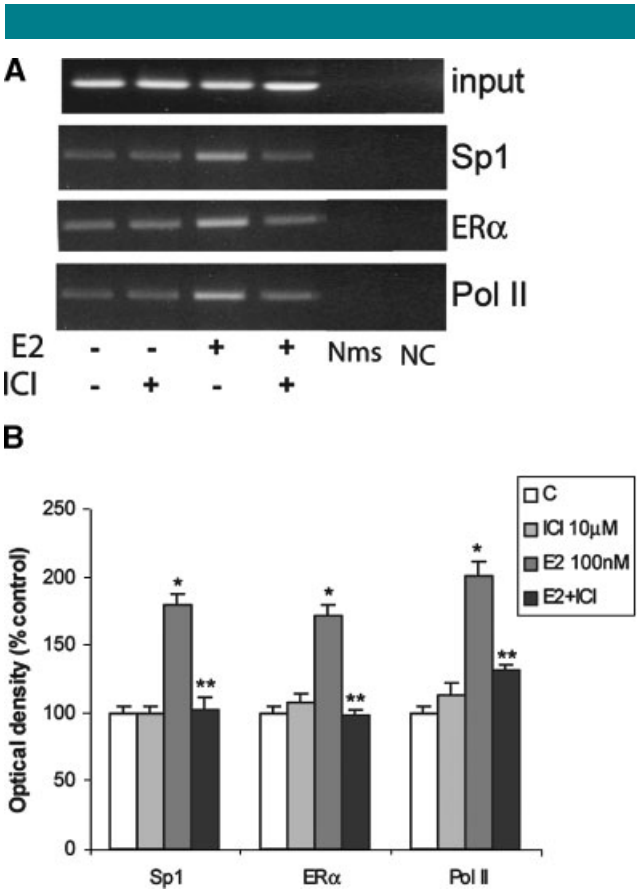

Fig. 5. 17β-Estradiol increases Sp-1/ERα recruitement to FasL promoter. A: Soluble precleared chromatin was obtained from TM4 cells treated for 1 h with 100 nM E2, 10 μM ICI and E2 + ICI or left untreated (C) and immunoprecipitated (IP) with an anti-Sp-1, anti ERα, anti polymerase II antibodies and with a normal mouse serum (Nms) as negative control. The FasL promoter sequences containing Sp-1 were detected by PCR with specific primers, as described in "Materials and Methods". To control input DNA, FasL promoter was amplified from 30 μl of initial preparations of soluble chromatin (before immunoprecipitations). PCR products obtained at 25 cycles are shown. Sample without the addition of DNA was used as negative control (NC). This experiment was repeated three times with similar results. B: The histograms represent the means ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. *$P < 0.01$ compared to control; **$P < 0.01$ compared to E2-treated samples.

demonstrated that nuclear extracts of TM4 Sertoli cells contain high levels of Sp-1 and Sp-3 that specifically bind to the GGGCGG consensus sequence present in the FasL gene, and overexpression of Sp-1 but not Sp-3 is able to increase the basal transcription of the FasL promoter (McClure et al., 1999). The latter observation fits with our functional studies demonstrating that Sp-1 is a crucial effector of estradiol signal in enhancing FasL gene expression. For instance, it is well known that ERs can transactivate gene promoters without directly binding to DNA but instead through interaction with other DNA-bound factors in promoter regions lacking TATA box. This has been most extensively investigated in relationship to protein complexes involving Sp-1 and ERα at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors. Sp-1 protein plays an important role in the regulation of mammalian and viral genes, and recent results have shown that E2 responsiveness of c-fos, cathepsin D, retinoic acid receptor α1 and insulin-like grow factor-binding protein 4 gene expression in breast cancer cells is linked to specific GC rich promoter sequences that bind ER/Sp-1 complex in which only Sp-1 protein binds DNA (Krishnan et al., 1994; Cowley et al., 1997; Porter et al., 1997; Sun et al., 1998; Qin et al., 1998; Saville et al., 2000).

In our work, the interaction between ERα and Sp-1 is clearly evidenced by gel mobility shift analysis and chromatin immunoprecipitation assay. Besides, the functional assays performed in ER-negative HeLa cells showed that ERα and not ERβ mediates the estrogen-induced increase in FasL gene expression. The specificity of ERα to induce transcription of FasL in TM4 was demonstrated using selective agonists for the ER subtypes. For instance we evidenced that only PPT was able to enhance FasL promoter activity.

Our results stemming from functional analysis, EMSA and ChiP assays led us to recruit FasL among those genes whose expression is upregulated by E2 through a direct interaction of ERα with Sp-1 protein.

In conclusion, the present study demonstrates that aromatizable steroids, normally present in the testicular milieu, through their conversion into E2 by aromatase activity, are able to increase FasL expression in TM4 Sertoli cells. The aromatase enzyme assures that estrogens through a short autocrine loop maintain Sertoli cells proliferation before their terminal differentiation. Thus, we propose that at the latter crucial maturative stage, FasL may achieve an intracellular content sufficient to protect Sertoli cells from any injury induced by Fas expressing immunocells, then potentiating the immunoprivileged condition of the testis.

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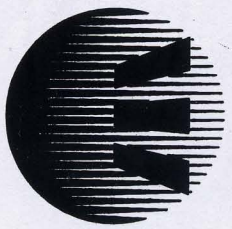

THE ENDOCRINE SOCIETY

Program & Abstracts

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of washed/concentrated epididymal sperms. In the immunohistochemistry, nals were found from the elongated spermatids and sperms in seminiferous immunocytochemical study revealed that the epididymal sperms were also and the intensity of signal was in the order of head, midpiece and tail. le of evidence on the presence of LH/hCG binding sites and their possible le reproductive tracts is availble, information about the origin of ligand et accumulated sufficiently. The present study indicates the motile sperms rce of LH ligand and might suggests an explanation for the production of receptor signaling in uterine cells during the male gamete transportation.
sent study was supported by KOSEF grant.

d Nonclassic Progesterone Receptors Are Both in Human Spermatozoa.

Martina Seyfert[1], Martin Wehling[1], Ralf M Losel*[1]. [1]*Dept of Clin nheim, Univ of Heidelberg, Mannheim, Germany.*

one is one of the physiological inducers of the acrosome reaction in matozoa. The receptor that responds to progesterone is not yet identified es differ in many aspects from the properties of the classic nuclear eptor which suggests the participation of a novel or nonclassic receptor. investigated the expression in human spermatozoa of a novel ing protein (hmPR1/PGMRC1) and its ortholog (hmPR2/PGMRC2) y been identified in liver microsomes and are considered receptor with the nuclear progesterone receptor. The purification procedure of optimized with special emphasis on the control of leukocyte and mination in single donor samples. The results indicate that all three ed in human sperm, as transcripts were detected in 46%, 42% and samples, respectively (n=24).
t by DFG and BMBF is gratefully acknowledged.

vel Junction Dynamic Regulator during in the Rat Testis.

Cheng[1]. [1]*The Population Council, New York, NY.*

shown that TGF-β3 regulates Sertoli cell tight junction (TJ) *in vivo*, which is mediated by p38 MAP kinase. Since the blood- is constituted by coexisting TJ and cell-cell adherens junctions cialization, ES), we sought to investigate if TGF-β3 regulates minately or indiscriminately. Using an *in vivo* model of AJ laboratory in which adult rats were treated with AF-2364 DD and Cheng CY, *Endocr Rev* 25:747-806, 2004), which longating/elongate spermatids, followed by spermatocytes, affecting Sertoli cell TJ function and BTB integrity *in vivo*, of germ cell loss were associated with a surge in TGF-β3 . Furthermore, the downstream Ras/ERK signaling pathway with an induction in intrinsic ERK activity. To further validate AJ dynamics in parallel to its effects on TJ, specific inhibitors) or TGF-β antagonists (e.g., TβRII/Fc chimera) were AF-2364 administration. Both reagents were shown to 364-induced germ cell loss from the epithelium without demonstrating unequivocally that TGF-β3 regulates AJ naling pathway. These results also suggest that there is TGF-β3 can select which signaling pathway is activated TJ is opened (via p38 MAPK) or the ES perturbed (via also illustrate that *in vivo* administration of TGF-β3 model to study TJ and AJ dynamics. When adult rats were estis via intratesticular injection, TGF-β3, but not the progressive loss of spermatids from the epithelium with a disruption of BTB integrity. Thereafter, both wk, and by 12-wk, the epithelium was indistinguishable y, these results illustrate that TGF-β3 released from epithelial cycle is a regulator of junction dynamics in esis.
, NICHD U01 HD045908

ulture System for the Study of

Helena Sim[1], Louisa Ludbrook[1], Michael J *Henry's Inst of Med Res, Clayton, Victoria, lec Biol, Monash Univ, Clayton, Victoria,*

one *SRY* gene, mammals form testes rather than cells in the urogenital ridge (UGR) committing other cell types along the male lineage. In XY humans, mutations in *SRY* cause male-to-female sex reversal in XY individuals. Despite 14 years since the discovery of SRY, its in vivo function has not been established, hampered in part by the lack of experimental cell culture systems. In the present study, the human cell line NT2/D1 was evaluated as a model for studying sex determination. Gene expression profiling revealed that NT2/D1 express 23 of 30 UGR genes tested including key sex determining genes such as *SOX9* and *AMH*. To identify genes controlled by SRY, we produced cells that over-express SRY and measured gene expression of 23 UGR genes. Three genes showed up-regulation. One of these, *SOX9*, showed elevated mRNA and protein expression. Four SRY clinical mutants that show varying degrees of DNA binding, bending and import defects failed to up-regulate *SOX9*. Up-regulation of *SOX9* in the testis is a key process of sex determination observed in a wide range of vertebrates, regardless of the presence/absence of SRY. This study is the first to establish a function in vivo assay for *SRY* function and also shows the first cellular evidence supporting the idea that, at least in humans, the role of SRY is to up-regulate *SOX9*. Detailed functional analysis of a urogenital ridge enhancer of *SOX9* and its activation by SRY are in progress.

P1-285

Fas Ligand Expression in TM4 Cells Is Up-Regulated by Estradiol through Estrogen Receptor α Interaction with SP-1.

Pietro Rizza[1], Stefania Catalano[1], Guowei Gu[1], Ines Barone[1], Cinzia Giordano[2], Stefania Marsico[1], Ivan Casauri[1], Sebastiano Ando*[2]. [1]*Pharmaco-Biol, Fac of Pharmacy, Univ of Calabria, Rende, Cosenza, Italy;* [2]*Cell Biol, Fac of Pharmacy, Univ of Calabria, Rende, Cosenza, Italy.*

The testis is an immunologically privileged site of the body, and Sertoli cells are believed to favor local immune tolerance to testicular autoantigens by segregating them within the blood-tubular barrier and by secreting immunosuppressive factors.

The expression of Fas ligand (FasL), a type II trans-membrane protein that belongs to the tumor necrosis factor family of cytokines, has been reported recently in Sertoli cells and induces apoptosis in cells expressing the Fas receptor.

Recent studies demonstrated that estradiol (E2) is able to regulate the expression of FasL in several cell lines.

In this study we used, as an experimental model, the mouse Sertoli cell line TM4, which conserves a large spectrum of functional features present in immature Sertoli cells, like the presence of aromatase, the enzyme that catalyzes the biosynthesis of estrogens from androgens which is mainly expressed during Sertoli cells functional maturation. Thus, in the present study we evaluated E2 effects on Fas/FasL expression in order to ascertain the role of *in situ* estradiol production in conferring the immuno-privileged status of Sertoli cells.

Our results demonstrated that Fas is absent in TM4 cells whereas FasL is present in terms of mRNA and protein content and that both are drastically upregulated by E2 exposure.

Functional assay using vector containing human FasL promoter, evidenced that E2 is able to activate this promoter. Transient transfection experiments performed with constructs containing different deleted segments of FasL promoter (-2365/-2; -318/-2; -237/-2) showed that the up-regulatory effects of E2 require the integrity of the FasL 5'-untranslated region located between -318 and -237. We focused our attention on Sp-1 binding site, present in this regulatory region, as possible effector of E2 signal according to previous findings.

Indeed, EMSA study evidenced how Sp-1-RE binding complex with nuclear extracts results in a single band, which was drastically incrased in TM4 cells exposed to E2 and supershifted in the presence of either anti-Sp-1 or anti- ERα antibodies.

All these findings make us to recruit FasL among those genes which expression is up-regulated by E2 through a direct interaction of ERα with Sp-1 protein. Besides, the enhanced FasL expression induced by E2 *in situ* production through aromatase activity may represent the intracrine mechanism contributing to the maintainment of Sertoli cells immunoprivilege.

P1-286 ♦

Overexpression of CXCL10 in Human Prostate LNCaP Cells Activates Its Receptor (CXCR3) Expression and Inhibits Cell Proliferation.

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Prostate cancer is the leading cause of death among men in the United States and Western countries. Genes, dietary factors and lifestyle-related factors contribute to the development of prostate cancer. Chronic or recurrent inflammation plays a role in the development of many types of cancer including prostate cancer. CXCL10 (interferon-γ inducible protein-10, IP-10) is a small secretory protein of 8.7 kD produced by macrophages and Leydig cells in testis. We reported previously that overexpression of CXCL10 in pcDNA3.1 vector with CMV promoter inhibited steroidogenic acute regulatory protein (StAR D1) expression, decreased progesterone synthesis and inhibited cell proliferation of mouse Leydig tumor MA-10 cells. Recently, it was found

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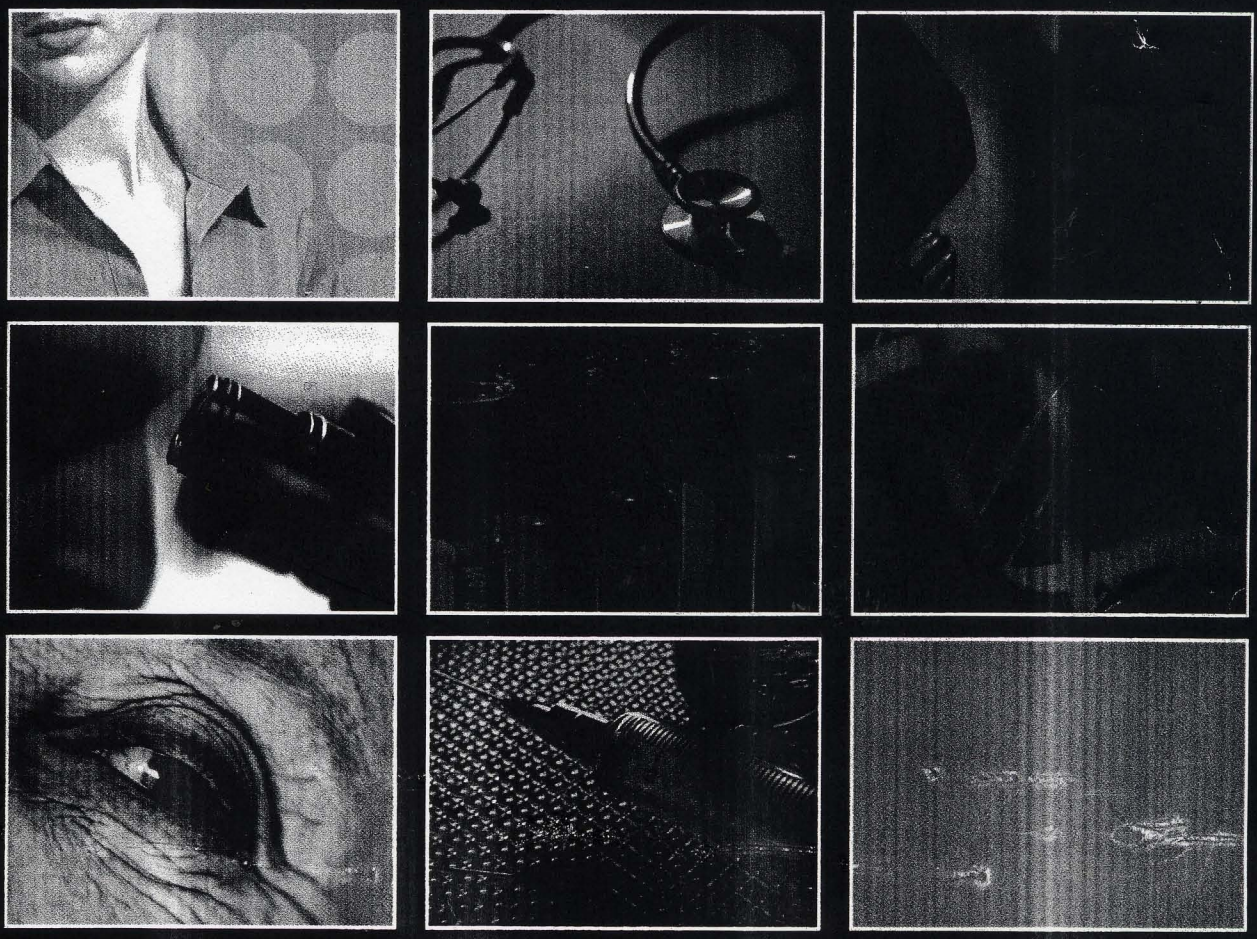

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6105

Bystander activity of Ad-mda7: human MDA-7 protein kills breast cancer cells via an IL-20 receptor-dependent pathway.

Zheng M, Bocangel D, Menander K, Hunt K, Chada S. Introgen Therapeutics Inc., Houston, TX; UTMD Anderson Cancer Center, Houston, TX

The melanoma differentiation-associated gene-7 (mda-7/IL24) is a unique member of the IL-10 family of cytokines, with ubiquitous tumor cell proapoptotic activity. Transduction of tumor or normal cells with an Adenoviral vector encoding the mda-7 gene (Ad-mda7) results in selective killing of tumor cells, with no effect on normal cells. However, both tumor and normal cells express high levels of MDA-7 protein after Ad-mda7 treatment and secrete glycosylated MDA-7 protein. Recent data indicate that secreted MDA-7 protein functions as both, a pro-Th1 cytokine and a potent antiangiogenic molecule. MDA-7 protein binds two distinct type II cytokine heterodimeric receptor complexes: IL-20R1/IL-20R2 (type 1 IL-20R) and IL-22R1/IL-20R2 (type 2 IL-20R). In this study we analyzed the activity of glycosylated secreted MDA-7 against human breast cancer cells. MDA-7 protein induced phosphorylation and nuclear translocation of STAT3 in breast tumor cells. MDA-7 protein also induced dose-dependent cell death in breast tumor cells, mediated via apoptosis. Antibodies against MDA-7 and the IL-20R1 receptor subunit were effective in blocking cell killing suggesting that the bystander apoptotic effect of MDA-7 is effected through type I IL-20R complexes. These results define a tumor-selective cytotoxic bystander role for secreted MDA-7 protein and identify a novel receptor-mediated death pathway in breast cancer cells.

6106

The tumour-suppressor function of breast myoepithelial cells is mediated in part through regulation of S100A9.

Mulligan KT, Wang H, Shaw JA, Hart IR, Jones JL. Institute of Cancer, Cancer Research UK Clinical Centre, Charterhouse Square, London, United Kingdom; University of Leicester, Leicester, United Kingdom

Cell-matrix interactions are known to be important in the control of many cellular functions including proliferation, apoptosis and tissue-specific gene expression. In normal breast, myoepithelial cells (MEC) form the major interface of the epithelial compartment with the extracellular matrix, however, this interface is disrupted with the development of invasive carcinoma.

We previously have demonstrated that primary MEC derived from normal breast exert a broad tumour-suppressor effect, reducing breast cancer cell proliferation and invasion and enhancing apoptosis. To address the mechanisms involved, we have performed cDNA microarray analysis on T47-D tumour cells co-cultured with MEC and compared the expression pattern to that obtained from T47-D cells grown alone. A number of potential tumour-suppressor genes were up-regulated in co-cultured T47-D cells including the calcium-binding protein S100A9 which showed an approximately 30-fold increase in the presence of MEC. S100A9 has been implicated in the control of cell growth, and therefore was a potential mediator of the MEC-induced reduction in proliferation. To investigate whether the MEC-mediated reduction in proliferation is mediated via S100A9, co-cultured T47-D cells were transfected with S100A9 siRNA. In the presence of S100A9 knockdown there was significant abrogation of MEC-mediated suppression of proliferation compared to control cells.

In conclusion, we demonstrate that normal breast MEC exert a broad tumour-suppressor role and that the suppression of breast cancer cell proliferation is mediated at least in part through the regulation of S100A9. Dissecting the molecular mechanisms involved in MEC-mediated tumour-suppression may help identify new prognostic markers and potential therapeutic targets.

6107

Evidence that PI3K/Akt pathway is involved in the short non genomic autocrine loop between 17-β estradiol and aromatase activity.

Catalano S, Barone I, Giordano C, Rizza P, Gu G, Mastroianni F, Bonofiglio D, Ando' S. Faculty of Pharmacy, Rende, CS, Italy

Background: Estrogens are strongly associated with breast cancer development and tumor growth in both premenopausal and postmenopausal women. The intratumoral conversion of androgens to estrogens by aromatase within the breast may be an important mechanism through which 17-β estradiol (E2) stimulates hormone-dependent breast cancer growth. The expression of aromatase in breast cancer tissue has been demonstrated by immunocytochemistry, RT-PCR and enzymatic activity. It has been shown how E2 may regulate aromatase activity in other vertebrates. The aim of the present study was to evaluate if E2/ER can modulate this enzymatic activity in human breast cancer cells.

Material and Method: In MCF-7 cells we examined, by the tritiated water release assay using 0.5μM [1β 3H] androst-4-ene-3-17 dione as substrate, aromatase activity in the presence or absence of E2, ICI 182,780, inhibitors or dominant-negative of MAPK and PI3K/Akt pathway and PTPase inhibitor. Western Blotting and immunoprecipitation assay were performed to evaluate Akt activation and phosphorylation status of aromatase protein upon E2 treatment.

Results: An increase of aromatase activity in MCF-7 cells, upon short exposure to E2 100nM, was observed without any change in the enzyme expression, whereas the addition of ICI 1μM reversed E2-induction (control=15.3±1.2; E2=36.4±1.9; E2+ICI=13.5±1.1 fmol/h/mg protein). The above reported effect was also reproduced in MCF-7 cells overexpressing aromatase ectopically. When we attempted to evaluate how an important pathway of cell survival, like PI3K/Akt, may influence aromatase activity, we revealed that either LY 294002 10μM or a dominant-negative of Akt ectopically expressed in MCF-7 abrogated the E2-induction. In the presence of constitutively active Akt we observed an increase in E2-stimulated aromatase activity. In ER-negative SKBR-3 cells, an up-regulatory effect of aromatase activity was induced by E2 only in the cells transfected with ERα, but not with ERβ. Finally, in the presence of sodium orthovanadate 10μM, a PTPase inhibitor, an increase of aromatase activity was noticeable, addressing how changes in phosphorylation status may influence aromatase activity.

Discussion: It is well known that aromatase is regulated at the transcriptional level through the alternative use of tissue specific promoters, while there are a few studies regarding post-translational regulation of aromatase activity. Our results for the first time demonstrated that E2/ERα up-regulates aromatase activity via interaction with the PI3K/Akt pathway. The phosphorylation processes of the aromatase protein sound to play a key role in the rapid changes in its enzymatic activity. All these data suggest the existence of a non genomic autocrine loop between E2 and aromatase activity in breast cancer cells.

6108

(18)F flurothymidine (FLT) for imaging of tumor cell proliferation; an in vitro-study for monitoring chemo-endocrine treatment in breast cancer cells.

Fersis N, Jusufoska A, Gargya B, Rupp C, Wallwiener D, Dittmann H. University of Heidelberg, Heidelberg, Germany; University of Tuebingen, Tuebingen, Germany

Objectives: F- FLT-PET is a promising tool for imaging primary breast cancer and metastases. Studies are needed to evaluate the clinical usefulness of FLT-PET for monitoring tumor response of neoadjuvant treated patients. Aim of the study was to evaluate whether FLT detects permutation of tumor cell proliferation as induced by chemo-endocrine treatment.

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P1-246

Evidence That Estradiol, through a Short Non Genomic Loop, Downregulates PTP1B and Enhances Aromatase Activity in MCF-7 Cells.

Ines Barone[1], Stefania Catalano[1], Cinzia Giordano[1], Ivan Casaburi[1], Guowei Gu[1], Pietro Rizza[1], Daniela Bonofiglio[1], Sebastiano Ando*[2,3]. *[1]Pharmacobiol, Univ of Calabria, Rende, Cosenza, Italy; [2]Cell Biol, Univ of Calabria, Rende, Cosenza, Italy; [3]Ctr Sanitario, Univ of Calabria, Rende, Cosenza, Italy.*

Estrogens play a crucial role in the development and progression of breast cancer. The intratumoral conversion of androgens to estrogens by aromatase within the breast may be an important mechanism of autocrine stimulation in hormone-dependent breast cancer. Estrogens, in addition to classic genomic actions, may elicit rapid "nongenomic" effects via a series of phosphorylation events. The involvement of protein phosphorylation in the regulation of aromatase activity has been evidenced, eventhough there are a few studies regarding post-transcriptional regulation of the enzyme. Thus, the aim of this study was to evaluate if estradiol (E2) can modulate aromatase activity in human breast cancer cells.

In MCF-7 cells wild-type and overexpressing aromatase gene, we demonstrated that E2 was able to enhance, at short time, aromatase activity without any change in the enzyme expression.

For the first time, we evidenced that the rapid changes in aromatase activity resulted from a direct phosphorylation of the protein itself. Indeed, E2 treatment induced a specific enhancement of tyrosine phosphorylation levels in His6-tagged aromatase purified protein. Sodium orthovanadate, the inhibitor of tyrosine phosphatases, increased basal and E2 induced enzymatic activity as well as tyrosine phosphorylation of aromatase purified protein. Mutagenesis studies confirmed the involvement of tyrosine residues in E2 modulation of aromatase activity.

Tyrosine phosphorylation is a reversible and dynamic process controlled by the activities of the protein tyrosine kinases (PTKs) and the competing actions of the protein tyrosine phosphatases (PTPs). In this respect, we identified PTP1B, the tyrosine phosphatase highly expressed in human breast cancer cell lines, as a crucial intermediate of E2 induction. We demonstrated a specific association between PTP1B and aromatase at protein-protein level and a reduction of aromatase activity in basal and E2-treated in MCF-7 cells overexpressing PTP1B. Moreover, E2 induced a significant increase in serine phosphorylation of endogenous PTP1B and a decrease of its catalytic activity as evidenced by *in vitro* phosphatase assay.

Taken together, our results suggest that E2, through a non transcriptional event, can downregulate PTP1B and then increase aromatase enzymatic activity, enhancing local estrogen production. This short autocrine loop furthermore gives a great emphasis to the role of aromatase in promoting breast cancer cell growth.

P1-247

Estrogen Inhibits DNA-Damage Signaling to Cell Cycle Checkpoints and DNA Repair in Breast Cancer.

Mahnaz Razandi[2], Ali Pedram[1], Albert J Evinger[1], Eva Lee[3], Ellis R Levin*[1,2,3]. *[1]Med, Univ of California, Irvine, Irvine, CA; [2]Med, Long Beach VA Med Ctr, Long Beach, CA; [3]Biochem, Univ of California, Irvine, Irvine, CA.*

The hallmarks of cancer mainly center on the abnormal growth and survival of transformed cells. In addition, the inability to repair DNA lesions in a timely fashion importantly promotes the developmental biology of tumors. Single- and double-stranded DNA breaks cause the activation of the ATR and ATM kinase signal cascades, respectively. In response to UV radiation or hydroxyurea (HU)-induced DNA damage, ATR is activated and signals to cell cycle checkpoints and DNA repair. We found that exposure of MCF-7 cells to UV activated ATR kinase-dependent phosphorylation of p53 (ser15), and Chk1 (ser345) in 5 minutes. The activating phosphorylations were substantially blocked by 10 nM E2, reversed by ICI182780. Targeting the E domain of ERα to the plasma membrane supported this action of E2 in ER-null breast cancer cells, while nuclear targeted-E domain did not. An ERα but not ERβ agonist blocked ATR activation in MCF-7 cells. ATR activation depends upon the physical association with ATRIP and TopBP1 proteins. E2 prevented UV-induced ATR:ATRIP and ATR:TopBP1 associations. The latter occurred through E2 stimulating the rapid activation of AKT-induced phosphorylation of TopBP1 at ser1149. E2 also prevented Claspin protein association with Chk1, required for Chk1 activation by ATR. This occurred by E2 promoting the AKT-induced phosphorylation of Chk1 at ser280, dissociating Claspin from Chk1, leading to the inhibition of the activating Chk1 phosphorylation at ser345. ATR-induced Chk1 kinase phosphorylation of the CDC25C phosphatase prevents Cdk1/Cyclin B activation, and mediates the G2/M checkpoint that prevents passage of mutations to daughter cells. All these actions were induced by UV or HU and were prevented by E2. E2 also delayed DNA repair, shown by the Comet assay and DNA 6-4 photo-adduct formation in MCF-7 cells exposed to UV or HU. In summary, we identify novel and potentially important functions of E2 that promote the development of breast cancer.

Supported by VA Merit Review and NIH Grant CA100366 (to ERL).

P1-248

Re-Activation of the p53 Pathway in Mutant p53 Expressing T47-D Breast Cancer Cells Suppresses Their Estrogen-Dependent Proliferation and Prevents In Vivo Tumor Formation.

Salman M Hyder*[1], Cynthia Besch-Williford[2], Indira Benakanakere[1], Yayun Liang[1]. *[1]Biomed Scis & Dalton Cardiovasc Res Ctr, Univ of Missouri, Columbia, MO; [2]Vet Pathbiol, Univ of Missouri, Columbia, MO.*

Over 50% of breast cancers express mutant p53 protein (mtp53) and the majority are estrogen-responsive. With this in mind we initiated studies to determine whether re-introduction of wild-type p53 (wtp53) functions in tumor cells prevents their hormone-dependent progression. Procedures to replace defective p53 functions in breast tumor cells are actively being pursued in clinical trials and thus it is essential to determine whether wtp53 is able to over-ride the effects of mtp53 protein and suppress uncontrolled cellular proliferation. We undertook to examine suppression of mtp53 activity in T47-D cells (mutation in the DNA-binding region). First, we re-expressed wtp53 protein in T47-D cells and selected stable clones that showed increased p53 binding to a p53 DNA binding site. Second, we used a small molecule drug, PRIMA-1 (p53 reactivation and induction of massive apoptosis) to convert endogenous mtp53 protein in T47-D cells into a functional DNA-binding form. We demonstrate that expression of wtp53 is able to over-ride the functions of naturally occurring inactive mtp53 protein in T47-D cells, thereby suppressing cell proliferation in regular media, and estrogen-dependent proliferation in media containing charcoal treated serum. Growth inhibition is not due to absence of estrogen receptor-alpha or estrogen receptor-beta though the receptor levels for estrogen receptor alpha were drastically reduced in wtp53 expressing cells. Focused microarray analysis of wtp53 expressing cells revealed suppression of PCNA cell-cycle regulatory gene both the mRNA and protein levels and cells failed to grow in vivo in nude mice that were implanted with slow-release estradiol pellets. Furthermore, xenografts obtained with parental T47-D cells expressing mtp53 grew poorly in estrogen-supplemented nude mice treated with PRIMA-1 which was administered to activate the p53 pathway. PRIMA-1 treated tumors exhibited a low proliferation index, strongly suppressed VEGF levels and induction of estrogen receptor-beta in tumor cells. Expression of estrogen receptor-alpha or progesterone receptors remained unaffected. These data indicate that alteration of the p53 signal transduction pathway can prevent proliferation of T47-D breast cancer cells both in vivo and in vitro. Thus activation of a functional p53-mediated signal transduction pathway in human breast cancer cells could be an effective strategy to suppress growth of hormone-dependent human breast cancers.

This research was supported by NIH grant CA-86916; Dept of Defense Breast Cancer Research Program W81XWH-05-1-0416; PDF0600723 and BCTR0600704 from Susan G Komen Breast Cancer Foundation, and by funds from the Research Animal Diagnostic Laboratory.

P1-249

Breast Cancer Androgen Receptors Are Implicated in Anti-Estrogen Therapy Failure.

Djuana ME Harvell[1], Nicole NS Spoelstra[2], Meenakshi Singh[2], Christina Finlayson[2], Anthony D Elias[4], Kathryn B Horwitz[1], Jennifer K Richer*[2]. *[1]Dept of Med, Div of Endocrinology, Metab & Diabetes, Univ of Colorado Hlth Scis Ctr, Aurora, CO; [2]Pathol, Univ of Colorado Hlth Scis Ctr, Aurora, CO; [3]Surg, Univ of Colorado Hlth Scis Ctr, Aurora, CO; [4]Dept of Med, Div of Med Oncol, Univ of Colorado Hlth Sci Ctr, Aurora, CO.*

Known predictors of breast cancer responsiveness to endocrine therapies include tumor size, lower stage, and presence of estrogen (ER) and/or progesterone (PR) receptors. However, ~30% of patients with ER or PR-positive breast cancers fail to show a clinical response to endocrine therapy and are said to have *de novo* or intrinsic resistance. The primary purpose of this study was to identify biomarkers that would improve prediction of response to endocrine therapies. This study implements a global gene profiling approach examining patient-matched tumor samples pre- and post-treatment with the aromatase inhibitor exemestane, or exemestane plus tamoxifen, in the neoadjuvant setting. We identify genes associated with successful tumor shrinkage as achieved by estrogen blockade therapy. In all pre-treatment tumor biopsies, cell cycle and cell death related genes were similarly represented. In contrast, after treatment the number of cell cycle related genes decreased in responders, but dramatically increased in non-responders. Conversely, the percentage of cell death-related genes rose dramatically with treatment in responders, but fell in non-responders. In pre-treatment biopsies of responders, assembly and organizational genes predominated (45%), whereas in the non-responders this category represented only 10%, while tissue development/remodeling was the major (33%) category. In addition, prediction Analysis of Microarrays software (PAM) was used to identify 50 genes that can predict likelihood of response. Of these, five (S100A8, Vav3, GREB1, Clusterin, and Annexin II) are known to be either androgen receptor (AR) regulated or differentially expressed in "androgen-dependent" versus "androgen-independent" prostate cancers. In the breast cancers, AR protein dramatically decreased with treatment in responders, but remained steady in non-responders. These data suggest that more attention to measurement of AR levels in breast tumors is warranted and that measurement of serum androgens before and during AI treatment be incorporated in future clinical/translational studies. In summary, we identify predictor genes when combined with tumor grade, proliferation markers, and ER/PR status, may