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Inhibition of Estrogen-dependent Cyclin D1 expression by Androgen Receptor in MCF-7 breast cancer cell

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ABSTRACT

Hormones play a crucial role in endocrine-mediated tumorigenesis and have been shown to influence cancer cell growth and progression . However, androgens and estrogens exert opposing effects on the growth and development of malignant human breast tissue. The high frequency of androgen receptor (AR) expression in both primary breast tumours (70-90%) and in breast metastases (75%) suggests that androgens are important modulators of breast cancer cell proliferation. Emerging evidences indicate that androgens have inhibitory effects on the growth of normal breast epithelial cells and play a protective role in the pathogenesis of breast cancer growth.

DHT treatment resulted in gradual and progressive decreases in the proportion of cells in S phase with concomitant increases in G1 phase cells. The progression of normal cells through the G1/S phase transition is dependent on the accumulation of G1 cyclin proteins such us D-type cyclins. These cyclins link the extracellular signals to the cell-cycle machinery and of the three D-type cyclins, it is cyclin D1 that predominantly associated with human tumorigenesis.

Since DHT has been shown to impede progression of MCF-7 from G1 into S phase, we examined the possibility that androgens, through their own receptor, inhibit breast cancer cell proliferation by modulating cyclin D1 expression. In this report we demonstrate that in MCF-7 wild type and over-expressing AR, DHT treatment dramatically decreases the expression of cyclin D1 protein and the transcriptional activity of the cyclin D1 promoter induced by E2.

The molecular mechanism underlining this inhibitory effect seems to involve the competition for the steroid receptor coactivator AIB1, that is important in the functional coupling of the ER with the cyclin D1 promoter. Physical squelching of the AR/ER common coactivator AIB1 may be responsible of the AR-mediated negative modulation of the ER-dependent signalling pathway and inhibition of breast cancer cells proliferation. Indeed, AIB1 sequestration by AR results in the

activation of AR-responsive gene pathways, such as p21 whereas negatively modulates estrogen-dependent cyclin D1 expression, which in turn impacts on MCF-7 cells proliferation.

INTRODUCTION

INTRODUCTION

The development of breast cancer often depends on the action of sex steroid hormones. Nuclear hormone receptors are members of the nuclear receptor super-family and act as ligand-inducible transcription factors, controlling the expression of target genes by interacting, directly or indirectly, with a number of co-regulatory complexes involved in chromatin remodeling and histone modification.

The high frequency of androgen receptor (AR) expression in both primary breast tumours (70-90%) and in breast metastases (75%) (*Lea et al.*, 1989; *Moinfar et al*., 2003; *Riva et al*., 2005) suggests that androgens are important modulators of breast cancer cell proliferation. Emerging evidences indicate that androgens have inhibitory effects on the growth of normal breast epithelial cells and play a protective role in the pathogenesis of breast cancer growth (*Birrell et al*., 1998; *Labrie et al.*, 2003; *Somboonporn & Davis*, 2004; *Yeh et al.*, 2003). Androgens are able to counterbalance positive growth stimuli in the breast since they antagonize the estrogen-induced proliferative effects which are intimately linked to breast carcinogenesis (*Murphy & Watson*, 2002). *In vivo* studies in rhesus monkey evidenced that blocking the action of endogenous androgens results in a significant increase in mammary epithelial cell proliferation, whereas, in ovariectomized animals, low doses of testosterone completely inhibit estrogen-induced mammary cell proliferation. (*Dimitrakakis et al*., 2003; *Murphy & Watson*, 2002).

In breast tumors expression of the AR correlates with increased patient survival (*Agoff et al*., 2003; *Schippinger et al.*, 2006). The expression of the androgen-regulated prostate-specific antigen (PSA) (*Lu et al.*, 1999) in breast cancer specimens and nipple aspirate, has been positively correlated with a low tumor grade, smaller tumors and a better prognosis (*Kimura et al.*, 1993; *Sauter et al.*, 2004; *Sauter et al.*, 2002; *Soreide et al.*, 1992). Moreover, several events involved in breast cancer genesis or progression have been shown to alter AR expression or function. In BRCA1-mutated tumors, loss of AR expression, and thus loss of AR signaling, supports neoplastic transformation of mammary epithelial cells (*Berns*

et al., 2003). In addition, in HER2-positive breast cancers, generally associated with a worse outcome, a trend toward a loss of AR signaling has been demonstrated (*Kollara et al.*, 2001).

In vitro, androgens, through AR, mainly exert an inhibitory effect on AR-positive breast cancer cell proliferation. The non-aromatisable androgen 5-α-dihydrotestosterone (DHT) inhibits both basal and estradiol-induced proliferation in estrogen-receptor positive breast cancer cell lines (*Andò et al.*, 2002; *Birrell et al.*, 1998; *de Launoit et al.*, 1991; *Greeve et al.*, 2004). Furthermore, coadministration of testosterone suppresses the estradiol-mediated induction of MYC (*Zhou et al.*, 2000) and over-expression of the AR in MCF-7 cells markedly decreased estrogen receptor α (ERα) transcriptional activity (*Andò et al.*, 2002; *Lanzino et al.*, 2005) consistent with the notion that androgens inhibit estrogen signalling pathways.

Regulation of mammalian cell proliferation by extracellular mitogens is controlled by receptor-initiated signalling pathways that ultimately converge on the cell cycle machinery, thereby mediating a G1/S transition (*Pardee AB*, 1974). Indeed, the stimulation of growth arrested cells in response to various mitogenic factors results in the induction of D-type cyclins, that link the extracellular signals to the cell cycle machinery. Of the three D-type cyclins, it is cyclin D1 that is predominantly associated with human tumorigenesis.

Specifically, in mammary epithelial cells growth factors and estrogens exert their proliferative effects by induction of the G1 progression and G1/S transition through a mechanism involving the transcriptional control of cyclin D1 gene (*CCND1)* (*Altucci et al.*, 1996; *Musgrove et al.*, 1993; *Richer et al.*, 2002). Evidence for a biologically relevant role for cyclin D1 in breast tumorigenesis includes several findings: mammary gland-targeted cyclin D1 over-expression resulted in mammary hyperplasia and adenocarcinoma in transgenic mice (*Wang et al.*, 1994); cyclin D1 antisense blocked ErbB2-induced mammary tumor growth in vivo (*Lee et al.*, 2000), and cyclin D1-deficient mice were resistant to ErbB2- or Ras-induced mammary tumorigenesis (*Yu et al.*, 2001). The correlation between CCND1 expression levels and cellular proliferation in breast cancer cells has been further confirmed by CCND1 silencing experiments, underlining cyclin

D1 as a potential therapeutic target for breast cancer (*Arnold & Papanikolaou,* 2005; *Grillo et al.*, 2006). The involvement of the *CCND1* gene in the estrogen proliferative effects on breast cancer cells is highlighted by several lines of evidence. Estrogens induce cyclin D1 gene in hormone responsive breast cancer cells (*Altucci et al.*, 1996); conversely, treatment of these cells with cyclin D1 antisense oligonucleotides blocks hormone-dependent cell proliferation (*Carroll et al.*, 2000). Cyclin D1 induction by estrogens in breast cancer cells shortens cell cycle G1-phase and can rescue growth factor deprived and antiestrogen-arrested cells enabling them to complete the cell cycle (*Bonapace et al*., 1996; *Musgrove et al.*, 1994).

Since DHT has been shown to inhibit progression of MCF-7 from G1 into S phase (*Greeve et al.*, 2004), we examined the possibility that androgens, through their own receptor, inhibit breast cancer cell proliferation by modulating cyclin D1 expression. In this report we demonstrate that in MCF-7 wild type and MCF-7 cells over-expressing the AR, DHT treatment dramatically decreases estradiol-induced expression of cyclin D1 protein by inhibiting cyclin D1 promoter transcriptional activity. Moreover, since ER-mediated gene transcription is regulated not only by binding to estrogens but also by other post-translational events such as the interactions with coregulatory proteins, we investigated the role of the steroid receptor coactivator AIB1, that is important in the functional coupling of the ER with the cyclin D1 promoter. Our data suggest that competition for AIB1 interaction, may represent a possible mechanism through which AR can modulate ER-mediated signalling pathway and inhibit breast cancer cells proliferation.

MATERIALS and METHODS

Materials **-** Dulbecco's modified Eagle's medium (DMEM/F-12), Lglutamine, Eagle's non-essential amino acids, penicillin, streptomycin, calf serum, bovine serum albumin, and phosphate-buffered saline were purchased from Eurobio (Les Ullis Cedex, France). Triazol reagent was obtained from Invitrogen, and FuGENE 6 was from Roche Applied Science. TaqDNA polymerase, 100-bp DNA ladder, dual luciferase kit, and thymidine kinase *Renilla* luciferase plasmid were provided by Promega (Madison, WI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and sodium orthovanadate were purchased from Sigma. Antibodies against p21, Cyclin D1, AIB1, tubulin, β-actin and salmon sperm DNA/ protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz CA). Biotinylated horse anti-goat IgG and ABC complex/horseradish peroxidase were provided by Vector Laboratories. Chromogen, 3-diaminobenzidine tetrachloride dihydrate, was purchased from Bio-Optica. ECL System was purchased from Amersham Biosciences. VCX500 ultrasonic processor was provided by Sonics (Newtown, CT).

Cell Culture and Proliferation assay **-** Breast cancer epithelial cell line MCF-7 were gifts from Dr. B. Van der Burt (Utrecht, The Netherlands) and grown in DMEM/F-12 medium containing 5% fetal calf serum, 1% L-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/ml penicillin/streptomycin in a 5% CO2 humidified atmosphere. Before each experiments cells were serum-starved for 24h in phenol red-free DMEM (PRF). Hormone stimulation was performed in DMEM containing 5% charcoal-treated fetal calf serum (PRF-CT) to reduce the endogenous steroid concentration.

Cell proliferation assay was performed as follow: MCF-7 and MCF-7 AR-transfected cells were seeded on six well plates (10^5 cells/well), grown for 2 days in complete medium, starved PRF medium for 24 h and then exposed to E2 and DHT in PRF-CT medium (for details, see the figure legends). After 3 days cells were harvested by trypsin and counted by trypan blue exclusion, using Burker's chamber.

Plasmids - The plasmid WWP-Luc containing human $p21^{WAF1/Cip1}$ promoter was kindly given by Dr. Wafik El-Deiry (Howard Hughes Medical Institute, Philadelphia); cyclin D1 (D1Δ-2966pXP2-Luc) promoter construct was a gift from Dr. Weisz A. (University of Naple, Italy). Expression vector for the wild-type AIB1 was a gift from Dr. O'Malley B. (Houston University, USA). Plasmid encoding full-length androgen receptor, pcDNA3-AR (AR), was a gift from Dr.McPhaul MJ (Texas University, USA).

Transfection Assay - MCF-7 were transfected using FuGENE 6 Reagent as recommended by the manufacturer. All transfection mixtures contained an appropriate amount of the various plasmids as indicated in the figure legends. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. pRL-TK-Luc, a plasmid encoding *Renilla* luciferase, was used to assess transfection effiency. Upon transfection, the cells were shifted to PRF for 24h and then treated with 10^{-7}M E2 or/and 10^{-7}M DHT or left untreated for 24 h. Luciferase activity in cell lysates was measured using the Dual luciferase assay System following the manufacturer's instructions. The firefly luciferase data for each sample were normalized to *Renilla* luciferase activity and expressed as relative luciferase units.

For whole cell extracts, cells were plated on a 100mm dish and transfected with an appropriate amount of the various plasmids as indicated in the figure legends. Upon transfection cells were shifted to PRF for 24 h and then treated with 10^{-7} M E2 and/or 10^{-7} M DHT or untreated in PRF-CT as indicated in the figure legends.

AIB1 Knockdown - AIB1 expression in MCF-7 cells was reduced using the Stealth™ Select RNAi (Invitrogen) following manifacture's instructions (MCF-7/siAIB1). In 35mm plates, AIB1 RNAi duplex (100pmol/well) was mixed with Lipofectamine 2000 (1:1), and incubated for 15 min at RT and than transfected into MCF-7 cells. After 6 h, the cells were shifted in complete medium for 24 h and then treated as indicated in the figure legend. As negative control, MCF-7 cells were transfected with no specific siRNA (Scrambled). The efficiency

of the knockdown was determined by Western analysis using corresponding specific antibody.

Western Blotting and Immunoprecipitation Analysis - Whole cell extracts were prepared by lysing cells in buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 3 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate) and centrifugation to remove the insoluble material. For Western blotting assay, 20-50 μg of the protein lysates were separated on SDS-10% polyacrylamide gels and electroblotted to nitrocellulose membrane. Membranes were incubated 1 h with 5% non fat dry milk in 0.2% Tween 20 in Tris-buffered saline (TBS-T) at room temperature and then probed with an appropriate dilution of the various antibodies as indicated in the figure legends, followed by anti-goat or anti-mouse horseradish peroxidase-conjugated antibody. Blots were developed using the ECL Plus Western blotting detection system according to the manufacturer's instructions.

For immunoprecipitation assay, 500 μg of the protein lysates were incubated for 2 h with protein A/G-agarose beads at 4 °C and centrifuged at 12,000 x *g* for 5 min, to avoid non-specific binding. The supernatants were incubated overnight with 10 μl of the antibodies (as indicated in the figure legends) (Santa Cruz) and 500 μl of HNTG (IP) buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin) for each. Immune complexes were recovered by incubation with protein A/G-agarose. The beads containing bound proteins were washed three times by centrifugation in IP buffer, then denatured by boiling in Laemmli sample buffer, and analyzed by Western blot to identify the coprecipitating effector proteins. Immunoprecipitation with protein A/G alone was used as the negative control. Membranes were stripped of bound antibodies by incubation in glycine 0.2 M, pH 2.6, for 30 min at room temperature. Prior to reprobing with different primary antibodies, stripped membrane were washed extensively in TBS-T and placed in blocking buffer (TBS-T containing 5% milk) overnight.

Reverse Transcription-PCR - Total cellular RNA was extracted using Triazol reagent as suggested by the manufacturer. 2 μg of total RNA were reverse-transcribed using 200 IU of Moloney murine leukemia virus-reverse transcriptase in a reaction volume of 20 μl (0.4 μg of oligo(dT), 0.5 mM deoxy-NTP, and 24 IU of RNasin) for 30 min at 37 °C, followed by heat denaturation for 5 min at 95 °C. The primers used are as follows: Cyclin D1 (353bp): forward, 5'-TCTAAGATGAAGGAGACCATC, and reverse, 5'-GCGGTAGTAGGAGAGGAAGTT-3'; Ribosomal RNA 36B4 (408-bp) was amplified as internal control: forward, 5'-CTCAACATCTCCCCCTTCTC-3', and reverse, 5'-CAAATCCCATATCCTCGTCC-3'; p21WAF1/Cip1 (270bp), forward 5'-GCTTCATGCCAGCTACTTCC-3' and reverse 5'-CTGTGCTCACTTCAGGGTCA-3'. PCR amplifications was performed in 10 mM Tris-HCl, pH 9.0, containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM MgCl2, and 0.25 mM each dNTP.

Chromatine Immunoprecipitation Assay (ChIP) - We followed the ChIP methodology described by Shang *et al.* with minor modifications. MCF-7 and MCF-7/AR cells were grown in 100-mm plates. 90% confluent cultures were shifted to PRF for 24 h and then treated with 10^{-7} M E2 and /or 10^{-7} M DHT for 15 min or were left untreated in PRF-CT. Following treatment, the cells were washed twice with phosphate-buffered saline and cross-linked with 1% formaldehyde at 37 °C for 10 min. Next the cells were washed twice with phosphate-buffered saline at 4 °C, collected, and resuspended in 200 μl of Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1) and left on ice for 10 min. Then the cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4 °C for 10 min at 14,000 rpm. Supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 μl of sonicated salmon sperm DNA/protein A-agarose for 1 h at 4 °C

The precleared chromatin was immunoprecipitated for 12 h either with anti AIB1 antibodies or with normal mouse IgG (Santa Cruz Biotechnology) as the negative

control. After that, 60 µl of salmon sperm DNA/protein A-agarose was added, and precipitation was 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-Cl, pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with Elution buffer (1% SDS, 0.1 M NaHCO3). The eluates were reverse cross-linked by heating at 65°C for 12 h and digested with 0.5 mg/ml proteinase K at 45°C for 1 h. DNA was obtained by phenol/chloroform/isoamylalchol (25:24:1) extractions. 2 µl of 10 mg/ml yeast tRNA were added to each sample, and DNA was precipitated with ethanol for 12 h at -20 °C and resuspended in 20 µl of TE buffer. 5 µl of each sample were used for PCR with the following promoter primers: (sense) 5'-GGCGATTTGCATTTCTATGA-3' and (antisense) 5'–CAAAACTCCCCTGTAGTCCGT-3' for Cyclin D1 (233bp); (sense) 5'-CAGCGCACCAACGCAGGCG-3' and (antisense) 5'-CAGCTCCGGCTCCACAAGGA-3' to amplify a 296bp of the ARE-containing p21 promoter.

Statistical Analysis - Each datum point represents the mean ±S.E. of three different experiments. Data were analyzed by analysis of variance test using the STATPAC computer program.

RESULTS

RESULTS

Androgen receptor inhibits serum-induced as well as estrogen-dependent MCF-7 cells proliferation.

We previously demonstrated that MCF-7 cells are androgen responsive and that DHT treatment induces a transient increase in AR protein levels (*Lanzino et al.*, 2005) similar to that seen in other cell types (*Kemppainen et al.*, 1992; *Yeap et al.*, 1999; *Zhou et al.*, 1995).

Thus, we investigated the role of the AR on the ER-positive MCF-7 cells proliferation. To this aim MCF-7 cells were serum starved; after 48hours medium was replaced with 2.5% Dextran Charcoal Treated DMEM/F12 in absence or presence of 10^{-7} M E2 and 10^{-7} M DHT, alone or in combination, for 3 days (Fig 1A). In addition, to better evidence a role for AR in modulating MCF-7 cells proliferation, serum starved MCF-7 cells were transfected with an expression plasmid encoding the full length AR (MCF-7/AR), or with the empty vector, and then treated with 10^{-7} M E2 and 10^{-7} M DHT, alone or in combination, or left untreated, for 3 days (Fig 1B).

As expected, cells grown in presence of estradiol proliferated, with a twofold increase in average cell number, over 3 days of culture (Fig. 1A). DHT treatment, instead, markedly inhibited serum- as well as E2- induced MCF-7 cell proliferation and, by the end of the treatment, the mean number of DHT-treated cells was less than 40% of respective controls (Fig 1A).

Interestingly, AR over-expression resulted in a more significant decrease of estradiol-induced MCF-7 cell proliferation, when compared to that of untransfected cells, reaching a ~50% inhibition upon combined DHT treatment. In both, Fig. 1A and B, inhibition of basal and E2-dependent MCF-7 cells growth was no longer detected following addition of the androgen antagonist hydroxyflutamide (OHFl), further evidencing the involvement of AR in this process.

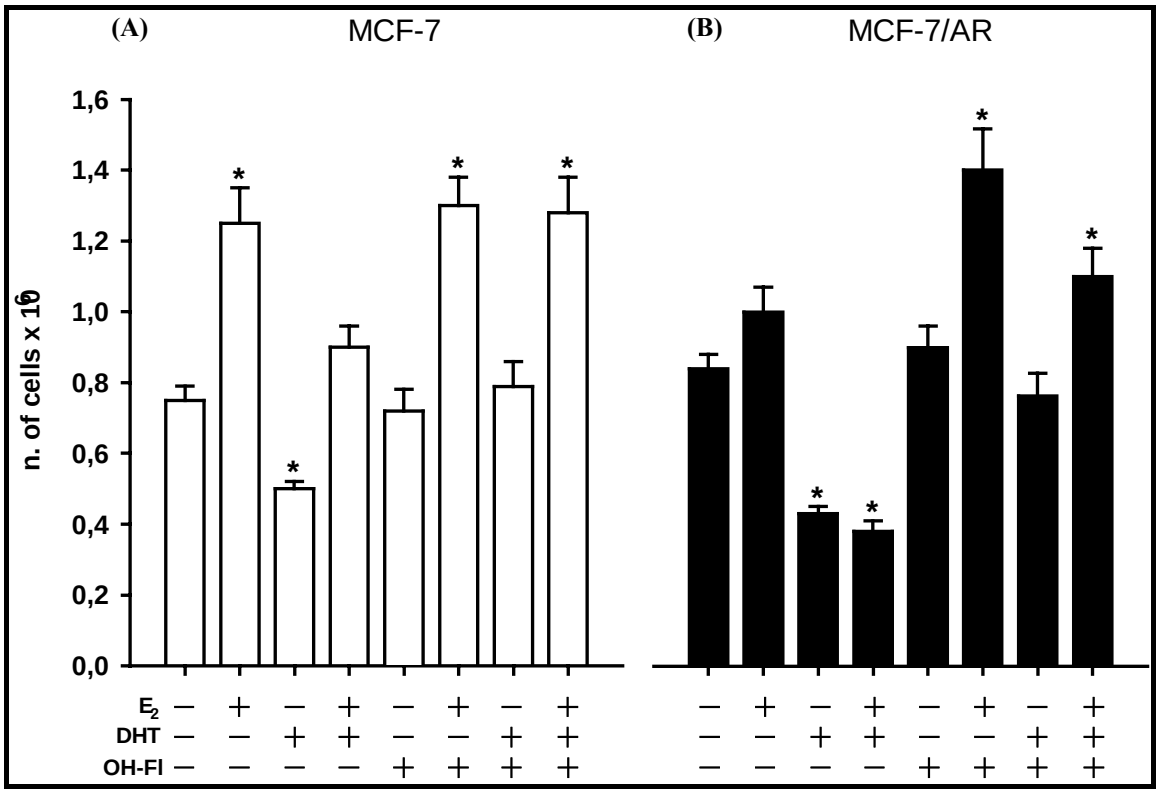

Fig.1: Over-expressed androgen receptor inhibits estrogen-dependent MCF-7 cells proliferation.

A) *MCF-7 cells synchronized in PRF were treated with 10^{-7} M E_2, 10^{-7} M DHT, and 10^{-6} M OH-Fl in PRF-CT for 3 days.* ***B)*** *MCF-7 cells synchronized in PRF were transfected. Upon transfection, cells were treated with 10^{-7} M E_2, 10^{-7} M DHT, and 10^{-6} M OH-Fl in PRF-CT for 3 days. These data represent a mean±s.d. of three independent experiments, each in duplicate. Data were statistically analysed by T student test, * = $p<0.05$*

DHT, through androgen receptor, decreases estrogen-dependent cyclin D1 expression and promoter activity.

Since previous studies demonstrated that DHT treatment impedes G1/S transition in MCF-7cells (*Andò et al.,* 2001; *Greeve et al.*, 2004), we investigated whether increased cellular levels of AR might modulate cyclin D1 expression.

Syncronized MCF-7 cells were left untransfected (Fig. 2A) or transfected with an expression plasmid encoding the full length AR, in absence or presence of 10^{-7} M E2 and 10^{-7} M DHT, alone or in combination, for 48 hours.

In MCF-7 wild type DHT administration resulted in a marked reduction of E2-induced cyclin D1 protein and mRNA expression (Fig. 2, A and B). Instead, MCF-7 cells over-expressing the AR, exhibited a decrease in the E2-dependent expression of cyclin D1 protein and mRNA (Fig. 2, A and B) which were further inhibited following DHT treatment.

The down-regulatory effect of AR on cyclin D1 expression led us to examine the possibility that activated AR may negatively modulate the cyclin D1 promoter transcriptional activity.

As shown in Fig. 2C, a cyclin D1-promoter reporter construct driving luciferase expression (CD1(-2996)-Luc) was, as expected, induced by serum as well as E2 stimulation in MCF-7 cells, while DHT treatment was able to inhibit both basal and E2-dependent cyclin D1 promoter transcriptional activity. This inhibitory effect was better evidenced in MCF-7/AR in which the over-expression of AR, resulted in the complete loss of the transcriptional signal induced by E2 when compared with hormone stimulated activity in the absence of exogenous AR. Moreover, DHT administration was able to induce an additional 40% decrease in E2-dependent transcriptional signal on cyclin D1 promoter. This effect was partially reversed by the addition of OHFl (data not shown) suggesting that it was due to AR activation.

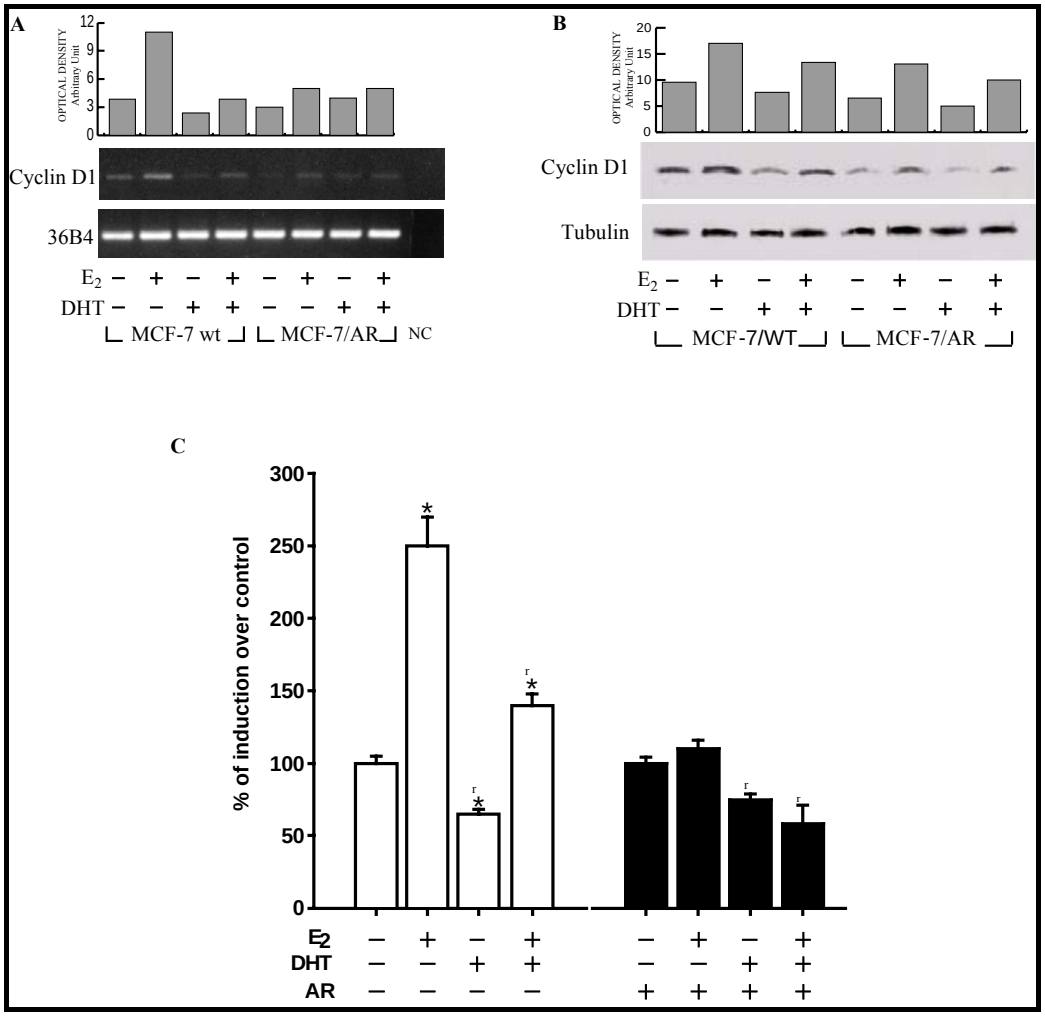

Fig. 2. Estrogen induction of cyclin D1 expression and promoter activity is reduced by over-expression of androgen receptor. *Total cellular RNA* ***(A)*** *and cytoplasmic proteins* ***(B)*** *were isolated from MCF-7 cells synchronized in PRF and left untransfected or transfected (as described in Material and Methods) with 3 μg pcDNA3-AR (AR) and then treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated, in PRF-CT for 24 h* ***(A)*** *or 48 h* ***(B)****.* ***(A)*** *The expression of cyclin D1 mRNA was evaluated by RT–PCR as described in Materials and Methods. 36B4 was amplified as control. MCF-7 RNA sample without the addition of reverse transcriptase was amplified as negative control (NC).* ***(B)****The expression of cyclin D1 was determined by western blotting (WB) using 50 μg of total proteins lysates. The expression of tubulin was assessed as control of protein loading. The results were obtained after repetitive stripping and reprobing of the same filters.*

Results, representative of three independent experiments, were quantified and reported as optical density (OD). ***(C)*** *MCF-7 cells, synchronized in PRF, were grown in PRF-CT for 3 days and transiently cotransfected with pCD1 prom-Luc (0.25μg /well) either in the absence or presence of pcDNA3-AR (AR) (0.5μg /well) as indicated. Upon transfection, cells were treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated in PRF-CT for 24 h. Firefly luciferase activity was analyzed and expressed as Relative Luciferase Activity with respect to the untreated samples, arbitrarily set at 100%. Results represent the mean±s.d. from five independent experiments each in duplicate.*

AIB1 rescues AR repression of estradiol-induced transcriptional activity of cyclin D1 promoter.

The AR interference on ER transcriptional response may implies that shared components of transcriptional machinery are involved (*Torchia et al.*, 1998), suggesting that AR and ERα might use a common pool of co-factors present in limiting cellular concentrations. In this concern, we investigated the role of the steroid receptor coactivator AIB1, that is important in the functional coupling of ERα with the cyclin D1 promoter (*Planas-Silva et al.*, 2001).

To assess whether AIB1 might be involved in the transcriptional interference of AR on ERα transcriptional signal, we examined the possibility that AIB1 overexpression could rescue AR repression of estradiol-induced activity on cyclin D1 promoter.

As shown in Fig. 3A, in the absence of exogenous AR expression, E2 treatment increased cyclin D1 promoter activity. When exogenous AR was simultaneously expressed, most of the activation by E2 was lost (as previously described). However, when the same experiment was repeated in the presence of a progressive increase in the amount of ectopic AIB1, cyclin D1 promoter activity was restored. Thus, AIB1 over-expression is able to abrogate completely the inhibitory effect induced by overexpressed AR and to restore estradiol-induced activity of cyclin D1 promoter in MCF-7 cells.

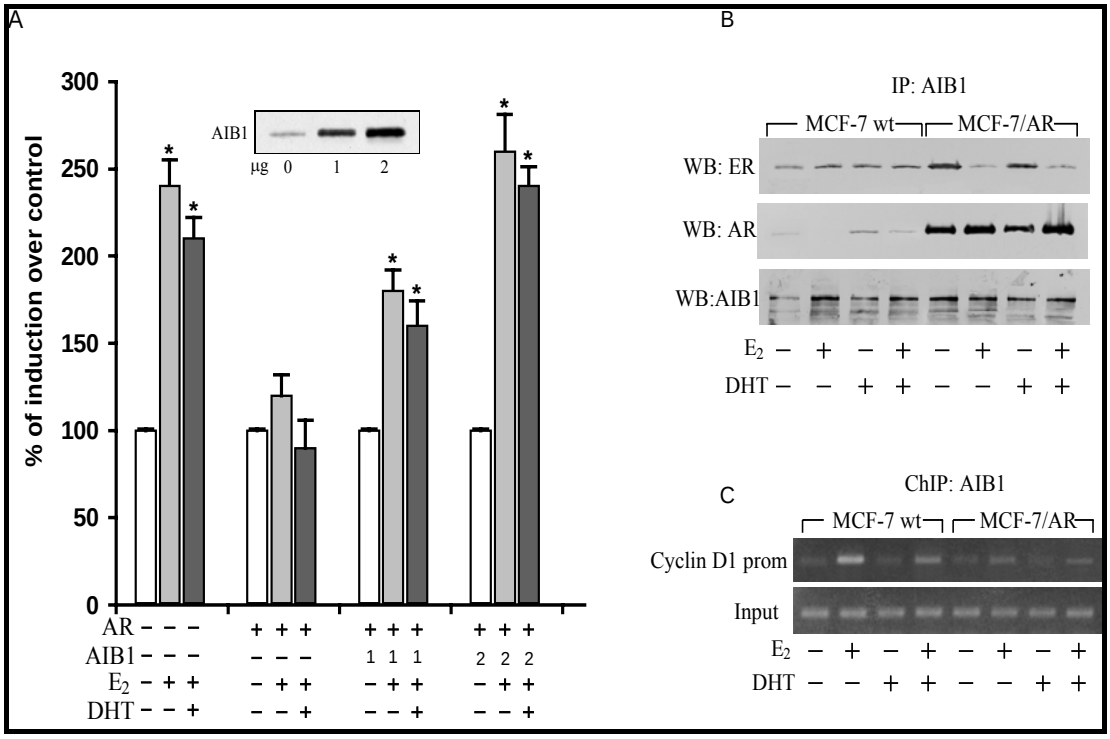

Fig.3 Over-expressed androgen receptor competes with ERα for AIB1 interaction.
(A) *MCF-7 cells, synchronized in PRF, were grown in PRF-CT for 3 days and transiently cotransfected with the promoter of cyclin D1 (0.25µg /well) either in the absence or presence of 0.5 µg pcDNA3-AR (AR) and increasing amounts (given in µg/well) of full-length AIB1 expression plasmid, as indicated. Upon transfection, cells were treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated in PRF-CT for 24hours. Firefly luciferase activity was analyzed and expressed as Relative Luciferase Activity with respect to the untreated samples, arbitrarily set at 100%. Results represent the mean±s.d. from five independent experiments each in duplicate.* ***(B)*** *Total cell extracts from MCF-7wt and MCF-7 overexpressing AR (5µ/dish) were immunoprecipitated (IP) with an anti-AIB1 antibody and immunoblotted (WB) to detect ER and AR protein levels. In control samples, the primary Abs were substituted with non-immune IgG. The results were obtained after repetitive stripping and reprobing of the same filters. Results are representative of three independent experiments.* ***(C)*** *ChIP was carried out on MCF-7 cells synchronized in PRF, grown in PRF-CT for 3 days and transfected with pcDNA3-AR (AR) (5µ /dish) or with the empty vector. Cells were treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated in PRF-CT for 15 min and DNA-associated proteins were precipitated using AIB1 antibody. The estrogen-responsive sequence of cyclin D1 promoter was detected by PCR with specific primers listed in Materials and Methods. 30 µl of initial preparations of soluble chromatin were amplified to control input DNA. In control samples (N) non-immune IgG was used instead of the primary Abs.*

AIB1 interaction with either AR or ERα is related to the intracellular content of both steroid receptors

As AIB1 has been reported to be able to bind and coactivate both ERα and AR (*Anzick et al.*, 1997; *Louie et al.*, 2003) we examined the possibility that the ability of AIB1 to preferentially interact with ERα and/or AR might be related to the intracellular levels of the two steroid receptors. To this aim, AIB1/ERα and/or AIB1/AR complexes formation was analyzed by co-immunoprecipitation experiments.

Total cellular extracts from MCF-7 and MCF-7/AR were subjected to immunoprecipitation with specific AIB1 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and then immunoblotted with antibody against ERα, AR and AIB1. Interestingly, in MCF-7 cells expressing high levels of endogenous ERα and low levels of AR (*Lanzino et al.*, 2005), AIB1 coimmunoprecipitates predominantly with ERα. In contrast in these cells, AR overexpression induces a prevalent interaction between AIB1 and AR (Fig. 3B).

The biological correlation of this AR/ERα competition for a shared coactivator, was investigated to test whether this mechanism might affects ERα activity on endogenous cyclin D1 promoter in the context of its local chromatin structure. Thus, to examine the possibility that AR may antagonize ERα function at the estrogen responsive region of cyclin D1 promoter through inhibiting AIB1 recruitment, we over-expressed AR in MCF-7 cells and assessed cyclin D1 promoter occupancy by chromatin immunoprecipitation (ChIP) assay. An AIB1 specific antibody was used to immunoprecipitate the protein-DNA complex. The presence of the specific promoter sequence in the chromatin immunoprecipitates, was analyzed by PCR with specific pairs of primers spanning the estrogen-responsive region (-245 to -24 bp) of the cyclin D1 promoter (Castro-Rivera E *et al*, 2001)

As indicated in Fig 3C, in MCF-7 cells E2-induced recruitment of AIB1 on cyclin D1 proximal promoter was decreased following addition of DHT. AR ectopic expression resulted in a significant reduction of AIB1 recruitment to the estrogen-responsive region of the cyclin D1 promoter in both basal and E2-induced states,

when compared to MCF-7 untransfected cells. Moreover, combined treatment with DHT caused a further reduction in the AIB1 occupancy of this promoter region.

To further confirm the significance of the modulation of the AIB1/ERα interaction for cyclin D1 functional activity in response to estradiol, we used a AIB1 siRNA approach, to selectively reduce AIB1 gene expression in MCF-7 cells.

The AIB1 siRNA concentration used in our experiment (100 pmol/well) produced a >80% reduction in cellular AIB1 protein levels, which were still repressed after 72 hours. A control scrambled sequence siRNA had no effect on AIB1 expression (Fig. 4A). The expression of either unrelated proteins such as GAPDH and actin or the related p160 coactivator SRC1 was unaltered by AIB1 siRNA (Fig 4B) showing that it was an effective and selective method of long-term suppression of endogenous AIB1 content, useful to determine the impact of reducing endogenous AIB1 on cyclin D1 expression in MCF-7 cells. Treatment with AIB1 siRNA completely negated the increase in cyclin D1 expression induced by estradiol either at level of protein (Fig 4B) or mRNA (Fig 4C) expression. Interestingly, these patterns of cyclin D1 expression reflect the ones observed following AR over-expression (Fig. 2A, B), supporting the hypothesis that AIB1 sequestration by AR might be an effective mechanism to explain the AR-mediated reduction of cyclin D1 gene activity.

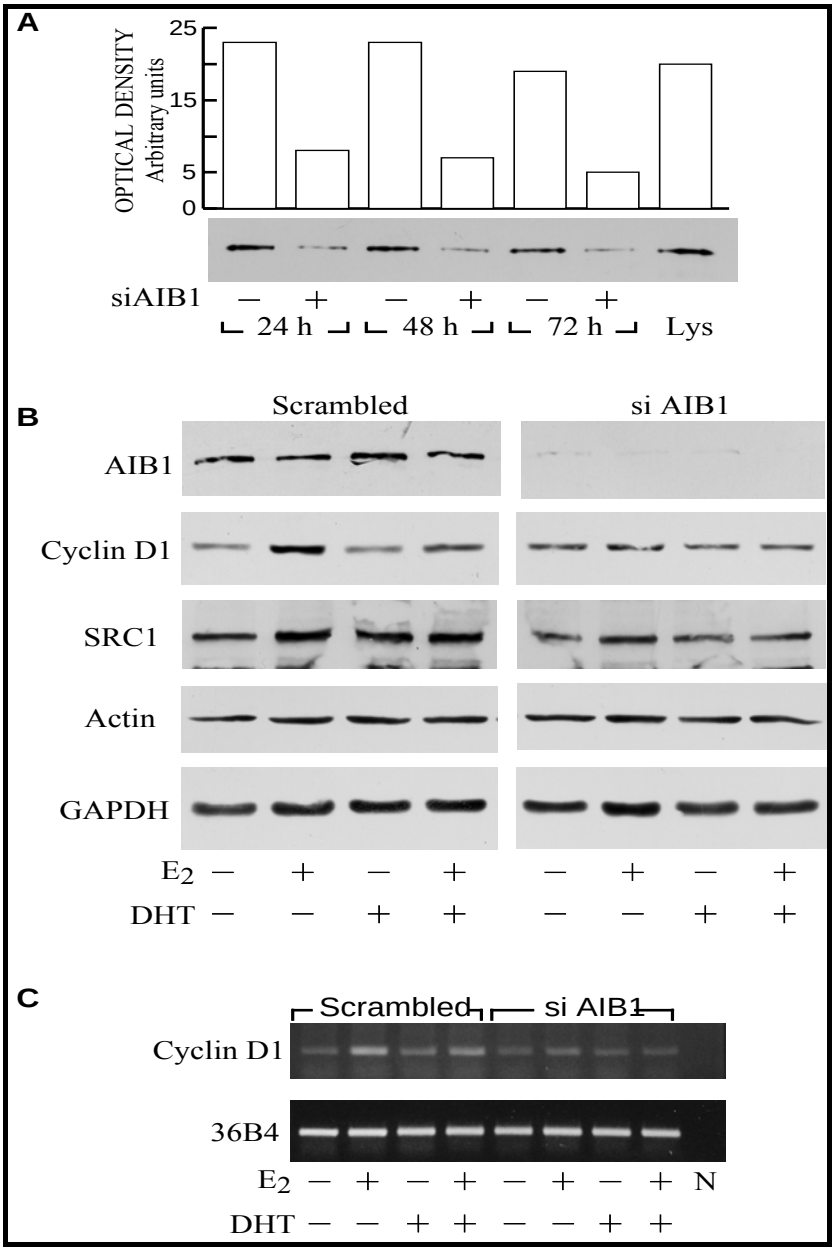

Fig.4: **AIB1 silencing decreases E2-dependent cyclin D1 expression.** ***(A)*** *Total cellular lysates were isolated from MCF-7 cells co-transfected with 100 pmo/welll of AIB1 siRNA or 100pmol scrambled control siRNA (Scrambled) over a 72 h time-course in PRF-CT. The expression of AIB1 was determined by western blotting using 70 μg of total proteins lysates.* ***(B) and (C)*** *MCF-7 cells, transfected with 100pmol/well AIB1 siRNA or 100 pmol/well scrambled control siRNA (Scrambled) were treated for 24h with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated in PRF-CT.* ***(B)*** *The expression of AIB1, cyclin D1, SRC-1, actin and GAPDH was assessed by WB on 50 μg of protein lysates. The results were obtained after repetitive stripping and reprobing of the same filters. Results are representative of three independent experiments.* ***(C)*** *The expression of cyclin D1 mRNA was evaluated by RT–PCR as described in Materials and Methods. 36B4 was amplified as control. MCF-7 RNA sample without the addition of reverse transcriptase was amplified as negative control (NC).*

AR-AIB1 complex is recruited on p21 promoter gene and positively modulates its expression

To ascertain if the AIB1 sequestration by AR might potentiate the induction of the expression of the endogenous androgen target gene $p21^{Waf\text{-}1/Cip\text{-}1}$, (*Lu et al.*, 2000) which has been shown to be the major cyclin-dependent kinase-inhibitor protein in the human estrogen-dependent MCF-7 breast cancer cell line (Planas-Silva & Weinberg, 1997).

The association of AIB1 to the *p21* gene promoter was examined in MCF-7 and MCF-7 over-expressing the AR, using ChIP assay. An AIB1 specific antibody was used to immunoprecipitate the protein-DNA complex. The presence of the specific promoter sequence in the chromatin immunoprecipitates, was analyzed by PCR with specific pair of primers spanning the classical androgen-responsive region of the p21 promoter. (*Lu S. et al.*, 1999)

It is worth to note that in MCF-7/AR cells, treatment with DHT, either in absence or presence of E2, induces an increase in the occupancy by AIB1 of the androgen-responsive region of the *p21* gene promoter, when compared to MCF-7 untransfected cells (Fig. 5A). Moreover, this DHT-induced increase of AIB1 recruitment to the *p21* gene promoter coincided with an increase in p21 mRNA expression (Fig. 5B) and protein levels (Fig. 5C).

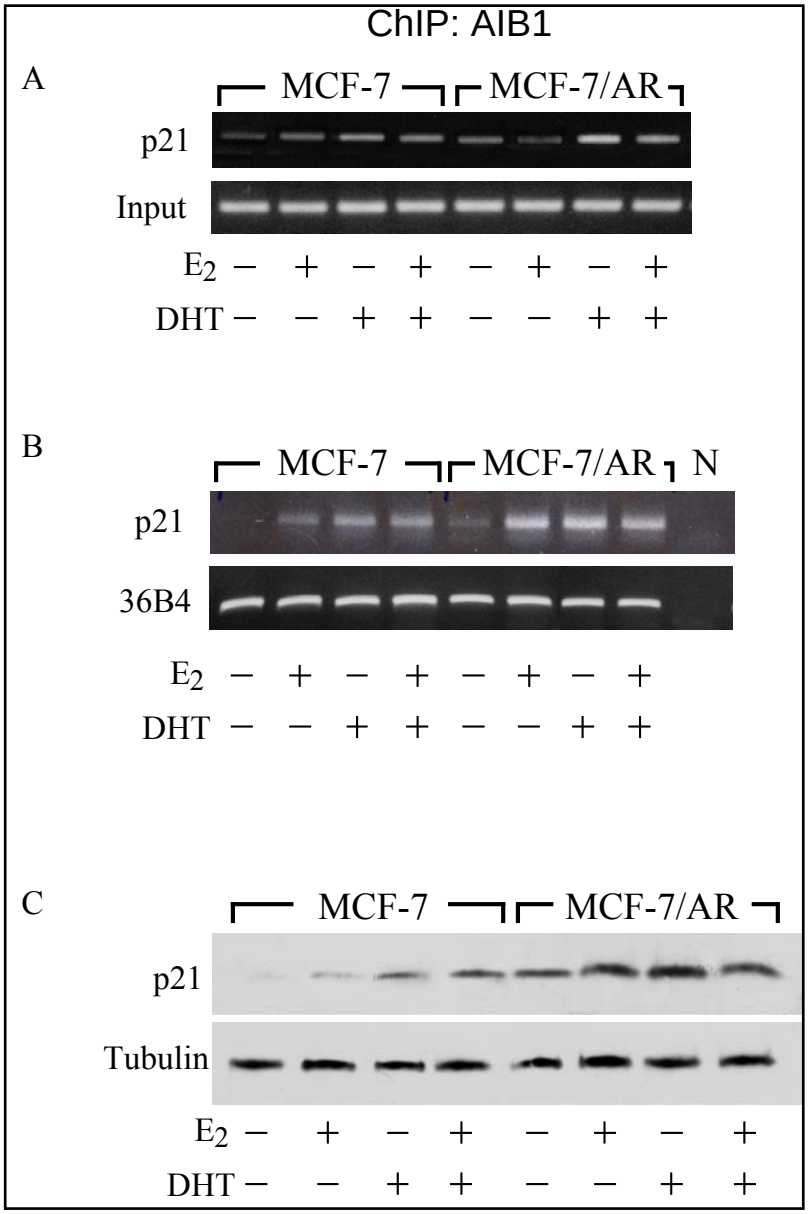

Fig 5. Sequestrated AIB1 enhances AR-mediated induction of p21 gene expression. **(A)***ChIP was carried out on MCF-7 cells synchronized in PRF, grown in PRF-CT for 3 days and transfected with pcDNA3-AR (AR) (5μg /dish) or with the empty vector. Cells were treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated in PRF-CT for 2 h and DNA-associated proteins were precipitated using AIB1 antibody. The androgen-responsive element containing sequence of p21 promoter was detected by PCR with specific primers listed in Materials and Methods. 30 μl of initial preparations of soluble chromatin were amplified to control input DNA. In control samples (N) non-immune IgG was used instead of the primary Abs.* ***(B) and (C)*** *Total cellular RNA and cytoplasmic proteins were isolated from MCF-7 cells synchronized in PRF, left untransfected or transfected (as described in Material and Methods) with 3 μg pcDNA3-AR (AR) and then treated with 10^{-7} M E_2 and/or 10^{-7} M DHT, or left untreated, in PRF-CT for 24 h* ***(B)*** *or 48 h* ***(C)****.* ***(B)*** *The expression of p21 mRNA was evaluated by RT–PCR as described in Materials and Methods. 36B4 was amplified as control. MCF-7 RNA sample without the addition of reverse transcriptase was amplified as negative control (NC).* ***(B)****The expression of p21 was determined by western blotting (WB) using 50 μg of total proteins lysates. The expression of tubulin was assessed as control of protein loading. The results were obtained after repetitive stripping and reprobing of the same filters.*

DISCUSSION

DISCUSSION

Estrogens play a central role in the proliferation and the differentiation of normal mammary epithelial cells as well as the development and progression of breast cancer *(Feigelson & Henderson*, 1996; *Korach*, 1994; *Nilsson et al.*, 2001). Indeed, human breast tumorigenesis is promoted by enhanced activity of the estrogen receptor (ER) that regulates the transcription of target genes, which in turn directs cellular proliferation (*Bonapace et al*., 1996; *Cicatiello et al.*, 2004; *Shang et al.*, 2000; *Yanagisawa et al.*, 2002). Among these genes, the *cyclin D1* gene play a pivotal role highlighted by several line of evidences. In cyclin D1 knockout mice, mammary gland development is profoundly impaired and more evident during pregnancy when ovarian steroids fail to induce their massive proliferative changes (*Fantl et al.*, 1995; *Sicinski et al.*, 1995). Cyclin D1 over-expression has been reported in about 50% of invasive breast cancer (*Michalides et al.*, 1996; *Weinstat-Saslow et al.*, 1995; *Zukerberg et al.*, 1995), and strongly correlated with ER levels *(Hui et al.*, 1996; *Jares et al.*, 1997; *Zukerberg et al.*, 1995). Interestingly, *cyclin D1* gene amplification has been found only in about 13-15% of breast tumors (*Fantl et al.*, 1990; *Gillett et al.*, 1994), suggesting that pathogenic activation of cyclin D1 can occur via additional mechanism, such as promoter deregulation or aberrant hormonal signalling. In breast cancer several factors, including estrogens, have been demonstrate to contribute to cyclin D1 protein over-expression (*Altucci et al.*, 1996; *Musgrove et al.*, 1993). The mechanism by which estrogens regulate CCND1 levels in hormone-responsive breast cancer cells is mainly transcriptional. Although no estrogen-responsive element- (ERE)–related sequence has been identified in the cyclin D1 promoter, several potential estrogen-responsive sites have been mapped in the cyclin D1 proximal promoter (*Altucci et al.*, 1996; *Castro-Rivera et al.*, 2001; *Liu et al.*, 2002; *Park et al.*, 2005; *Sabbah et al.*, 1999).

In the present study we provide evidences that the non aromatisable androgen DHT, through its own receptor, inhibits estrogen-dependent MCF-7 cells proliferation by down-regulating cyclin D1 expression. More specifically, in

MCF-7 cells the over-expression of AR negatively modulates the ability of ERα to direct transcription of the cyclin D1 promoter. It is recognized that ERα-mediated transcription is a highly complex process involving a multitude of coregulatory factors and cross-talk among distinct signalling pathways (*Hall et al.*, 2001; *Lanzino et al.*, 2005). The negative interference of AR on ERα transcriptional activity suggests that shared cofactors might be involved in such inhibitory effect and led us to explore the role of the steroid receptor coactivator AIB1, whose abnormal expression is associated with malignancies in estrogen target tissues. Interestingly, the role of AIB1 is not restricted to nuclear receptor signalling since it has bee demonstrated to be an important factor for growth-mediated signalling pathways (*Oh Annabel*, 2004).

AIB1 was originally identified on the basis of its frequent amplification and over-expression in ovarian and breast cancers (*Anzick et al.*, 1997; *Bautista et al.*, 1998; *List et al.*, 2001). In addition, AIB1 amplification correlates with ER positivity of primary breast tumors as well as with tumor size (*Bautista et al.*, 1998; *Kurebayashi et al.*, 2000). This interest has been further heightened by a recognition that AIB1 has an unique role in regulating ERα signalling. It plays an essential role in the modulation of ERα transcriptional activity (*Shao et al.*, 2004; *Tikkanen et al.*, 2000) and represent a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells (*List et al.*, 2001) since AIB1 levels specifically influence the ability of ER to interact with the cyclin D1 promoter in an estrogen-dependent manner (*Planas-Silva et al.*, 2001). Thus, we hypothesized that AIB1 might participate in the interplay between AR and ERα in a crucial manner, modulating cyclin D1 expression and then, regulating breast epithelial cancer cells proliferation and tumor progression.

In this study we demonstrated that exogenously expressed AIB1 reverses the AR-induced inhibition of E2-dependent transcriptional activity of cyclin D1 promoter in MCF-7 cells suggesting that the transcriptional interference between AR and ERα on cyclin D1 promoter might be mediated through competition for limiting amounts of AIB1 in the cell. It has been proposed that coactivators preferentially interact with receptors dependent on cell type, ligand and promoter context, which could contribute to the specificity of the physiological response (*Tikkanen et al.*,

2000). The ability of AIB1 to modulate AR/ERα interplay seems to be dependent on the steroid receptor content since in MCF-7 expressing high levels of endogenous ERα and low levels of AR, AIB1 co-immunoprecipitates predominantly with ERα. In contrast, AR over-expression induces a dominant interaction of AIB1 with AR. More interestingly, in MCF-7/AR cells, ChIP assay demonstrates a significant decrease in AIB1 occupancy of the estrogen-responsive region of cyclin D1 promoter arguing for the negative modulation of cyclin D1 mRNA and protein levels. Indeed these results are consistent with previous findings that loss of AIB1 affects ERα-mediated signaling by both directly inhibiting transcriptional initiation and blocking ERα turnover, which may further compromise transcriptional regulation by the receptor (*Shao et al.*, 2004). It is reasonable to speculate that a similar mechanism might explain the inhibition of serum-induced Cyclin D1 expression observed upon DHT treatment and AR over-expression. Indeed, competition for AIB1 interaction might be able to interfere with serum-growth factors signalling since AIB1 is required for IGF-1-induced proliferation signalling, cell survival, and gene expression in human breast cancer cells (*Oh A. et al.*, 2004).

It is worth to note that, in MCF-7 cells, over-expression of AR induce a strong increase of AIB1 recruitment on the androgen-responsive region of p21 gene promoter which coincides with a marked enhancement of p21 mRNA and protein levels. This observation is consistent with the inhibitory role of DHT and AR on MCF-7 cell proliferation, since p21 is thought to be an important regulator of breast cancer cells growth (*Prall et al.*, 1997) and represents the major cyclin-dependent kinase-inhibitor protein in the human estrogen-dependent MCF-7 breast cancer cell line (*Planas-Silva & Weinberg*, 1997; *Skildum et al.*, 2002). It is generally accepted that p21 is an important regulatory switch with a dual functionality in vivo: at lower concentrations it acts as assembly factor necessary for the association of D-type cyclins with Cdk4/6 leading to cell cycle progression, whereas at higher concentrations it causes cell cycle arrest and favours differentiation in certain cell types (*LaBaer et al.*, 1997; *Sherr & Roberts*, 1999). Moreover, there is a significant correlation between p21 immuno-reactivity and well-differentiated histological grade in ER-positive breast carcinoma (Oh et

al., 2001), whereas a reduced expression of p21 is associated with a high risk of breast cancer recurrence (Wakasugi et al., 1997). Interestingly, p21 has been recently shown to reduce the estrogen-induced cyclin D1 mRNA (Fritah et al., 2005). Thus, we may speculate the existence of a positive feed-back loop between AR and p21 in the modulation of cyclin D1 expression.

In conclusion, physical squelching of the AR/ER common coactivator AIB1 may represent a possible mechanism through which AR can negatively modulate ER-mediated signalling pathway and inhibit breast cancer cells proliferation. Indeed, AIB1 sequestration by AR results in the activation of AR-responsive gene pathways, such as p21 whereas negatively modulates estrogen-dependent cyclin D1 expression, which in turn negatively impact on MCF-7 cells proliferation. The biological significance of the AR-induced inhibition of cyclin D1 expression is highlighted by clinical studies using tamoxifen as an adjuvant therapy in ER-positive breast cancer, showing a higher response and better survival rate in cancers with cyclin D1 low/moderate expression than those with high expression of cyclin D1 (*Jirstrom et al.*, 2005; *Stendahl et al.*, 2004). In this context, the expression and the functional activity of the AR in breast tissues and tumors, by opposing estrogen signalling, might play a critical role in regulating cellular proliferation and tissue homeostasis. Thus, the widespread expression of AR in primary and metastatic breast tumors suggest the possibility of targeting the AR signalling pathway in treatment of early stage and advanced disease.

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Evidences that Leptin Up-regulates E-Cadherin Expression in Breast Cancer: Effects on Tumor Growth and Progression

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Abstract

Leptin, a cytokine mainly produced by adipocytes, seems to play a crucial role in mammary carcinogenesis. In the present study, we explored the mechanism of leptin-mediated promotion of breast tumor growth using xenograft MCF-7 in 45-day-old female nude mice, and an *in vitro* model represented by MCF-7 three-dimensional cultures. Xenograft tumors, obtained only in animals with estradiol (E_2) pellet implants, doubled control value after 13 weeks of leptin exposure. In three-dimensional cultures, leptin and/or E_2 enhanced cell-cell adhesion. This increased aggregation seems to be dependent on E-cadherin because it was completely abrogated in the presence of function-blocking E-cadherin antibody or EGTA, a calcium-chelating agent. In three-dimensional cultures, leptin and/or E_2 treatment significantly increased cell growth, which was abrogated when E-cadherin function was blocked. These findings well correlated with an increase of mRNA and protein content of E-cadherin in three-dimensional cultures and in xenografts. In MCF-7 cells both hormones were able to activate E-cadherin promoter. Mutagenesis studies, electrophoretic mobility shift assay, and chromatin immunoprecipitation assays revealed that cyclic AMP–responsive element binding protein and Sp1 motifs, present on E-cadherin promoter, were important for the up-regulatory effects induced by both hormones on E-cadherin expression in breast cancer MCF-7 cells. In conclusion, the present study shows how leptin is able to promote tumor cell proliferation and homotypic tumor cell adhesion via an increase of E-cadherin expression. This combined effect may give reasonable emphasis to the important role of this cytokine in stimulating primary breast tumor cell growth and progression, particularly in obese women. [Cancer Res 2007;67(7):3412–21]

Introduction

Leptin is an adipocyte-derived hormone (1) that, in addition to the control weight homeostasis by regulating food intake and energy expenditure (2, 3), is implicated in the modulation of many other processes such as reproduction, lactation, hematopoiesis, immune responses, cell differentiation, and proliferation (4, 5). The activities of leptin are mediated through the transmembrane leptin receptor (ObR; refs. 6, 7) by activation of the Janus-activated kinase/signal transducers and activators of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways (8, 9).

Epidemiologic studies show a positive association between obesity and an increased risk of developing different cancers (10, 11). Several lines of evidence suggest that leptin and ObR are involved in the development of normal mammary gland and in mammary carcinogenesis (12–14). It has been recently reported that in primary breast tumors, leptin was detected in 86.4% of cases examined, and its expression was highly correlated with ObR (15). This indicates that leptin can influence breast cancer cells not only by endocrine and/or paracrine actions but also through autocrine pathways.

In epithelium and epithelium-derived tumors, cell-cell adhesion and tumor mass mostly depend on E-cadherin, a 120-kDa transmembrane molecule (16, 17). As it might be expected, E-cadherin seems to have a major influence on primary cancer development and evolution. Alteration in the function of E-cadherin and the cadherin-catenin complex has been implicated in cancer progression (18), invasion (19–21), and metastasis (22, 23).

In this study, we explored a new aspect of the involvement of leptin in initial steps of mammary tumorigenesis. Specifically, we asked whether leptin can affect primary tumor mass either *in vivo* in MCF-7 cell tumor xenograft or *in vitro* in MCF-7 three-dimensional cultures. Our results showed that leptin is able to promote tumor cell proliferation and homotypic tumor cell adhesion via an increase of E-cadherin expression. These combined effects may give reasonable emphasis to the important role of this cytokine in stimulating local primary breast tumor cell growth and progression, particularly in obese women.

Materials and Methods

Plasmids. The plasmids containing the human E-cadherin promoter or its deletions were given by Dr. Y.S. Chang (Chang-Gung University, Republic of China; ref. 24). pHEGO plasmid containing the full length of estrogen receptor α (ERα) cDNA was provided by Dr. D. Picard (University of Geneva). pSG5 vector containing the cDNA-encoding dominant-negative STAT3, which is a variant of the transcription factor STAT3 lacking an internal domain of 50 bp located near the COOH terminus ($STAT^-$), was given by Dr. J. Turkson (University of South Florida, College of Medicine, Tampa, FL). pCMV5myc vector containing the cDNA-encoding dominant-negative extracellular signal-regulated kinase 2 K52R ($ERK2^-$) was provided by Dr. M. Cobb (Southwestern Medical Center, Dallas, TX).

Site-directed mutagenesis. The E-cadherin promoter plasmid-bearing cyclic AMP–responsive element binding protein (CREB)–mutated site (CREB mut) was created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA). We used as template the human E-cadherin

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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promoter, and the mutagenic primers were as follows: 5′-AGGGTGGATCACCTGAtacCAGGAGTTCCAGACCAGC-3′ and 5′-GCTGGTCTGGAACTCCTGgtaTCAGGTGATCCACCCT-3′. The constructed reporter vector was confirmed by DNA sequencing.

Cell lines and culture conditions. MCF-7, HeLa, and BT-20 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 containing 5% calf serum and BT-20 cells were cultured in MEM supplemented with 10% fetal bovine serum, 1% Eagle's nonessential amino acids, and 1% sodium pyruvate (Sigma, Milan, Italy). Cells were cultured in phenol red–free DMEM (serum-free medium), containing 0.5% bovine serum albumin, 24 h before each experiment. All media were supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma).

***In vivo* studies.** The experiments *in vivo* were done in 45-day-old female nude mice (*nu/nu Swiss*; Charles River, Milan, Italy). At day 0, the animals were fully anesthetized by i.m. injection of 1.0 mg/kg Zoletil (Virbac) and 0.12% Xylor (Xylazine) to allow the s.c. implantation of estradiol (E_2) pellets (1.7 mg per pellet, 60-day release; Innovative Research of America, Sarasota, FL) into the intrascapular region of mice. The day after, exponentially growing MCF-7 cells (5.0×10^6 per mouse) were inoculated s.c. in 0.1 mL of Matrigel (BD Biosciences, Bedford, MA). Leptin treatment was started 24 h later, when animals were injected i.p. with either solutions: recombinant human leptin (230 μg/kg) diluted in saline + 0.3% bovine serum albumin (BSA) or saline + 0.3% BSA only (control). The treatment was done for 5 days a week until the 13th week. Tumor development was followed twice a week by caliper measurements along two orthogonal axes: length (L) and width (W). The volume (V) of tumors was estimated by the following formula: $V = L \times (W^2) / 2$. At the time of killing (13 weeks), tumors were dissected out from the neighboring connective tissue, frozen in nitrogen, and stored at −80°C. All the procedures involving animals and their care have been conducted in conformity with the institutional guidelines at the Laboratory of Molecular Oncogenesis, Regina Elena Cancer Institute in Rome.

Three-dimensional spheroid culture and cell growth. The cells were plated in single-cell suspension in 2% agar–coated plates and untreated or treated with 1,000 ng/mL leptin and/or 100 nmol/L E_2 for 48 h. To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; Chemicon International, Temecula, CA) or EGTA to a final concentration of 4 mmol/L. To generate three-dimensional spheroids, the plates were rotated for 4 h at 37°C. The three-dimensional cultures were photographed using a phase-contrast microscope (Olympus, Milan, Italy). The extent of aggregation was scored by measuring the spheroids with an ocular micrometer. The spheroids between 25 and 50, 50 and 100, and >100 μm (in the smallest cross-section) were counted in 10 different fields under ×10 magnification.

Cell number was determined, after trypsinization of spheroids, by direct cell counting at 48 h of treatments.

E-cadherin adhesion assay. MCF-7 cells were pretreated with leptin (1,000 ng/mL) and/or E_2 (100 nmol/L) for 48 h and then plated on six-well plates coated with 1.5 μg/mL recombinant human E-cadherin/Fc chimeric. Before the experiment, the wells were blocked with 1% BSA for 3 h at 37°C and then washed with PBS.

After washing out nonadherent cells, adherent cells were incubated 3 h in medium containing 500 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution. The reaction product was measured at 570 nm.

Total RNA extraction and reverse transcription-PCR assay. Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA). Reverse transcription was done using RETROscript kit (Ambion, Austin, TX). The cDNAs were amplified by PCR using the following primers: 5′-TCTAAGATGAAGGAGACCATC-3′ and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1), 5′-TGGAATCCAAGCAGAATTGC-3′ and 5′-TATGTGGCAATGCGTTCTCTATCCA-3′ (E-cadherin), and 5′-CTCAACATCTCCCCCTTCTC-3′ and 5′-CAAATCCCATATCCTCGT-3′ (36B4). The PCR was done for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) and E-cadherin (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) and 15 cycles (94°C for 1 min, 59°C for 1 min, and 72°C for 2 min) to amplify 36B4, in the presence of 1 μL of first-strand cDNA, 1 μmol/L each of the primers mentioned above, deoxynucleotide triphosphate (0.5 mmol/L), Taq DNA polymerase (2 units per tube; Promega, Madison, WI) in a final volume of 25 μL.

Western blot analysis. Equal amounts of total protein were resolved on an 8% to 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with the appropriated antibody. The antigen-antibody complex was detected by incubation of the membrane at room temperature with a peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

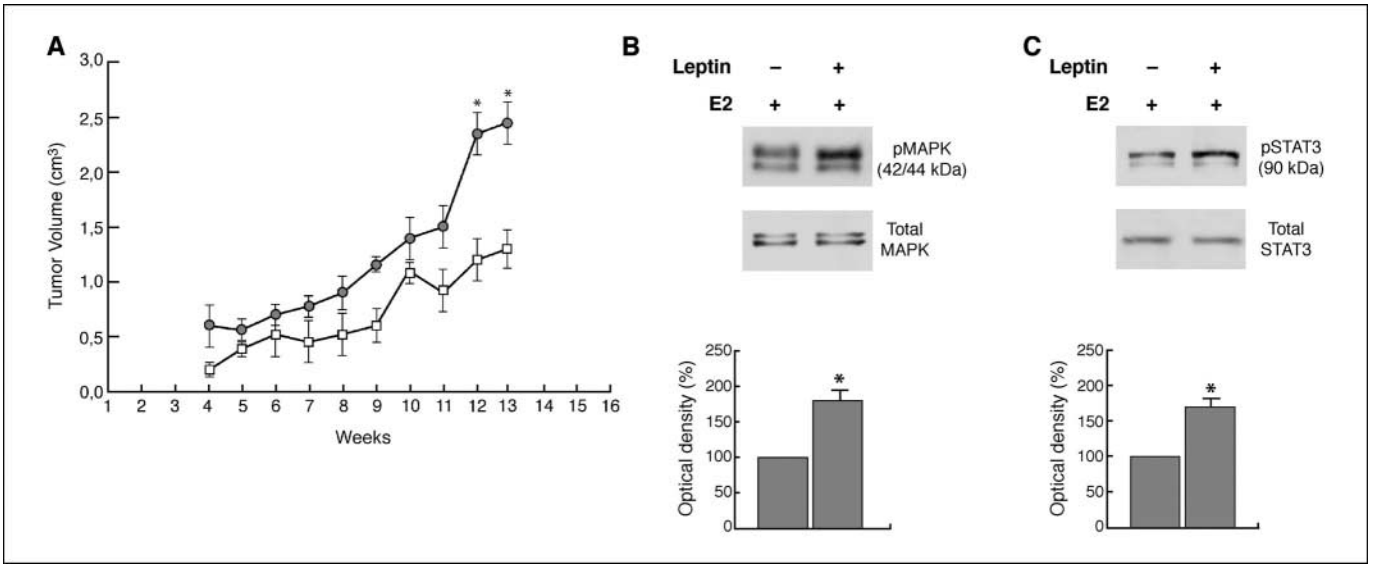


Figure 1. Effect of leptin on growth of MCF-7 cell tumor xenografts. *A,* xenografts were established with MCF-7 cells in female mice implanted with E_2 pellet. One group was treated with 230 μg/kg leptin (●, $n = 5$) and a second group with vehicle (□, $n = 5$). *, $P < 0.05$, treated versus control group. Representative Western blot on protein extracts from xenografts excised from control mice and mice treated with leptin showing MAPK (*B*) and STAT3 (*C*) activation. The immunoblots were stripped and reprobed with total MAPK and STAT3, which serve as the loading control. *pMAPK,* phosphorylated MAPK; *pSTAT,* phosphorylated STAT. *Columns,* mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; *bars,* SE. *, $P < 0.05$.

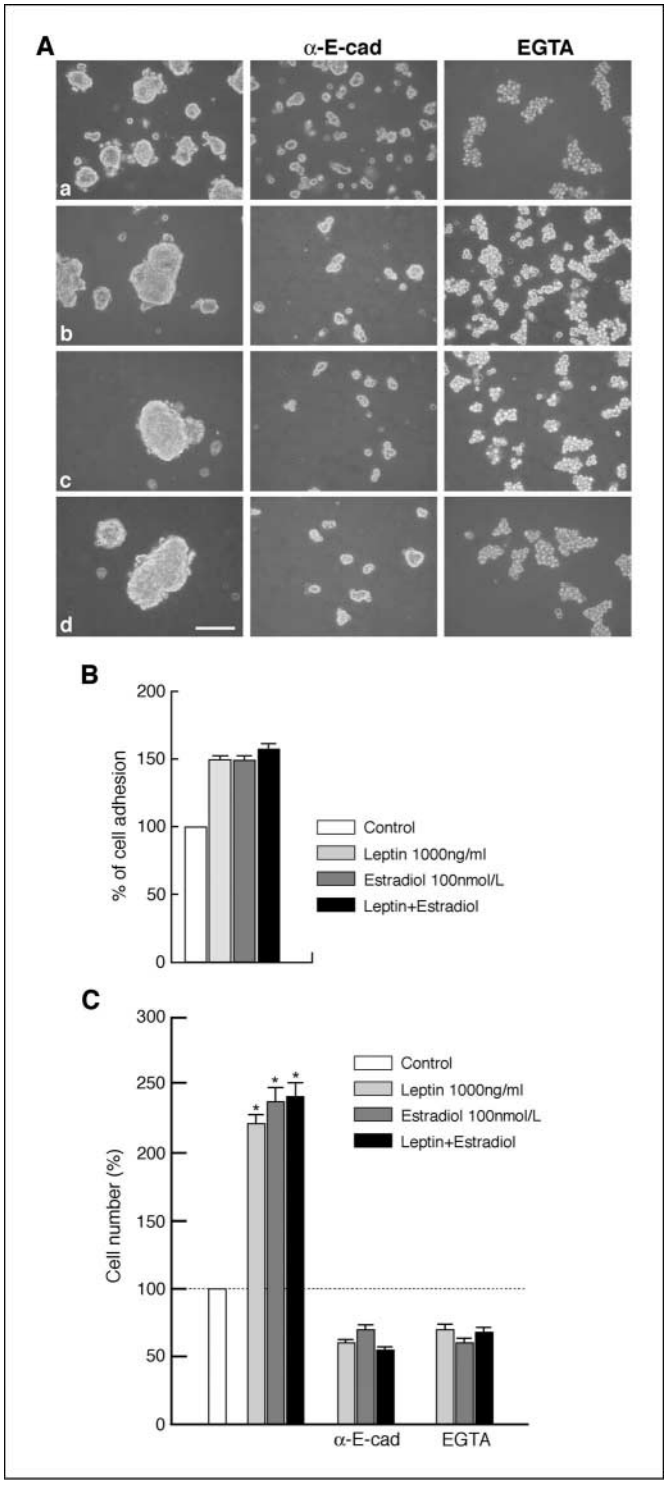


Figure 2. Leptin-enhanced cell-cell adhesion and proliferation depend on E-cadherin function. *A,* E-cadherin–positive MCF-7 cells were seeded in 2% agar–coated plates and cultured as three-dimensional spheroids (*a–d*). To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mmol/L). Cells were untreated (*a*) or treated with leptin (*b*), E_2 (*c*), and leptin plus E_2 (*d*) for 48 h and then photographed under phase-contrast microscopy. Bar, 50 μm. *B,* six-well plates were coated with E-cadherin/Fc recombinant protein, and binding of cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Columns,* mean of five wells; *bars,* SE. *C,* proliferation of MCF-7 cells treated with leptin and/or E_2 for 48 h in the absence or presence of E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mmol/L). *Columns,* average of three experiments; *bars,* SE. Representative results. *, $P < 0.05$.

Transfection assay. MCF-7 cells were transfected using the FuGENE 6 reagent (Promega) with the mixture containing 0.5 μg of human E-cadherin promoter constructs. HeLa cells were transfected with E-cadherin promoter (0.5 μg per well) in the presence or absence of HEGO (0.2 μg per well) or cotransfected with STAT3 or ERK2 dominant negative (0.5 μg per well). Twenty-four hours after transfection, the cells were treated with 1,000 ng/mL leptin and/or 100 nmol/L E_2 for 48 h. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK *Renilla* luciferase plasmid (5 ng per 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 were prepared from MCF-7 as previously described (25). 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: CRE, 5′-TGGATCACCTGAGGTCAGGAGTTCCAGACC-3′; Sp1, 5′-ATCAGC-GGTACGGGGGGCGGTGCTCCGGGG-3′. *In vitro* transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system (Promega). 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 MCF-7 nuclear protein or an appropriate amount of CREB protein or Sp1 human recombinant protein (Promega), 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. The specificity of the binding was tested by adding to the mixture reaction–specific antibodies (anti-CREB and anti-Sp1). Mithramycin A (100 μmol/L; ICN Biomedicals, Inc., Costa Mesa, CA) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extracts. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris borate-EDTA for 3 h at 150 V.

Chromatin immunoprecipitation assay. We followed chromatin immunoprecipitation (ChIP) methodology described by Morelli et al. (26). MCF-7 cells were untreated or treated with 1,000 ng/mL leptin and/or 100 nmol/L E_2 for 1 h. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with anti-CREB or anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Pellets were washed as reported (26), eluted

Table 1. Effect of leptin on cell aggregation in MCF-7 breast cancer cells

MCF-7	Spheriods		
	25 ≤ 50 μm	50 ≤ 100 μm	>100 μm
Control	30 ± 1.2	0.6 ± 0.2	0.0 ± 0.0
Leptin	6 ± 0.8	26 ± 1.8	85 ± 2.5
E_2	7 ± 0.6	32 ± 2.1	78 ± 3.2
Leptin + E_2	3 ± 0.9	40.5 ± 2.3*	80.7 ± 2.9

NOTE: MCF-7 cells were cultured as three-dimensional spheroids in serum-free medium. The extent of aggregation was scored by measuring the spheroid diameters with an ocular micrometer. The values represent a sum of spheroids in 10 optical fields under ×10 magnification. The results are mean ± SE from at least three experiments. Representative three-dimensional cultures are shown in Fig. 2*A*.

*$P < 0.05$ versus leptin and E_2.

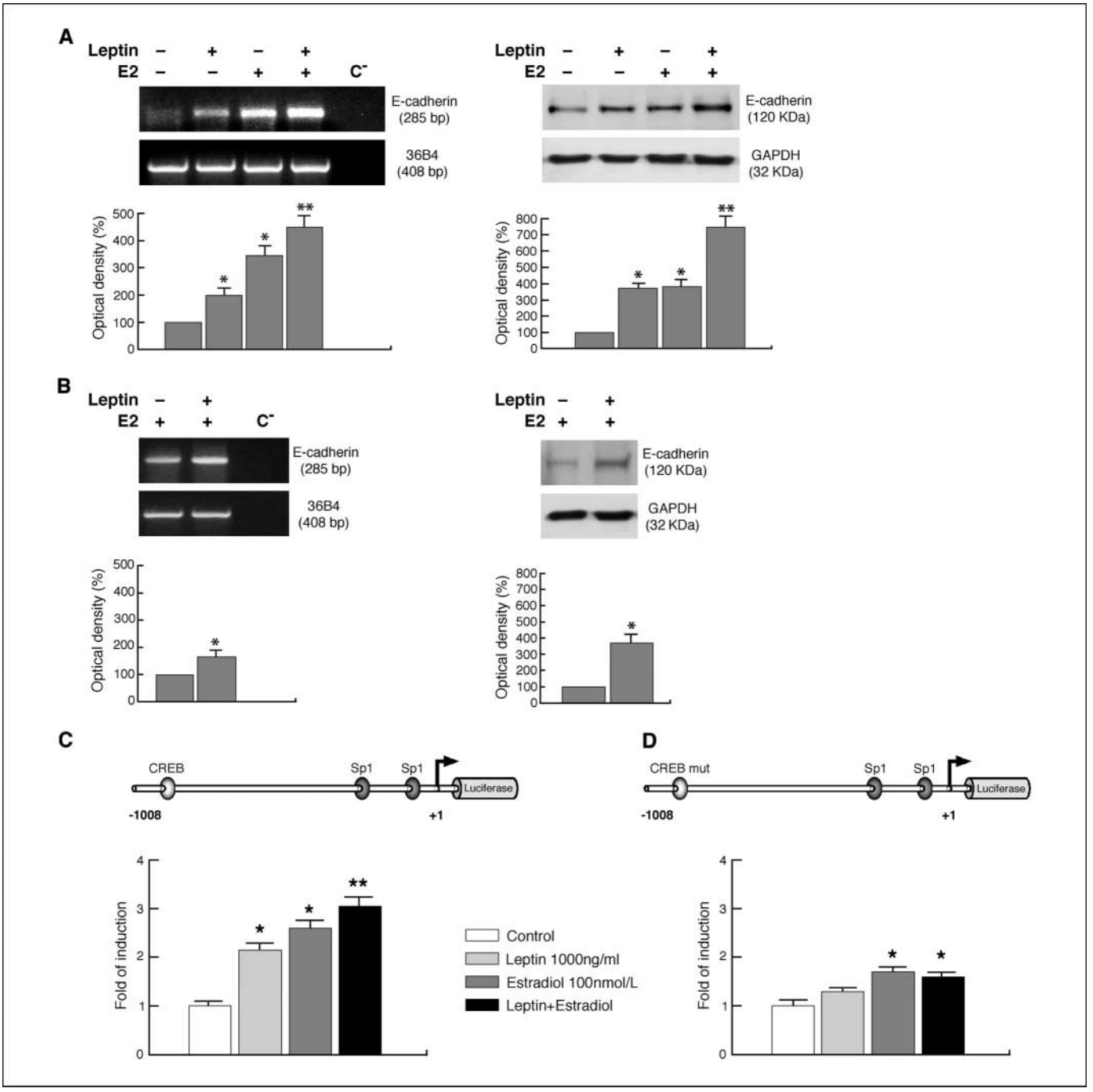


Figure 3. Leptin up-regulates E-cadherin expression in MCF-7 spheroids and xenografts. Reverse transcription-PCR of E-cadherin mRNA was done in MCF-7 three-dimensional cultures stimulated for 48 h with 1,000 ng/mL leptin and/or 100 nmol/L E_2 (*A*) as well as in xenografts (*B*). 36B4 mRNA levels were determined as a control. *C*$^-$, RNA sample without the addition of reverse transcriptase (negative control). Protein extracts obtained from MCF-7 spheroids (*A*) and xenografts (*B*) were immunoblotted with a specific antibody against human E-cadherin. Representative results. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *Columns,* mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; *bars,* SE. MCF-7 cells were transiently transfected with a luciferase reporter plasmid containing the human E-cadherin promoter full-length p-1008/+49 (*C*) or mutated in the CREB site (CREB mut; *D*). Schematic representation of human E-cadherin promoter constructs. The +1 position represents the transcriptional initiation site. The cells were left untreated (control) or treated in the presence of 1,000 ng/mL leptin and/or 100 nmol/L E_2. *Columns,* mean of three separate experiments; *bars,* SE. In each experiment, the activities of the transfected plasmid were assayed in triplicate transfections. *, $P < 0.05$; **, $P < 0.01$ compared with control.

with elution buffer (1% SDS and 0.1 mol/L $NaHCO_3$), and digested with proteinase K (26). DNA was obtained by phenol/chloroform extractions and precipitated with ethanol; 5 μL of each sample were used for PCR with CREB primers (5′-TGTAATCCAACACTTCAGGAGG-3′ and 5′-TTGAGACG-GAGTCTCGCTCT-3′) and Sp1 primers (5′-TAGCAACTCCAGGCTAGAGG-3′ and 5′-AACTGACTTCCGCAAGCTCACA-3′). The PCR conditions were 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min for 30 cycles.

Statistical analysis. Data were analyzed by ANOVA using the STATPAC computer program. Statistical comparisons for *in vivo* studies were made by Wilcoxon-Mann-Whitney test.

Results

Effects of leptin on breast cancer cell tumor growth. To determine *in vivo* the influence of leptin on breast cancer cell tumor growth, we used 45-day-old female nude mice bearing, into the intrascapular region, MCF-7 cell tumor xenografts with or without estrogen pellets. Tumors were obtained only in animals with estrogen pellet implants, which were in general larger in animals treated with leptin at the dose of 230 μg/kg (Fig. 1*A*). Particularly, 13 weeks of leptin parenteral administration increased the tumor volume to 100% the size of E_2 treatment. Besides, leptin significantly enhanced phosphorylation of tumor-derived MAPK and STAT3, suggesting that concentration and dosing schedule of leptin were appropriated for *in vivo* stimulation (Fig. 1*B* and *C*).

Leptin enhances cell-cell adhesion and cell proliferation. We did three-dimensional MCF-7 cultures to evaluate *in vitro* the effects of leptin on cell aggregation. It has been reported that multicellular spheroid culture can more closely mimic some *in vivo* biological features of tumors and improve the relevance of *in vitro* studies (27–30).

Our results evidenced that leptin and/or E_2 treatment for 48 h enhances cell-cell adhesion of MCF-7 cells compared with untreated cells (Fig. 2*A*). The combined exposure to both hormones switches cell aggregation towards the formation of spheroids exhibiting prevalently a diameter larger than 100 μm (Table 1).

E-cadherin is a major type of adhesion molecule, which forms Ca^{2+}-dependent homophilic ligations to facilitate cell-cell contact in epithelial cells (16, 17). Thus, to study whether E-cadherin was responsible for leptin-enhanced cell-cell adhesion, we supplemented the cell culture medium with function-blocking E-cadherin antibody or EGTA, a calcium-chelating agent. As shown in Fig. 2*A*, in the presence of the antibody, MCF-7 cells formed small aggregates showing limited intercellular contact, whereas EGTA treatment prevented cell-cell adhesion, and cells remained rounded and singled suspended.

In addition, the role of E-cadherin was confirmed using an adhesion assay in which cells were allowed to adhere to E-cadherin/Fc protein–coated dishes. This assay showed a greater binding of cells pretreated with leptin and/or E_2 for 48 h with respect to untreated cells (Fig. 2*B*). The adhesion was blocked using either a function-blocking E-cadherin antibody or EGTA (data not shown). Thus, the increased aggregation observed in the presence of leptin and/or E_2 was dependent on E-cadherin.

In three-dimensional cultures, we also observed a significant increase of cell growth upon leptin and/or E_2 treatment. The leptin-induced cell proliferation was completely abrogated when E-cadherin function was blocked (Fig. 2*C*).

Furthermore, in MCF-7 spheroids and in xenografts, we observed an increase of cyclin D1, a regulator of cell cycle progression, in terms of mRNA and protein content in the presence of leptin and/or E_2 (Supplementary Fig. S1).

Leptin up-regulates E-cadherin expression. To investigate if an enhanced expression of E-cadherin occurred in the above-mentioned conditions, we did reverse transcription-PCR and Western blotting analysis. Our results showed that either leptin or E_2 and, in higher extent, the exposure to both hormones increased expression of E-cadherin in terms of mRNA and protein content (Fig. 3*A*). The latter results were also evident in MCF-7 xenografts (Fig. 3*B*).

To evaluate whether both hormones were able to activate E-cadherin promoter, we transiently transfected MCF-7 cells with human E-cadherin promoter plasmid (p-1008/+49). A significant increase in promoter activity was observed in the transfected cells exposed to leptin and/or E_2 for 48 h (Fig. 3*C*).

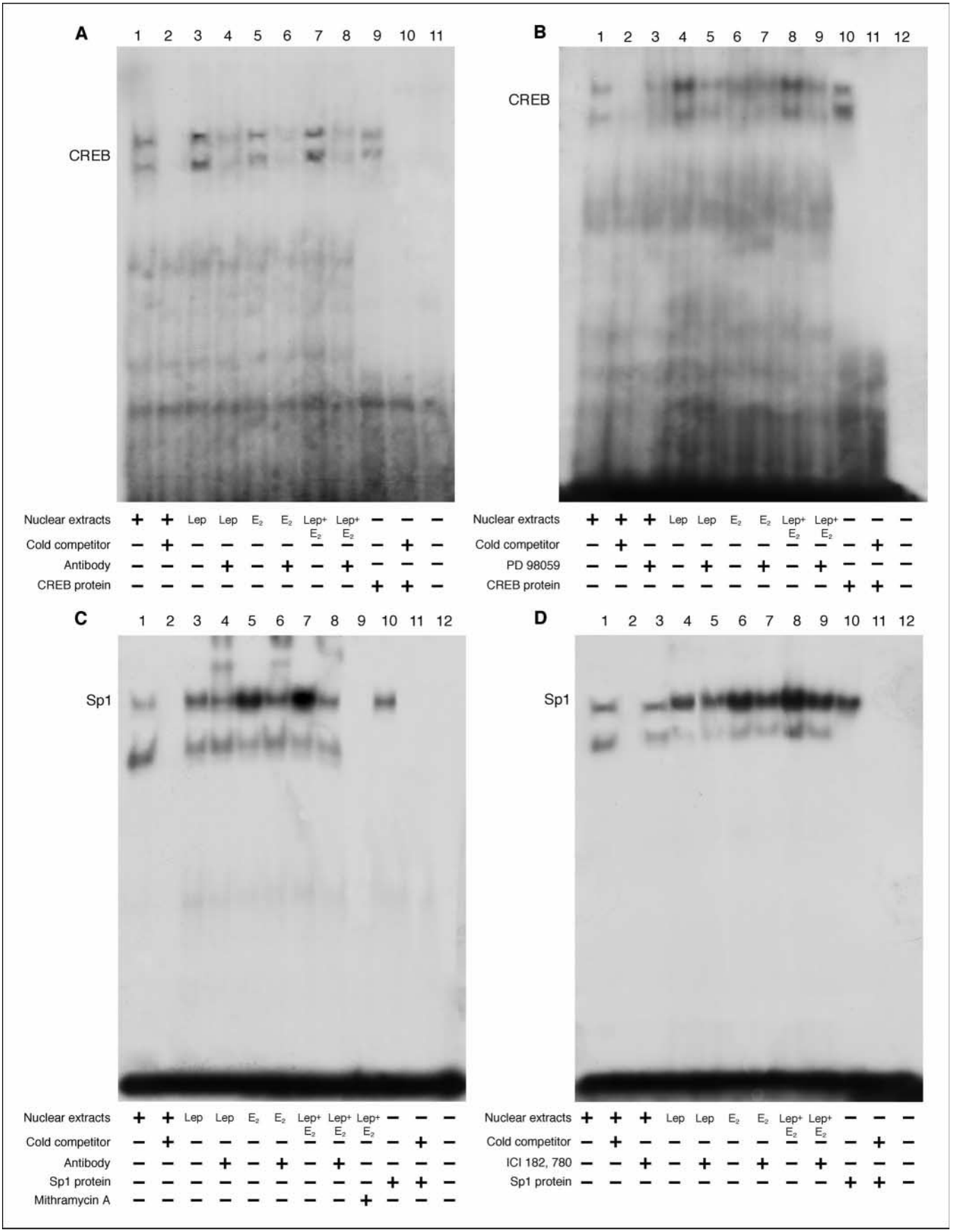
In contrast, we observed that leptin was unable to activate the constructs containing different deleted segments of human E-cadherin promoter (p-164/+49 and p-83/+49) with respect to the full length, whereas E_2 induced activation in the presence of p-164/+49 construct (Supplementary Fig. S2).

Leptin enhances CREB-DNA and Sp1-DNA binding activity to E-cadherin promoter. The role of leptin and E_2 on the transcriptional activity of the *E-cadherin* gene was explored analyzing the nucleotide sequence of the *E-cadherin* gene promoter. We evidenced, upstream to the initiation transcription site, one CRE (−925/−918) and two Sp1 (−144/−132 and −51/−39) as putative effectors of leptin and estrogens. For instance, in MCF-7 cells transiently transfected with E-cadherin promoter plasmid-bearing CREB-mutated site (CREB mut), we observed that the stimulatory effect of leptin was abrogated, whereas the activation of E_2 still persisted, although in a lower extent with respect to the intact promoter (Fig. 3*D*).

To characterize the role of these motifs in modulating E-cadherin promoter activity, we did electrophoretic mobility shift assay (EMSA). Nuclear extracts from MCF-7 cells, using as probe a CRE-responsive element, showed two protein-DNA complexes (Fig. 4*A, lane 1*), which were abolished by the addition of a nonradiolabeled competitor (Fig. 4*A, lane 2*). Leptin treatment induced a strong increase in CREB DNA-binding activity (Fig. 4*A, lane 3*), which was immunodepleted in the presence of CREB antibody (Fig. 4*A, lane 4*). Using transcribed and translated *in vitro* CREB protein, we obtained two bands migrating at the same level as that of MCF-7 nuclear extracts (Fig. 4*A, lane 9*). In the presence of the MAPK inhibitor PD98059, the complex induced by leptin treatment was reduced (Fig. 4*B, lanes 5* and *9*). These findings addressed a specific involvement of leptin signaling in the up-regulation of E-cadherin expression.

Using a DNA probe containing an Sp1 site, we observed in MCF-7 nuclear extracts, a specific protein-DNA complex that was slightly enhanced by leptin, increased upon E_2 exposure and furthermore by the combined treatments (Fig. 4*C, lanes 1, 3, 5*, and *7*). In the presence of Sp1 human recombinant protein, we observed a single complex that causes the same shift with respect to the band revealed in MCF-7 nuclear extracts (Fig. 4*C, lane 10*). The addition

Figure 4. Effects of *in vitro* leptin treatment on CREB-DNA and Sp1-DNA binding activity in MCF-7 cells. Nuclear extracts from MCF-7 cells were incubated with a double-stranded CREB-specific (*A* and *B*) or Sp1-specific (*C* and *D*) consensus sequence probe labeled with [γ-^{32}P]ATP and subjected to electrophoresis in a 6% polyacrylamide gel (*lane 1*). *A,* we used as positive control a transcribed and translated *in vitro* CREB protein (*lane 9*). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (*lanes 2* and *10*). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin (*Lep*) and/or 100 nmol/L E_2 for 48 h incubated with probe (*lanes 3, 5*, and *7*, respectively). The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (*lanes 4, 6*, and *8*). *B,* MCF-7 cells were serum starved overnight with 10 μmol/L PD 98059 (*lanes 3, 5, 7*, and *9*). *Lanes 11* (*A*) and *12* (*B*) contain probe alone. *C,* Sp1 human recombinant protein was used as positive control (*lane 10*). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (*lanes 2* and *11*). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin and/or 100 nmol/L E_2 for 48 h incubated with probe (*lanes 3, 5*, and *7*, respectively). The specificity of the binding was tested by adding to the reaction mixture a Sp1 antibody (*lanes 4, 6*, and *8*). The formation of DNA-Sp1 complexes was blocked by the addition of 100 μmol/L mithramycin A (*lane 9*). *D,* the pure antiestrogen ICI 182,780 (1 μmol/L) was added in leptin-treated (*lane 5*) and/or E_2-treated (*lanes 7* and *9*) MCF-7 nuclear extracts. *Lane 12* contain probe alone.


A
1 2 3 4 5 6 7 8 9 10 11
CREB
Nuclear extracts + + Lep Lep E2 E2 Lep+ E2 Lep+ E2 – – –
Cold competitor – + – – – – – – – + –
Antibody – – – + – + – + – – –
CREB protein – – – – – – – – + + –
B
1 2 3 4 5 6 7 8 9 10 11 12
CREB
Nuclear extracts + + + Lep Lep E2 E2 Lep+ E2 Lep+ E2 – – –
Cold competitor – + – – – – – – – – + –
PD 98059 – – + – + – + – + – – –
CREB protein – – – – – – – – – + + –
C
1 2 3 4 5 6 7 8 9 10 11 12
Sp1
Nuclear extracts + + Lep Lep E2 E2 Lep+ E2 Lep+ E2 Lep+ E2 – – –
Cold competitor – + – – – – – – – – + –
Antibody – – – + – + – + – – – –
Sp1 protein – – – – – – – – – + + –
Mithramycin A – – – – – – – – + – – –
D
1 2 3 4 5 6 7 8 9 10 11 12
Sp1
Nuclear extracts + + + Lep Lep E2 E2 Lep+ E2 Lep+ E2 – – –
Cold competitor – + – – – – – – – – + –
ICI 182, 780 – – + – + – + – + – – –
Sp1 protein – – – – – – – – – + + –

of mithramycin A (100 μmol/L), which binds to GC boxes and prevents sequential Sp1 binding, to nuclear extracts treated with leptin and E_2 blocked the formation of DNA-Sp1 complexes (Fig. 4*C, lane 9*). The original band DNA-protein complex was supershifted by Sp1 antibody (Fig. 4*C, lanes 4, 6*, and *8*). In all hormonal treatments done, the pure antiestrogen ICI 182,780 reduced the Sp1-DNA binding complex (Fig. 4*D, lanes 5, 7*, and *9*), evidencing that leptin induced an activation of ERα, as we previously showed (25).

Effects of leptin on CREB and Sp1 recruitment to the E-cadherin promoter. To corroborate EMSA results, we did ChIP assay. We found that the stimulation of MCF-7 cells with leptin increased the recruitment of CREB to *E-cadherin* gene promoter (Fig. 5*A*). Furthermore, we observed that leptin or E_2 stimulated the recruitment of Sp1 to the E-cadherin promoter, and the combined treatment induced an additive effect (Fig. 5*B*). The latter event suggests that leptin and E_2 may converge in activating ERα to recruit Sp1 on E-cadherin promoter.

Involvement of ERα in the leptin-induced up-regulation of E-cadherin expression. Stemming from the data provided by EMSA and ChIP assays, we evaluated the involvement of ERα in the enhanced E-cadherin expression induced by leptin. Our results showed that in three-dimensional cultures, in the presence of the pure antiestrogen ICI 182,780, the up-regulatory effect of leptin on E-cadherin protein expression still persisted, whereas the stimulatory effects of E_2 was abrogated (Fig. 6*A*).

In addition, the specific role of leptin signaling in up-regulating E-cadherin expression was also confirmed by functional studies in ERα-negative HeLa cells. We evidenced that leptin was able to activate E-cadherin promoter (Fig. 6*B*), which was abrogated in the presence of ERK2 and STAT3 dominant negative (Fig. 6*C*), sustaining furthermore the involvement of leptin signaling. It is worth to note how the ectopic expression of ERα in HeLa cells was able to potentiate the effect of leptin (Fig. 6*B*). To test the activity of the transfected ERα, we did Western blotting analysis for phosphorylated ERα, whereas for dominant-negative *ERK2* and *STAT3* genes, we evaluated the expression of c-*fos*, as target of both pathways (31–33). Moreover, in BT-20 cells lacking of ERα, leptin-enhanced E-cadherin protein content was reduced in the presence of either ERK2 or STAT3 dominant negative. In the same cells, cotransfected with ERα and ERK2 or STAT3 dominant negative, E_2 alone or in combination with leptin was unable to maintain the up-regulatory effect on E-cadherin expression (Supplementary Fig. S3).

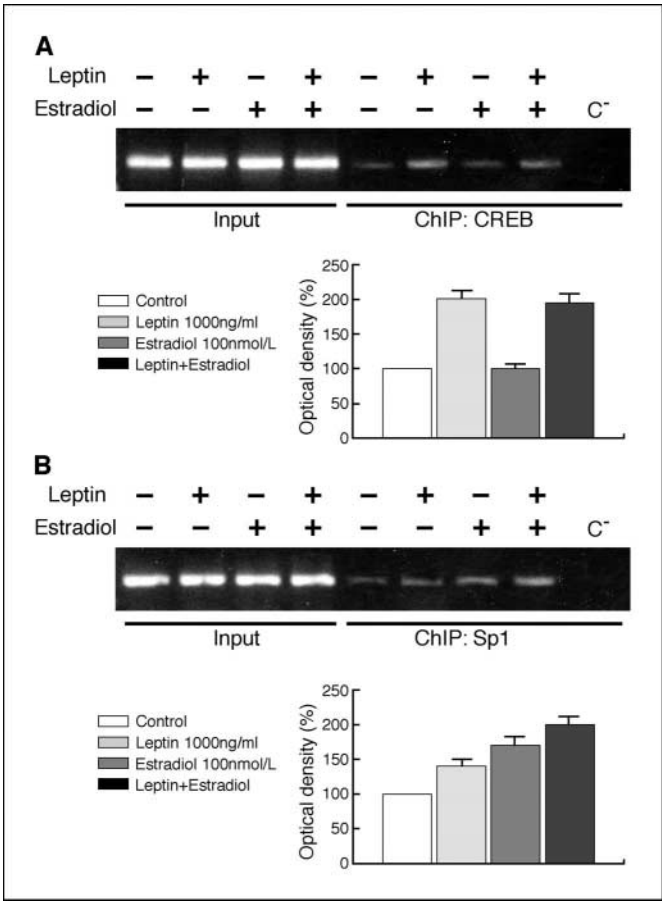


Figure 5. Recruitment of CREB and Sp1 to the E-cadherin promoter in MCF-7 cells. The cells were treated for 1 h with 1,000 ng/mL leptin and/or 100 nmol/L E_2 or left untreated. The precleared chromatin was immunoprecipitated with specific antibodies [i.e., anti-CREB for CREB immunoprecipitates (*A*) or anti-Sp1 for Sp1 immunoprecipitates (*B*)]. E-cadherin promoter sequences containing CREB or Sp1 sites were detected by PCR with specific primers, as detailed in Materials and Methods. To determine input DNA, the E-cadherin promoter fragment was amplified from 5 μL purified soluble chromatin before immunoprecipitation. PCR products obtained at 30 cycles. ChIP with non-immune IgG was used as negative control (*C*⁻). This experiment was repeated three times with similar results. Most representative experiment. *Columns,* mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; *bars,* SE.

Discussion

Leptin stimulates cell growth, counteracts apoptosis, and induces migration and angiogenic factors in different cellular cancer models (10). For instance, hyperleptinemia is a common feature of obese women who have a higher risk of breast cancer than women with normal weight (34), but the association between circulating leptin and breast cancer is still not clear. It has been reported that in interstitial fluid of the adipose tissue, leptin concentration is higher than the circulating levels (35). Thus, we may reasonably assume that in the presence of an abundant adipose tissue surrounding epithelial breast cancer cells, the paracrine leptin effects become crucial in affecting local and primary tumor progression.

The aim of this study was to evaluate whether leptin can influence local primary breast cancer development and progression, using an *in vivo* model of MCF-7 xenografts implanted in female nude mice and an *in vitro* system represented by MCF-7 three-dimensional cultures. Our results showed in MCF-7 xenografts that leptin treatment significantly potentiated the E_2-increased tumor size. In the same view, *in vitro* studies revealed that the combined exposure to both hormones enhanced cell-cell aggregation with respect to the separate treatments.

E-cadherin is an intercellular adhesion molecule generally implicated as tumor suppressor in several types of epithelial tumors, based on findings that the expression of this homotypic adhesion molecule is frequently lost in human epithelial cancers (18, 20, 21). However, it has well been shown in ovarian epithelial tumors that E-cadherin expression is much more elevated than normal ovaries, suggesting that E-cadherin can play a role in the development of ovarian carcinomas (36). For instance, it is worth to mention that E-cadherin may serve not only as an intercellular adhesion molecule, but it may also trigger intracellular activation of proliferation and survival signals (37).

In our study, the increased cell-cell aggregation, observed in MCF-7 three-dimensional cultures upon leptin and/or E_2 treatments, seems to be dependent on E-cadherin molecule that has an indispensable role in this process. Indeed, the addition of a function-blocking E-cadherin antibody or a calcium-chelating agent (EGTA) blocked cell-cell adhesion induced by both hormones. Besides, we showed by adhesion assay a greater binding of cells pretreated with leptin and/or E_2 on E-cadherin/Fc protein–coated dishes.

In the same experimental conditions, an increased proliferative rate was observed upon leptin or E_2 exposure, which was completely abrogated when E-cadherin function was blocked.

An important cell cycle regulator, such as cyclin D1, resulted to be up-regulated in three-dimensional cultures and in xenografts.

Besides, in both models, we showed that leptin and/or E_2 enhanced E-cadherin expression in terms of mRNA, protein content, and promoter activity.

The analysis of E-cadherin promoter sequence revealed the presence of CRE and Sp1 sites as potential target of leptin and E_2 signals. It is well documented how leptin and E_2 through nongenomic effects are able to activate the MAPK pathway that induces activation of CREB kinase, a member of the $p90^{RSK}$ family that corresponds to RSK2 and thereby phosphorylates CREB Ser^{133} (38–40). This well fits with our functional studies showing that leptin was no longer able to activate the *E-cadherin* gene promoter mutated in the CREB site, whereas E_2 maintained an activatory effect although in a lower extent with respect to the intact promoter. The latter data suggest that the activatory effect of E_2 may persist through its binding to Sp1-DNA complex.

The important role of the Sp1-responsive element in activating E-cadherin promoter was shown by EMSA and ChIP assays. Our results evidenced that E_2, as extensively documented, acts in a nonclassic way through the interaction of ERα with Sp1 (41–45). It is worth to note that upon leptin exposure, we also observed an increase in Sp1-DNA binding activity, clearly reduced in the presence of the pure antiestrogen ICI 182,780, as well as an enhanced recruitment of Sp1 to E-cadherin promoter. These observations are supported by our previous findings reporting that leptin is able to transactivate, in a unliganded-dependent manner, ERα through MAPK signal (25).

A cross-talk between leptin and E_2 has been well documented in neoplastic mammary tissues and breast cancer cell lines (15, 25, 46, 47). For instance, E_2 up-regulates leptin expression in MCF-7 cells (15), whereas leptin is an amplifier of E_2 signaling through a double mechanism: an enhanced aromatase gene expression (46) and a direct transactivation of ERα (25). Thus, we investigated whether the up-regulatory effect induced by leptin on E-cadherin expression can be modulated by ERα. We found that E-cadherin protein seems up-regulated still by leptin in the presence of the pure antiestrogen ICI 182,780. Moreover, in HeLa cells, leptin was able to activate E-cadherin promoter, which was abrogated in the presence of ERK2 or STAT3 dominant negative, suggesting that

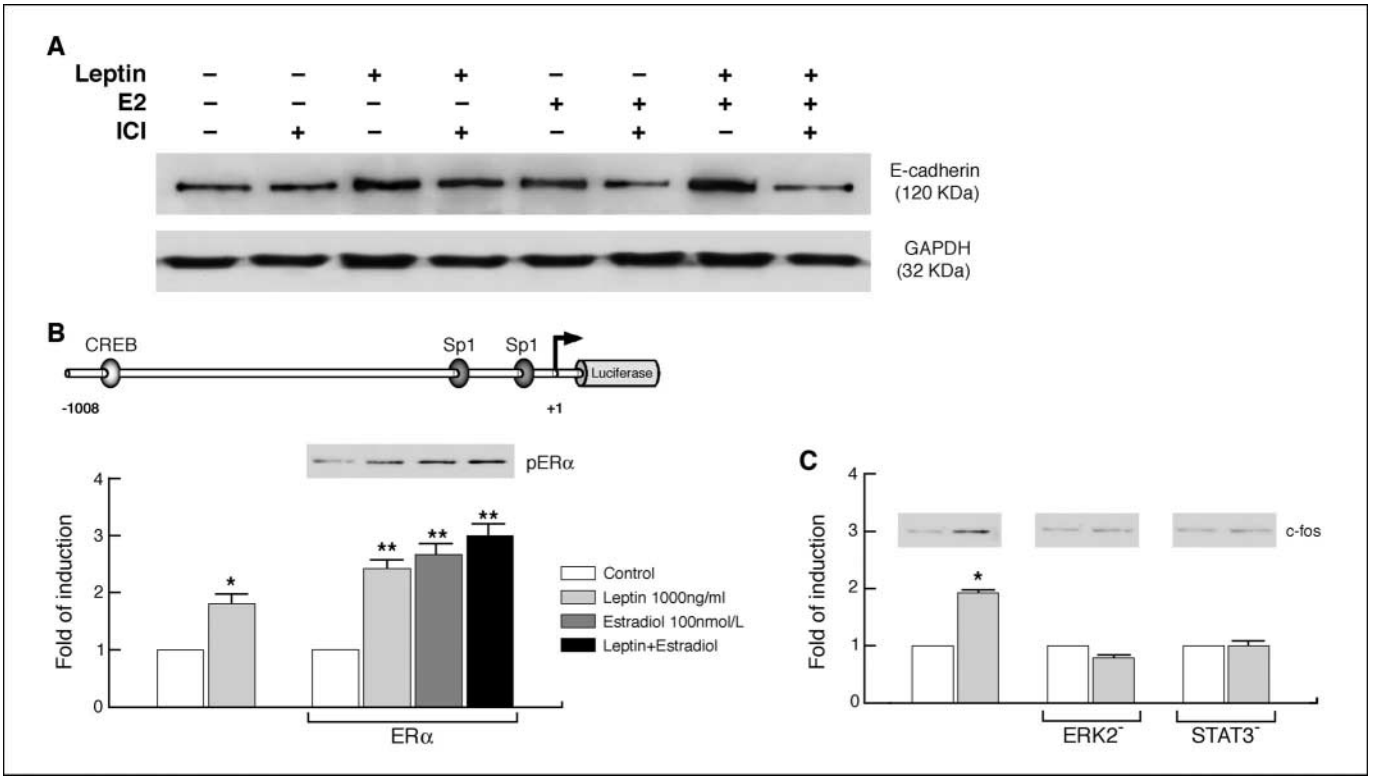


Figure 6. Influence of ERα on leptin-induced upregulation of E-cadherin expression. *A,* MCF-7 spheroids were preincubated with 1 μmol/L ICI 182,780 (*ICI*) for 1 h and then treated with leptin (1,000 ng/mL) and/or E_2 (100 nmol/L) for 48 h. Total proteins (50 μg) were immunoblotted with a specific antibody against human E-cadherin. GAPDH serves as loading control. *B,* ER-negative HeLa cells were transfected with a plasmid containing E-cadherin promoter or cotransfected with E-cadherin promoter and pHEGO. Transfected cells were treated with leptin (1,000 ng/mL) and/or E_2 (100 nmol/L) for 48 h. *Columns,* means of three separate experiments; *bars,* SE. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *Inset,* Western blot analysis for phosphorylated ERα (*pERα*) using anti–phosphorylated ERα (Ser^{118}). *, $P < 0.05$; **, $P < 0.01$, compared with control. *C,* HeLa cells were transiently transfected with dominant-negative ERK2 or STAT3 plasmid and then treated for 48 h with leptin. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *Inset,* Western blot analysis for c-*fos*. *, $P < 0.05$, compared with control.

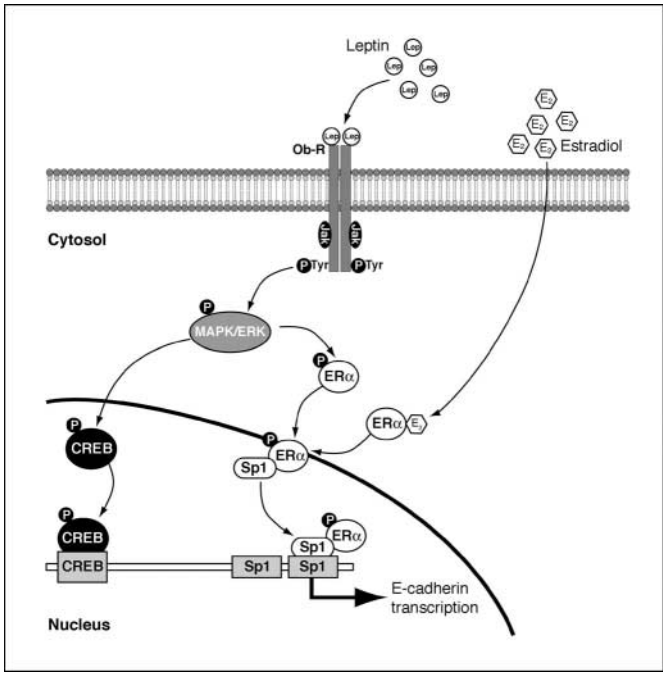


Figure 7. Hypothesized model of leptin signaling in modulating E-cadherin expression in breast cancer. Interaction of leptin (*Lep*) with its specific receptor (ObR) induces, through MAPK activation, phosphorylation of CREB and its transactivation. Leptin may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. Both CREB and Sp1 transcriptional factors bind on E-cadherin promoter at specific responsive sequences and induce an enhanced E-cadherin expression.

leptin signaling is involved in enhancing E-cadherin expression. These latter data are supported by Western blotting analysis done in BT-20 cells lacking of ERα in which ERK2 and STAT3 dominant negative reversed leptin-enhanced E-cadherin protein content. The up-regulatory effect induced by E_2 on E-cadherin expression in the presence of ectopic ERα seemed inhibited in the presence of ERK2 and STAT3 dominant negative. The latter findings may be a consequence of the enhanced expression of leptin receptor upon E_2 exposure (15), which may have an impaired signaling on E-cadherin expression. An additional explanation, which could coexist with the previous one, may be that both ERK2 and STAT3 dominant negative could interfere with ERα-Sp1 interaction at level of *E-cadherin* gene transcription (48).

A hypothetical model of the possible mechanism through which leptin and E_2 may functionally interact in modulating E-cadherin expression in breast cancer is shown in Fig. 7. Leptin through MAPK activation may phosphorylate CREB and induce its transactivation. For instance, CREB phosphorylated at Ser^{133} is often reported not only as an index of PKA but also as an effector of MAPK activation (49). Concomitantly, leptin in the presence of E_2 may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. On the other hand, it is well known that ERα, in the presence of its natural ligand, interacts with Sp1.

Thus, we may reasonably propose that upon leptin exposure, the increased E-cadherin–mediated cellular adhesion and activation of proliferation signals may enhance the transformation of normal epithelial cells to neoplastic cells and then stimulate the growth of tumor mass. Distinct from its role as a tumor suppressor, E-cadherin may function as tumor enhancer in the development of primary breast cancer.

In conclusion, all these data address how leptin and E_2 signaling may represent a target of combined pharmacologic tools to be exploited in the novel therapeutic adjuvant strategies for breast 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

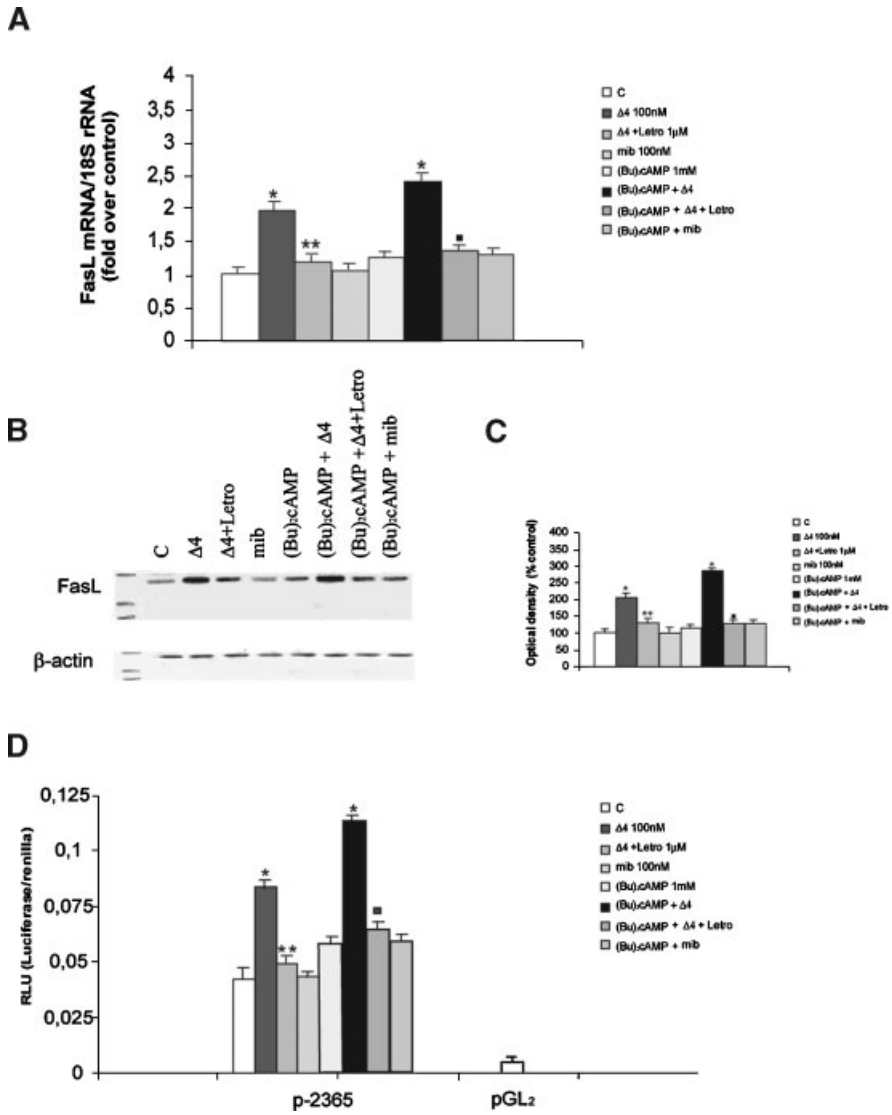

Fig. 1. Effects of Δ4 on FasL expression. A: Total RNA was obtained from TM4 cells untreated (control, C) or treated for 24 h with Δ4 (100 nM) mibolerone (mib 100 nM), $(Bu)_2$cAMP (1 mM), $(Bu)_2$cAMP + Δ4 and $(Bu)_2$cAMP + mib. One micromolar of aromatase inhibitor letrozole (Letro) was used. Real 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

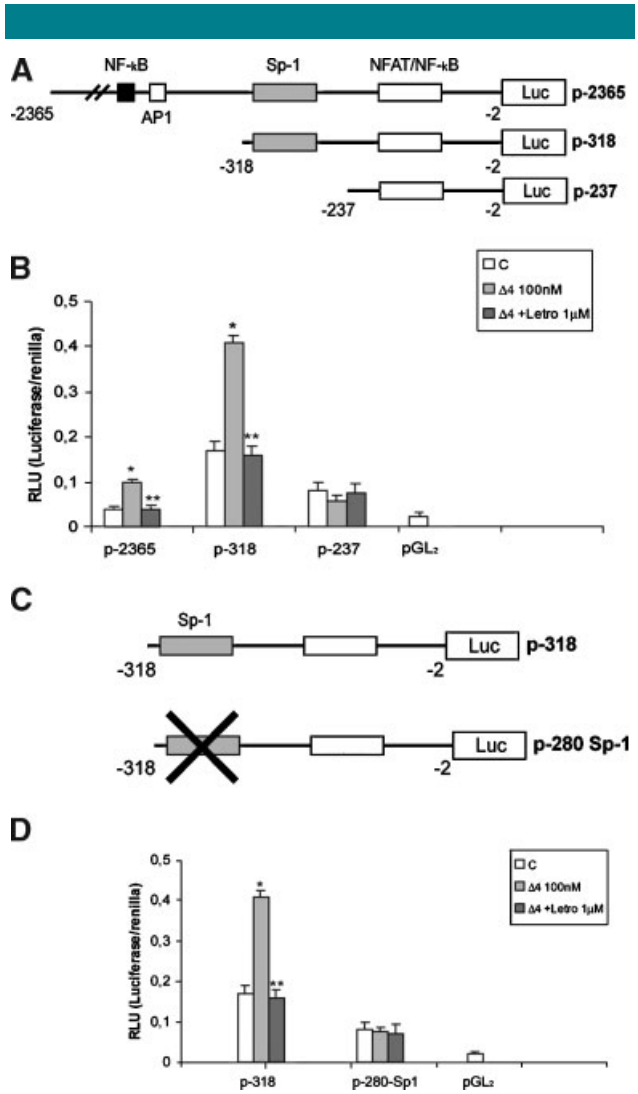

Fig. 2. Effects of estradiol "in situ" production on expression of human FasLpromoter/luciferase reporter gene construts in TM4 cells. A: Schematic map of the FasL promoter fragments used in this study. All of the promoter constructs contain the same 3′ boundary (−2). The 5′ boundaries of the promoter 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

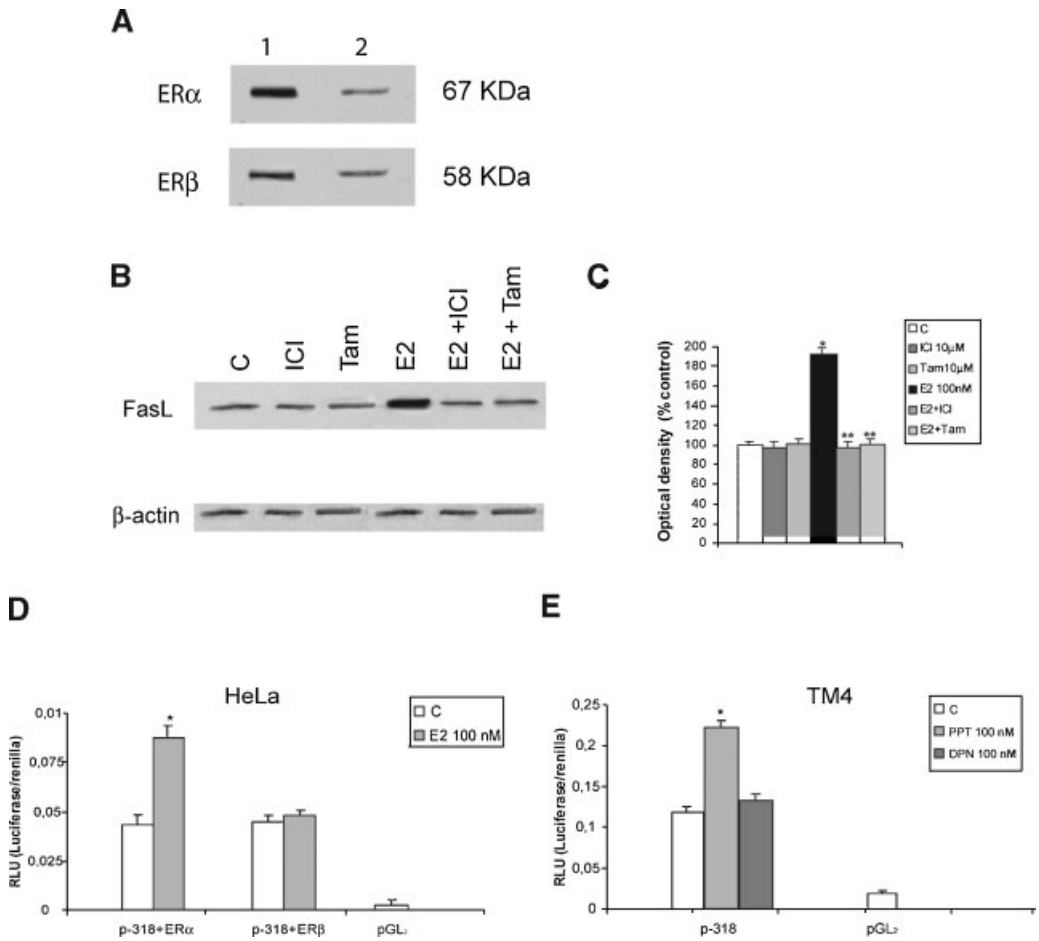

Fig. 3. 17β-Estradiol enhances FasL transcriptional activity through ERα. A: Lysates from TM4 cells were used to evaluate by Western blot analysis the expression of ERα and ERβ (lane 2). The human breast cancer cell line MCF-7 and human prostate cancer cell line LNCaP were used as positive control for 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

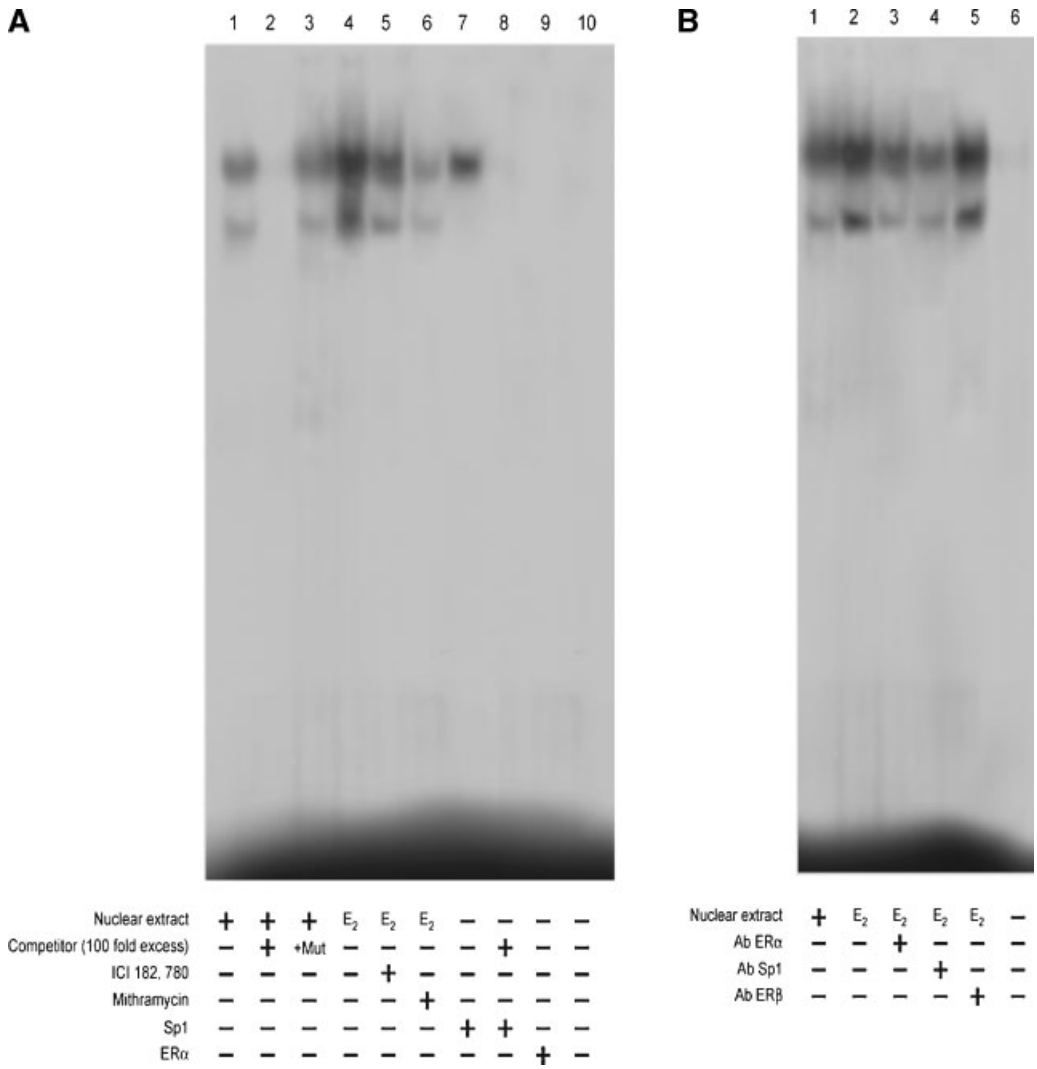

Fig. 4. Electrophoretic mobility shift assay of the Sp-1 binding site in the FasL promoter region. A: Nuclear extracts from TM4 cells were incubated with a double-stranded Sp1-specific consensus sequence probe labeled with [γ-^{32}P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 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

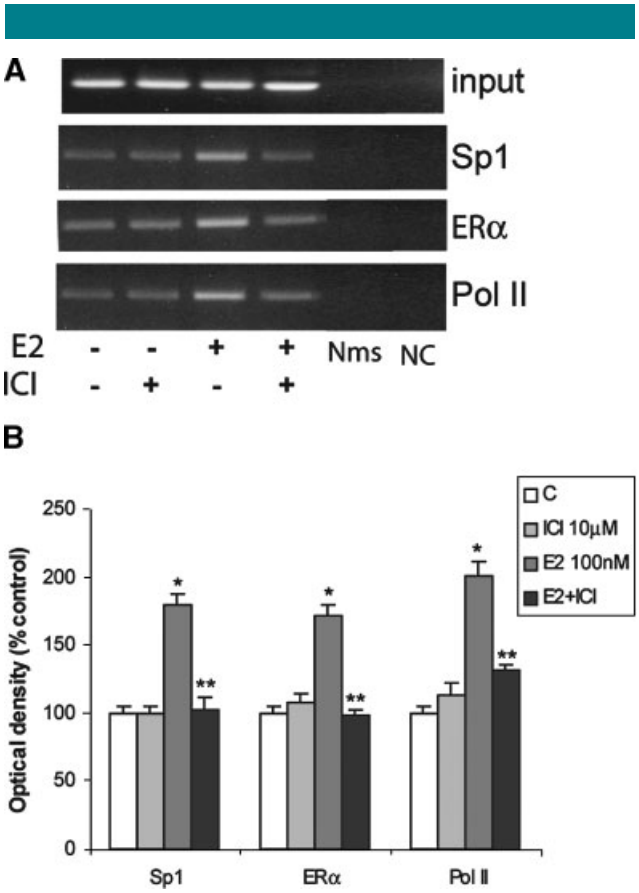

Fig. 5. 17β-Estradiol increases Sp-1/ERα recruitement to FasL promoter. A: Soluble precleared chromatin was obtained from TM4 cells treated for 1 h with 100 nM E2, 10 μM ICI and E2 + ICI or left untreated (C) and immunoprecipitated (IP) with an anti-Sp-1, anti ERα, anti polymerase II antibodies and with 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.

Acknowledgments

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Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway

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BACKGROUND: Results from mice lacking the androgen receptor (AR) showed that it is critical for the proper development and function of the testes. The aim of this study was to investigate whether a functional AR is present in human sperm. METHODS: The expression of AR and its effects on sperm were evaluated by RT-PCR, Western Blot, Immunocytochemistry, PI3Kinase and DNA laddering assays. RESULTS: We showed in human sperm that AR is located at the head region. Dihydrotestosterone (DHT), in a dose-dependent manner, leads to the rapid phosphorylation of the AR on tyrosine, serine and threonine residues and this effect was reduced by the AR antagonist hydroxyflutamide (OH-Flut). The effects of AR were evaluated on the phosphoinositide-3 kinase/protein kinase B (PI3K/AKT) pathway. Specifically, 0.1 and 1 nM DHT stimulated PI3K activity, whereas 10 nM DHT decreased PI3K activity and levels of p-AKT S473 and p-AKT T308, p-BCL2, and enhanced phosphatase and tensin homologue (PTEN) phosphorylation. In addition, 10 nM DHT was able to induce the cleavage of caspases 8, 9 and 3 and cause DNA laddering, and these effects were reversed either by casodex or OHFlut. By using wortmannin, a specific PI3K inhibitor, the cleavage of caspase 3 was reproduced, confirming that in sperm the PI3K/AKT pathway is involved in caspase activation. CONCLUSIONS: Human sperm express a functional AR that have the ability to modulate the PI3K/AKT pathway, on the basis of androgen concentration.

Keywords: androgen receptor; androgens; human sperm; male reproduction; PI3K/AKT

Introduction

A functional androgen receptor (AR) is required for male embryonic sexual differentiation, pubertal development and regulation of spermatogenesis in mammals. The role of AR during spermatogenesis has been the subject of intense interest for many years (Collins *et al.*, 2003). Several findings have shown that AR function is required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids (De Gendt *et al.*, 2004). Studies of androgen withdrawal and disruption of AR activity, either by surgical, chemical or genetic means, have demonstrated that spermatogenesis rarely proceeds beyond meiosis. In all of these model systems, very few round and even fewer elongated spermatids are observed, as clearly demonstrated in a previous study (Yeh *et al.*, 2002). However, the mechanisms by which androgens regulate male fertility are not fully understood and the sites of androgen action within the male reproductive system are not yet resolved.

Whereas few studies have raised the intriguing possibility that some germ cells may exhibit immunoreactive AR (Kimura *et al.*, 1993; Vornberger *et al.*, 1994), other reports point to Sertoli cells or Leydig cells or peritubular/myoid cells as the exclusive androgen target cells in the testis (Ruizeveld de Winter *et al.*, 1991; Iwamura *et al.*, 1994; Goyal *et al.*, 1996; Suarez-Quian *et al.*, 1999). Recently, the presence of the AR in human sperm was demonstrated by western blot and by immunofluorescence assay (Solakidi *et al.*, 2005).

It is generally accepted that androgens bind to intracellular ARs resulting in mRNA and protein synthesis (McPhaul and Young, 2001). Nevertheless, rapid responses to androgens have been observed in different tissues, that cannot be explained by involvement of mRNA and protein synthesis (Peterziel *et al.*, 1999; Castoria *et al.*, 2004). These rapid, nongenomic effects are also seen for other steroid hormones (Cato *et al.*, 2002) and their importance as a complementary route for cell regulation has recently become evident. Different nuclear receptors (Calogero *et al.*, 2000; Aquila *et al.*, 2004) have been found to be present in human spermatozoa, regulating cellular processes through nongenomic mechanisms. This may

represent an exclusive modality of action in spermatozoa since they are apparently transcriptionally inactive cells.

In addition to stimulating cell growth, androgens and/or AR play important roles in the promotion of cell apoptosis (Heisler *et al.*, 1997; Olsen *et al.*, 1998; Shetty *et al.*, 2002; King *et al.*, 2006). The term apoptosis defines programmed cell death, which is executed by the activation of caspases, a family of cytoplasmic cysteine proteases (Cohen, 1997) through two major pathways: the intrinsic and the extrinsic. The intrinsic pathway involves the cell sensing stress that triggers mitochondria-dependent processes, resulting in cytochrome *c* release and activation of caspase 9 (Olson and Kornbluth, 2001). The extrinsic pathway involves the final cleavage of caspase 8 (Schulze-Osthoff *et al.*, 1998). Both caspases 8 and 9 can be directly regulated through protein phosphorylation from protein kinase B (AKT) (Cardone *et al.*, 1998; Shim *et al.*, 2004). The phosphoinositide-3 kinase (PI3K) signalling pathway is an important intracellular mediator of cell survival and antiapoptotic signals (Parsons, 2004). PI3K activation leads to production of 3′-phosphoinositide second messengers, such as phosphatidylinositol 3,4,5-trisphosphate, which activate a variety of downstream cell survival signals. Accumulation of phosphatidylinositol 3,4,5-trisphosphate at the membrane recruits a number of signalling proteins containing pleckstrin homology domains, including AKT. On recruitment, AKT becomes phosphorylated and activated and exerts its antiapoptotic activity through inactivation of proapoptotic proteins. In addition, the PI3K pathway has also been shown to be negatively regulated by phosphatase and tensin homologue (PTEN), a lipid phosphatase that cleaves the D3 phosphate of the second messenger phosphatidylinositol 3,4,5-trisphosphate (Maehama and Dixon, 1998; Wu *et al.*, 1998). Recently in fibroblasts, it has been demonstrated that AR mediates androgen nongenomic function, and that androgen activates PI3K/AKT through the formation of a triple complex between AR, the regulatory subunit p85 of PI3K (PIK3R1) and SRC tyrosine kinase. Indeed, this interaction is dependent on androgen concentration, and a particularly high androgen concentration will dissociate the AR-SRC tyrosine kinase-PI3K complex (Castoria *et al.*, 2003).

The functional impact of programmed cell death in human sperm is poorly understood (Sakkas *et al.*, 2003). Up to now it has been unclear whether apoptosis in ejaculated spermatozoa takes place in a similar way as in somatic cells or whether spermatozoa, which are thought to have a transcriptionally inactive nucleus, undergo abortive forms of this process (Sakkas *et al.*, 2003). However, sperm constitutively express proteins required to execute apoptosis. Active caspases have been observed predominantly in the postacrosomal region (caspases 8, 1 and 3) and caspase 9 has been particularly localized in the midpiece, associated with mitochondria (Paasch *et al.*, 2004). Moreover, a wide spectrum of cell cytoskeletal proteins and membrane components are also targets of caspase 3 (Paasch *et al.*, 2004), and the proper regulation of the caspase cascade plays an important role both in sperm differentiation and testicular maturity (Said *et al.*, 2004). In addition, caspases have been implicated in the pathogenesis of multiple andrological pathologies such as impaired spermatogenesis, decreased sperm motility, increased levels of sperm DNA fragmentation, testicular torsion, varicocele and immunological infertility (Said *et al.*, 2004). Further studies are needed to evaluate the full significance of caspases activation in spermatozoa. A direct link between AR and sperm survival has not been investigated previously.

In the present study, we have demonstrated the presence of a functional AR in sperm. It emerges from our data that low androgen concentrations stimulate PI3K activity, which is inhibited at higher levels. Additionally, in the latter circumstance increases in PTEN phosphorylation and cleavages of caspases 8, 9 and 3 were evident.

Materials and Methods

Chemicals

PMN Cell Isolation Medium was from BIOSPA (Milan, Italy). Total RNA Isolation System kit, enzymes, buffers, nucleotides 100 bp ladder used for RT–PCR were purchased from Promega (Milan, Italy). Moloney Murine Leukemia Virus (M-MLV) was from Gibco BRL Life Technologies Italia (Milan, Italy). Oligonucleotide primers and TA Cloning kit were made by Invitrogen (Milan, Italy). Gel band purification kit was from Amersham Pharmacia Biotech (Buckinghamshire, UK). DMEM-F12 medium, BSA protein standard, laemmli sample buffer, prestained molecular weight markers, percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide, anti-rabbit IgG flourescein isothiocyanate (FITC) conjugated, Earle's balanced salt solution, Hoechst 33 342, steroids and all other chemicals were purchased from Sigma Chemical (Milan, Italy). RPMI 1640 medium was from Life Technologies, Inc. (Gaithersburg, MD) and DMEM (PRF-SFM) was from Eurobio (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus western blotting detection system, Hybond^{TM} ECL^{TM}, [γ-^{32}P]ATP and HEPES sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Goat polyclonal actin antibody (1–19), monoclonal mouse anti-AR (AR 441) and anti- PIK3R1 antibodies, monoclonal anti-p-tyrosine (PY99), normal mouse serum, peroxidase-coupled anti-rabbit and anti-goat and protein A/G-agarose plus were from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti-p-SRC tyrosine kinase was from Oncogene (Milan, Italy). Polyclonal rabbit anti-p-serine, anti-p-threonine, anti-p-AKT1/AKT2/AKT3 S473, anti-p-AKT1/AKT2/AKT3 T308, anti-p-BCL2, anti-p-PTEN, anti-caspases (8, 9 and 3) antibodies were from Cell Signaling (Milan, Italy). PY20 (Transduction Laboratories, Lexington, UK), anti-phospho-serine and anti-phospho-threonine Abs were from Zymed Laboratories (San Francisco, CA). Casodex (Cax) was from Astra Zeneca (Milan, Italy) and hydroxy-flutamide (OH-Flut) was from Schering (Milan, Italy). The specific caspases inhibitor Z-VAD-FMK (ZVF) was from R&D Systems (Milan, Italy). PCR 2.1 vector was from Promega and the sequencing was by MWG AG Biotech (Ebersberg, Germany).

Semen samples and spermatozoa preparations

Semen specimens from normozoospermic men were obtained after three days of sexual abstinence. The samples were ejaculated into sterile containers and left for at least 30 min in order to completely liquefy before being processed. Sperm from ejaculates with normal parameters of semen volume, sperm count, motility, vitality and morphology, according to the WHO Laboratory Manual (World Health Organization, 1999), were included in this study. In each experiment, three normal samples were pooled. Spermatozoa preparation was

performed as previously described (Aquila *et al.*, 2002). An independent observer, who observed several fields for each slide, inspected the cells. Percoll-purified sperm were washed with unsupplemented Earle's medium and were incubated in the same medium (uncapacitating medium) for 30 min at 37°C and 5% CO_2, without (control) or with treatments (experimental). Some samples were incubated in capacitating medium (CAP) (Earle's balanced salt solution medium supplemented with 600 mg BSA /100 ml and 200 mg sodium bicarbonate/100 ml). When the cells were treated with the inhibitors Cax, OH-Flut and ZVF, a pretreatment of 15 min was performed. The study was approved by the local medical Ethical Committees and all participants gave their informed consent.

LNCaP cells culture

LNCaP, human prostate adenocarcinoma cells, were grown in RPMI 1640 medium supplemented by 5% heat inactivated fetal bovine serum and Penicillin–Streptomycin 1%. Cultures were maintained at 37°C, 5% CO_2 and 100% humidity. In the experiments, steroids and growth factors were withdrawn from cells, and they were grown in phenol red-free DMEM containing 0.5% BSA and 2 mM L-glutamine for 24 h. LNCaP were treated for 30 min at 37°C and 5% CO_2, without (control) or with the indicated treatments (experimental).

RNA isolation and RT–PCR

Total RNA was isolated from human ejaculated spermatozoa and purified as previously described (Aquila *et al.*, 2002). Contamination by leucocytes and germ cells in the sperm preparations was assessed by amplifying *PTPRC* and *KIT* transcripts, respectively. The applied PCR primers and the expected lengths of the resulting PCR products are shown in Table 1. *AR* primers were chosen to amplify the region of the DNA binding domain plus the hinge region of the receptor. PCR was carried for 40 cycles using the following parameters: 95°C/1 min, 55°C/1 min, 72°C/2 min for *AR*; 95°C/1 min, 52°C/1 min, 72°C/2 min for *KIT*; 95°C/1 min, 55°C/1 min, 72°C/2 min for *PTPRC*. For all PCR amplifications, negative (reverse transcription-PCR performed without M-MLV reverse transcriptase) and positive controls (LNCaP for *AR*, human testis for *KIT* and human leucocytes for *PTPRC*) were included.

Table 1: Oligonucleotide sequences used for RT–PCR

Gene	Sequence (5′–3′)	Size of PCR product (bp)
AR	5′-TGCCCATTGACTATTACTTTCC-3′ 5′-TGTCCAGCACACACTACACC-3′	400
KIT	5′-AGTACATGGACATGAAACCTGG-3′ 5′-GATTCTGCTCAGACATCGTCG-3′	780
PTPRC	5′-CAATAGCTACTACTCCATCTAAGCCA-3′ 5′-ATGTCTTATCAGGAGCAGTACATG-3′	230

Gel extraction and DNA sequence analysis

The *AR* RT–PCR product was extracted from the agarose gel by using a gel band purification kit, and the purified DNAs were subcloned into PCR 2.1 vector and then sequenced.

Western blot analysis of sperm proteins

Sperm samples washed twice with Earle's balanced salt solution (uncapacitating medium), were incubated for 30 min without or with the treatments indicated in the figures. During western blot analysis, sperm samples were processed as previously described (Aquila *et al.*, 2002). The negative control was performed using a sperm lysate that was immunodepleted of AR (i.e. preincubation of lysates with anti-AR antibody for 1 h at room temperature and immunoprecipitated with protein A/G-agarose) (Aquila *et al.*, 2004). As internal controls, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti-actin antibody. As a positive control, LNCaP (prostate cancer cell line) was used. The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

Immunofluorescence assay

Sperm cells, were rinsed three times with 0.5 mM Tris–HCl buffer (pH 7.5) and were fixed using absolute methanol for 7 min at −20°C. AR staining was carried out, after blocking with normal human serum (10%), using the monoclonal anti-human AR (1 μg/ml) as primary antibody and an anti-mouse IgG FITC conjugated (4 μg/ml) as secondary antibody. To stain DNA in living cells, Hoechst 33 342 (Hoechst) was added at a final concentration of 10 μg/ml. The specificity of AR was tested by using normal mouse serum instead of the primary antibody; sperm cells incubated without the primary antibody were also used as negative controls. The cellular localization of AR and Hoecst was studied with a Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135 M inverted microscope with 600×. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap, and a minimum of 200 spermatozoa per slide were scored.

Immunoprecipitation of sperm proteins and LNCaP cells proteins

Spermatozoa were washed in Earle's balanced salt solution and centrifuged at 800*g* for 20 min. Sperm resuspended in the same uncapacitating medium and LNCaP cells were incubated without (control, UC) or in the presence of dihydrotestosterone (DHT) at increasing concentrations (0.1, 1, 10 and 100 nM) for 30 min. Other samples were pretreated for 15 min with 10 μM OH-Flut. In order to evaluate the rapid effect of DHT on AR, spermatozoa were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 at different times (5 and 30 min and 1 h). To avoid non-specific binding, sperm lysates were incubated for 2 h with protein A/G-agarose beads at 4°C and centrifuged at 12 000*g* for 5 min. The supernatants (each containing 600 μg total protein) were then incubated overnight with 10 μl anti-AR and 500 μl HNTG (IP) buffer (50 mM HEPES, pH 7.4; 50 mM NaCl; 0.1% Triton X-100; 10% glycerol; 1 mM phenylmethylsulfonylfluoride; 10 μg/ml leupeptin; 10 μg/ml aprotinin and 2 μg/ml pepstatin). Immune complexes were recovered by incubation with protein A/G-agarose. The beads containing bound proteins were washed three times by centrifugation in immunoprecipitation buffer, then denaturated by boiling in Laemmli sample buffer and analysed by western blot to identify the coprecipitating effector proteins. Immunoprecipitation using normal mouse serum was used as negative control. Membranes were stripped of bound antibodies by incubation in glycine (0.2 M, pH 2.6) for 30 min at room temperature. Before reprobing with the different indicated antibodies, stripped membranes were washed extensively in Tris buffered saline with Tween 20 (TBS-T) and placed in blocking buffer (TBS-T containing 5% milk) overnight.

Evaluation of sperm viability

Viability was assessed by using Eosin Y method. Spermatozoa were washed in uncapacitating medium and centrifuged at 800*g* for 20 min. To test androgen effects on sperm viability, spermatozoa

were incubated in unsupplemented Earle's medium at 37°C and 5% CO_2 without (control, UC) or in the presence of DHT at increasing concentrations (0.1, 10 and 100 nM) or 10 nM testosterone (T) for 2 h. In a different set of experiments, sperm were incubated in unsupplemented Earle's medium at 37°C and 5% CO_2 without (UC) or in the presence of 10 nM DHT or T at different times (0, 10 and 30 min, 2, 6 and 24 h). Some samples were pretreated for 15 min with 10 μM OH-Flut. 10 μl of Eosin Y [0.5% in phosphate-buffered saline (PBS)] were mixed with an equal volume of sperm sample on a microscope slide. The stained dead cells and live cells that excluded the dye, were scored among a total of 200 cells and by an independent observer. Further, viability was evaluated before and after pooling the samples.

PI3K activity

PI3K activity was performed as previously described (Aquila *et al.*, 2004). The negative control was performed using a sperm lysate, where p110 catalyzing subunit of PI3K was previously removed by preincubation with the respective antibody (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose. The PIK3R1 was precipitated from 500 μg of sperm lysates. The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4) and finally with 10 mM Tris, 100 mM NaCl and 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P] ATP and 10 μg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2) for 20 min at 37°C. Phospholipids were extracted with 200 μl $CHCl_3$/methanol. The labelled products of the kinase reaction, the PI phosphates, in the lower chloroform phase were spotted onto *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid-treated silica gel 60 thin-layer chromatography plates state running solvent used for TLC. Radioactive spots were visualized by autoradiography.

DNA laddering

DNA laddering was determined by gel electrophoresis. Spermatozoa were washed in Earle's balanced salt solution and centrifuged at 800*g* for 20 min, then were resuspended in the same uncapacitating medium and in different tubes containing no androgens (control, UC), T or DHT or estrogen or progesterone or wortmannin at the indicated concentrations for 30 min. Some samples were resuspended in CAP. Some samples were pretreated for 15 min with 10 μM Cax or 10 μM OH-Flut or ZVF alone or each combined with 10 nM DHT. After incubation cells were pelletted at 800*g* for 10 min. The samples were resuspended in 0.5 ml of extraction buffer (50 mM Trios-HCl [pH 8), 10 mM EDTA, O.5% sodium dodecyl sulphate (SDS)] for 20 min in rotation at 4°C. DNA was extracted with phenol/chloroform for three times and once with chloroform. The aqueous phase was used to precipitate acids nucleic with 0.1 volumes or of 3 M sodium acetate and 2.5 volumes cold EtOH overnight at −20°C. The DNA pellet was resuspended in 15 μl of H_2O treated with RNAse A for 30 min at 37°C. The absorbance of the DNA solution at 260 and 280 nm was determined by spectrophotometry. The extracted DNA (2 μg/lane) was subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and then photographed.

Statistical analysis

The experiments for RT–PCR, immunofluorescence and immunoprecipitation assays were repeated on at least four independent occasions, and western blot analysis was performed in at least six independent experiments, PI3K activity and DNA laddering assay were performed in at least four independent experiments. The data obtained from viability (six replicate experiments using duplicate determinations) were presented as the mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) followed by Newman–Keuls testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

Results

AR mRNA and protein were detected in human sperm

To determine whether mRNA for *AR* is present in human ejaculated spermatozoa, RNA isolated from percoll-purified sperm samples from normal men was subjected to reverse PCR. The nucleotide sequence of *AR* was deduced from the cDNA sequence of the human *AR* gene and our primers amplified a region from 1648 to 2055 bp corresponding to the DNA binding domain plus the hinge region of the AR. RT–PCR amplification of *AR* in human sperm revealed the expected PCR product size of 400 bp. This product was sequenced and found identical to the classical human *AR*. No detectable levels of mRNA coding either *PTPRC*, a specific marker of leucocytes, or *KIT*, a specific marker of germ cells, were found in the same semen samples (Fig. 1A), thus ruling out any potential contamination.

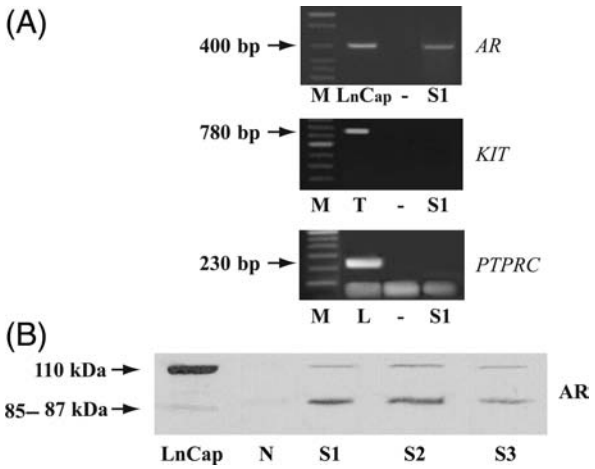

Figure 1: AR expression in human ejaculated spermatozoa (**A**) reverse transcription-PCR analysis of human *AR* gene, *KIT* and *PTPRC* in percolled human ejaculated spermatozoa (S1), negative control (no M-MLV reverse transcriptase added) (-), positive control (LnCap, prostate cancer cell; T, human testis and L, human leucocytes), marker (M). Arrows indicate the expected size of the PCR products; (**B**) western blot of AR protein by using a monoclonal antibody raised against the epitope 299–316 of the AR from human origin: extracts of percolled sperm, were subjected to electrophoresis on 10% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and probed with the above mentioned antibody. Expression of the receptors in three samples of ejaculated spermatozoa from normal men (S1, S2 and S3). LNCap cells were used as positive control. N, negative control performed as described in *Materials and Methods*. The experiments were repeated at least four times and the autoradiographs of the figure show the results of one representative experiment

The presence of AR protein in human ejaculated spermatozoa was investigated by western blot using a monoclonal antibody raised against the epitope mapping at the 299–316 aa in the N-terminus of AR from human origin (Fig. 1B). The antibody revealed the presence in sperm of two protein bands with molecular weights of 110 and 85–87 kDa, the latter expressed to a greater extent.

Immunolocalization of AR in human sperm

Using an immunofluorescence technique, we identified a positive signal for AR in human spermatozoa (Fig. 2A). No immunoreaction was detected either by replacing the anti-AR antibody by normal mouse serum (Fig. 2D) or when the primary antibody was omitted (data not shown), demonstrating the immunostaining specificity. AR immunoreactivity was specifically compartmentalized at the sperm head (Fig. 2A), where the DNA is packaged, as it can be seen in Fig. 2B in which the DNA is stained by Hoechst. Fig. 2C shows the merged images of Fig. 2A and B.

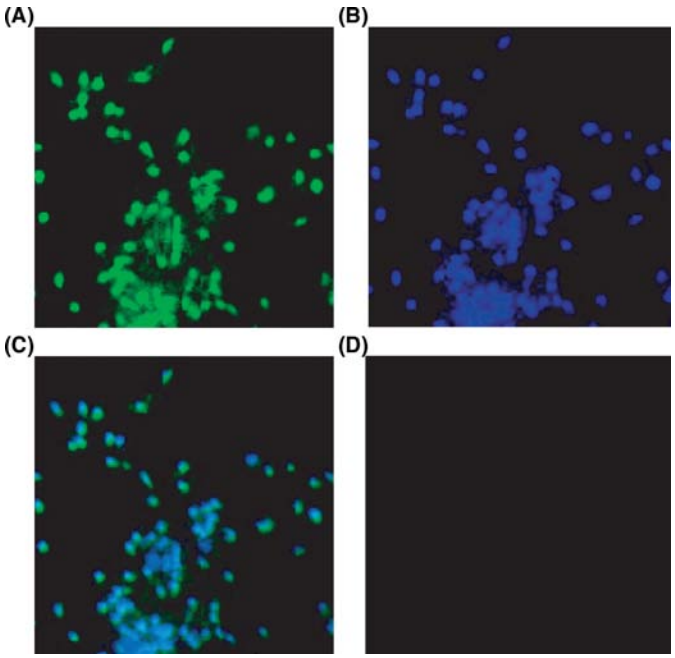

Figure 2: Immunolocalization of AR in human ejaculated spermatozoa

Spermatozoa were extensively washed and incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2. Spermatozoa were then fixed and analyzed by immunostaining as detailed in *Materials and Methods*. (**A**) AR localization in sperm; (**B**) staining with Hoechst of spermatozoa nuclei; (**C**) overlapping images of A and B; (**D**) sperm cells incubated replacing the anti-AR antibody by normal mouse IgG were utilized as negative control. The pictures shown are representative examples of experiments that were performed at least four times with reproducible results

AR is phosphorylated in human sperm

It was reported that the function of AR is strongly correlated with the phosphorylation status (Wang *et al.*, 1999), which is rapidly enhanced upon androgen exposure when it is able to activate signal transduction pathways. AR immunoprecipitates were blotted with three different antibodies: anti-p-tyrosine, anti-p-threonine and anti-p-serine. As shown in Fig. 3A, two major AR antibody reactive proteins corresponding to the 85–87 and 110 kDa were observed. To determine if the changes in phosphorylation status of AR under androgen treatments may occur in ejaculated sperm, these were exposed for 30 min to varying concentrations of DHT (0.1–10 nM). We observed that the AR phosphorylation was enhanced in a dose related manner (Fig. 3A) and was significantly reduced by OH-Flut, an AR antagonist. To investigate if the enhanced phosphorylation status may represent an early event, we performed a time course study revealing that AR phosphorylations occurred rapidly as they were observed from 0 to 15 min and then dropped significantly after 1 h (Fig. 3B). Moreover, all three phospho-antibodies demonstrated a prevalence for phosphorylation of the 110 kDa isoform. Furthermore, we repeated the experiments with the LNCaP cells to see whether they show similar results. As evidenced in Fig. 3C, in LNCaP cells, the major phosphorylation event appears to affect serine residues to a higher extent.

Androgens effect on sperm viability

To evaluate sperm viability under androgen treatment, we performed different sets of experiments. Sperm were incubated in the presence of 10 nM T or 10 nM DHT at the indicated times (Fig. 4A). Other samples were incubated in uncapacitating medium for 2 h in the absence or presence of different T or DHT concentrations (0.1–100 nM). As shown in Fig. 4B, the majority of cells remained viable in the control at 2 h. Cell viability significantly decreased with 10 and 100 nM T or DHT. Interestingly, the effect of androgen was reversed by using OH-Flut, addressing an AR mediated effect. It should be mentioned that the 100 nM androgen concentration is much higher than that commonly found circulating *in vivo* in man, while about 3 nM is detected in the seminal plasma of normal subjects (Luboshitzky *et al.*, 2002).

Androgen action on PI3K activity, p-AKT, p-BCL2 and p-PTEN is mediated by AR

As shown in Fig. 5A low androgen concentration (0.1 and 1 nM) induced PI3K activity, while it was reduced by using 10 and 100 nM DHT. Both 10 nM T and to a greater extent 10 nM DHT treatments decreased PI3K activity (Fig. 5A). The 10 nM DHT effect was reversed in the presence of OH-Flut. Concomitantly, we observed a reduction on the levels of the downstream p-AKT S473 and p-AKT T308 (Fig. 5B and C) as well as p-BCL2 (Fig. 5D), a known antiapoptotic protein (Ito et al., 1997). Specifically, DHT but not T had a significant inhibitory effect on p-AKT S473 and p-AKT T308 levels. Further, 10 nM of T or DHT significantly increased the phosphorylation of PTEN, a specific inhibitor of PI3K (Fig. 5E). All the above mentioned effects were reversed by Cax and OH-Flut, indicating that the effects of androgens are mediated by the classic AR in sperm. Recently, it was found that estradiol (E_2) enhances sperm survival signalling (Aquila *et al.*, 2004). Therefore, we aimed to evaluate whether, in sperm, a functional interaction exists between androgen and estrogen on PI3K activity. In sperm samples incubated with 100 nM E_2 combined with increasing DHT

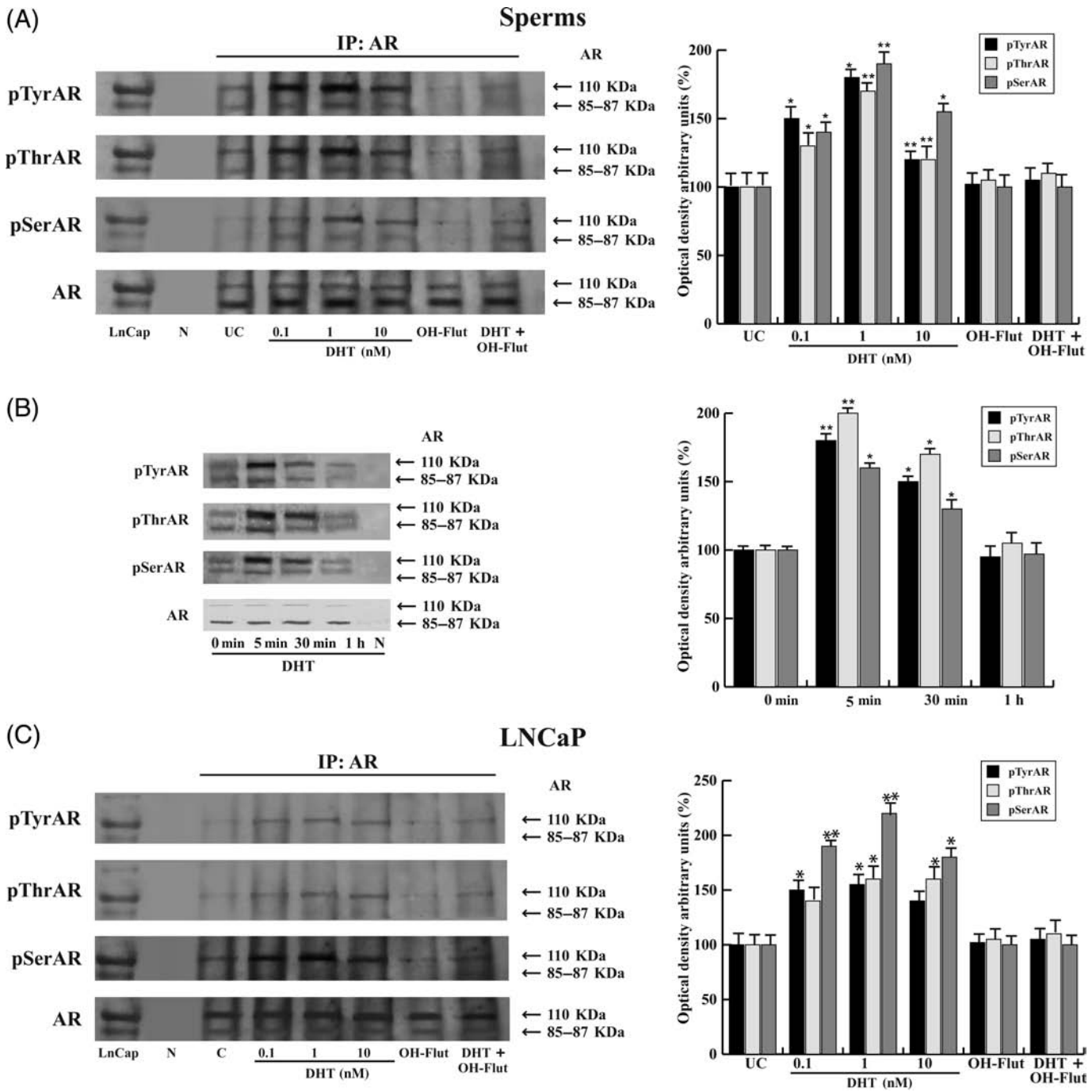

Figure 3: AR is phosphorylated in human sperm

AR phosphorylation was determined by immunoprecipitation using an AR specific antibody. The immunoprecipitates were blotted with three different antibody: anti-p-tyrosine (pTyrAR), anti-p-threonine (pThrAR) and anti-p-serine (pSerAR). (**A**) sperm were incubated without (control, UC) or in the presence of DHT at increasing concentrations (0.1, 1 and 10 nM) for 30 min. Some samples were pretreated for 15 min with 10 μM OH-Flut. Sperm lysates (600 μg) were immunoprecipitated using anti-AR and then blotted with specific antibodies raised to anti-p-tyrosine, anti-p-serine, anti-p-threonine, anti-AR. Immunoprecipitation by using normal mouse serum was used as negative control (N). The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. $*P < 0.05$, $**P < 0.01$ DHT-treated versus untreated cells. (**B**) time course of sperm incubated without (control, UC) or in the presence of 10 nM DHT. The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. $*P < 0.05$, $**P < 0.01$ DHT-treated versus untreated cells. (**C**) LNCaP cells were incubated without (control, C) or in the presence of DHT at increasing concentrations (0.1, 1 and 10 nM) for 30 min. Some samples were pretreated for 15 min with 10 μM OH-Flut. The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. $*P < 0.05$, $**P < 0.01$ DHT-treated versus untreated cells

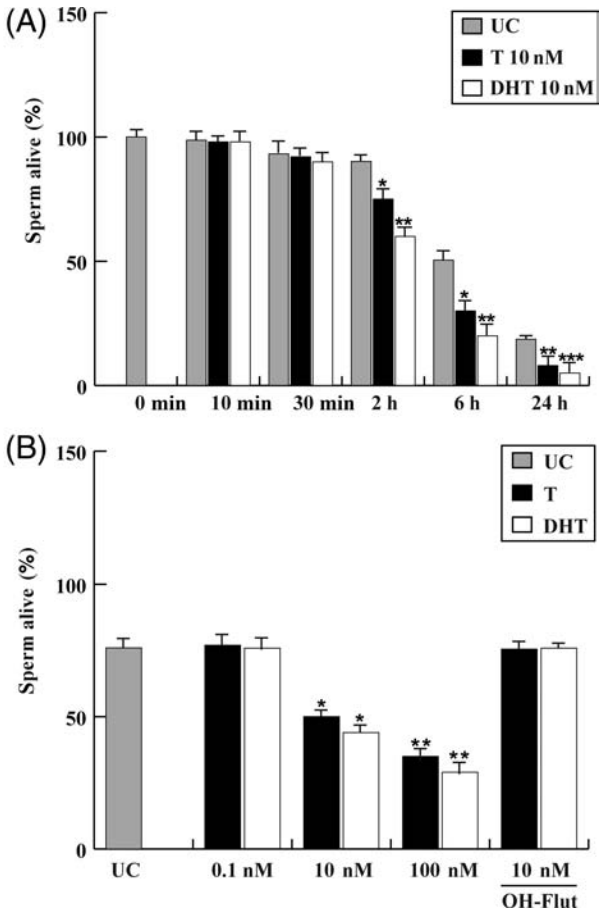

Figure 4: Effect of androgen on sperm viability
Viability was assessed by using Eosin Y as described in *Materials and Methods*. (**A**) time course of sperm incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 without (UC) or in the presence of 10 nM T or 10 nM DHT. (**B**) sperm were incubated without (UC) or in the presence of T or DHT at increasing concentrations (0.1, 10and 100 nM) for 2 h. Some samples were pretreated for 15 min with 10 μM OH-Flut and then treated with 10 nM T or DHT. All experiments were repeated at least six independent times with duplicate samples, and the values represent the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ versus control

concentrations, the E_2-induced PI3K activity progressively decreased (Fig. 5F).

Androgens induce AR, PIK3R1 and phospho-src tyrosine kinase coimmunoprecipitation in human sperm

It was reported that a triple complex between AR, PIK3R1 and SRC tyrosine kinase is required for androgen-stimulated PI3K/AKT activation (Castoria *et al.*, 2003; Sun *et al.*, 2003), therefore we investigated whether it also occurs in sperm. At the 0.1 nM DHT concentration, phospho-SRC tyrosine kinase coimmunoprecipitated with the two proteins immunodetected by the C-19 anti-AR antibody, migrating at 110 and 85–87 kDa. Remarkably, no association of phospho-SRC tyrosine kinase with AR occurred at the 100 nM DHT concentration. Fig. 6 shows immunocomplexes blotted with anti-AR (Fig. 6A) or phospho-SRC tyrosine kinase (Fig. 6B) or anti-PIK3R1 (Fig. 6C) antibodies. The possibility that androgen treatment could modify the AR level was excluded since in the immunoprecipitated proteins the same amount of AR was detected.

Since the coimmunoprecipitation of phospho-SRC tyrosine kinase and PI3-kinase with AR decreased as androgens concentration increased, we may suppose that this is the mechanism through which high DHT concentration reduced PI3k activity.

Androgens effects on caspases are mediated by AR

On the basis of the abovementioned results we sought to evaluate androgen action on the caspases family (Paasch *et al.*, 2004), since these proteins are involved in cell death. Particularly, caspase 3 which is the main effector of both caspases 8 and 9, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA strand breaks. Our study revealed activation of the caspases 8, 9 and 3 upon 30 min of 10 nM T or 10 nM DHT treatments. The DHT effect was reversed by both AR antagonists, 10 μM Cax or 10 μM OH-Flut (Fig. 7A). Notably, the effect on caspases was specific for androgen as it was not observed with E_2 or progesterone treatments. Particularly, progesterone treatment was performed because of the similarity in structure between progesterone receptor and AR. The cleavage of caspase 3 was increased by androgens in a dose-dependent manner (Fig. 7C). In the presence of wortmannin, a specific inhibitor of PI3K activity, the cleavage of caspase 3 was also observed, addressing a regulatory role of PI3K in caspase activation in sperm. Furthermore, in order to demonstrate a specific effect on caspase activation, an additional control experiment was included showing that activation of caspases by androgens can be inhibited with a specific caspases inhibitor such as ZVK. All these data were confirmed by DNA laddering assay (Fig. 7B and D).

Discussion

Androgens and AR have been shown to play critical roles in testis function (Collins *et al.*, 2003). AR has been detected in Sertoli, Leydig, peritubular myoid and spermatid cells (round and elongated) (Kimura *et al.*, 1993; Vornberger *et al.*, 1994; Suarez-Quian *et al.*, 1999). The currently prevailing view is that sperm does not contain AR and this stems from previous studies reporting that no AR immunostaining of germ cells was observed both in rat and in human testis (Suarez-Quian *et al.*, 1999). However, several studies reported that in spermatozoa, the binding capacity of androgens was greater than that of estrogens or progesterone (Hyne and Boettcher, 1977; Cheng *et al.*, 1981), and recently AR was shown to be present in sperm by western blot and immunofluorescence assays (Solakidi *et al.*, 2005).

In this study, we have demonstrated the presence of AR in human sperm at different levels: mRNA expression, protein expression and immunolocalization. By RT–PCR, we amplified a gene region corresponding to the DNA binding domain plus the hinge region of the human AR. This product was sequenced and found to be identical to the classical human *AR*. As it concerns the presence of mRNAs in mammalian ejaculated spermatozoa, originally it was hypothesized that these transcripts were carried over from earlier stages of

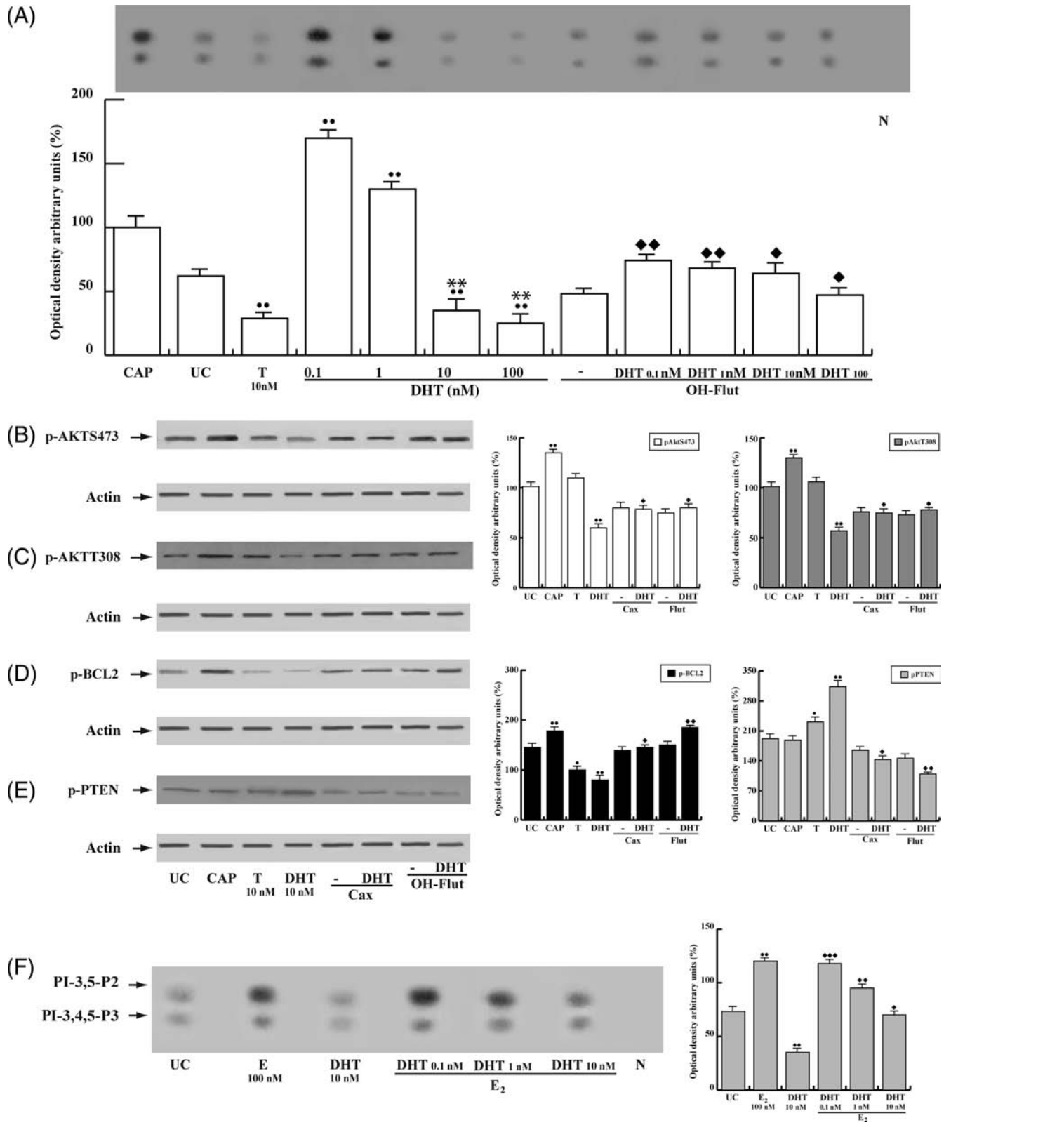

Figure 5: Androgens action on PI3K activity, p-AKT, p-BCL2 and p-PTEN is mediated by AR

Washed pooled sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2, in the absence (UC) or in the presence of 10 nM T or in the presence of DHT at increasing concentrations (0.1, 1, 10 and 100 nM) for 30 min. 500 μg of sperm lysates were used for PI3K activity in sperm incubated at the indicated DHT concentatrions in the absence or in the presence of 10 μM OH-Flut (**A**). The autoradiograph presented is representative example of experiments that were performed at least four times with repetitive results. The histograms indicated on the bottom of the figure are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. $^{\bullet\bullet}P < 0.01$ T- and DHT-treated versus untreated cells, $^{**}P < 0.01$ 10 and 100 nM DHT versus 0.1 and 1 nM DHT, $^{\blacklozenge\blacklozenge}P < 0.01$ and $^{\blacklozenge}P < 0.05$ 10 μM OH-Flut plus DHT-treated versus DHT-treated cells. 50 μg of sperm lysates were used for western blot analysis of p-AKT S473 (**B**) and

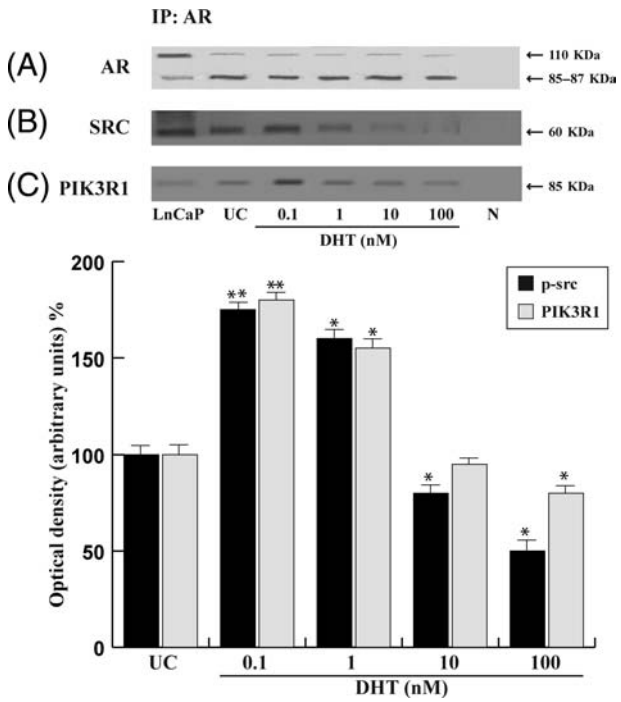

Figure 6: AR, PIK3R1 and phospho-SRC tyrosine kinase coimmunoprecipitate in human sperm
Washed spermatozoa from normal samples were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, without (UC) or in the presence of DHT at increasing concentrations (0.1, 1, 10 and 100 nM). 600 μg of sperm lysates were immunoprecipitated using anti-AR antibody and then blotted with specific antibodies raised to AR (**A**), p-SRC tyrosine kinase (**B**) and PIK3R1 (**C**). LnCap lysates were used as positive control (lane 1); Immunoprecipitation by using normal mouse serum was used as negative control (N). The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the left of the blot. *$P < 0.05$, **$P < 0.01$ DHT-treated versus untreated cells

spermatogenesis, however, new reports re-evaluate the significance of mRNA in these cells (Andò and Aquila, 2005; Miller *et al.*, 2005) and the issue is currently under investigation.

To date, multiple isoforms of the AR have been described and among them two proteins are well characterized: AR-B and AR-A (Wilson and McPhaul, 1996). They are believed to be derived from the same gene and differ only in the NH_2-terminal transactivation domain (Wilson and McPhaul, 1996). Our antibody against an epitope (aminoacids 299–311), i.e. common to both the AR-A and AR-B isoforms, detected two protein bands: one of 85–87 kDa and another one approximately of 110 kDa. Both, the AR-B and AR-A isoforms, are expressed in a variety of fetal and adult (male and female) human tissues and especially in reproductive tissues (Wilson and McPhaul, 1994). The B form migrates with an apparent mass of 110 kDa and constitutes >80% of the immunoreactive receptor in most cell types. The A form of the AR migrates with an apparent mass of 87 kDa. It was identified as an NH_2-terminally (from 1 to 187 aa) truncated protein of AR-B and it was first described in human genital skin fibroblasts. The detection of two distinct forms of the AR raised a number of issues. AR-A is expressed at low levels in many androgen-responsive tissues; however, it appears to have functions similar to those of the full-length AR-B isoform. Functional activities of cDNAs containing the two isoforms were assessed using cotransfection assays that employed two models of androgen-responsive genes (MMTV-luciferase and PRE2-tk-luciferase) in response to mibolerone, a potent androgen agonist, in three different cell lines (Gao and McPhaul, 1998). These studies demonstrated subtle differences in the activities of the A and B isoforms, which depended on the promoter and cell context. Additional studies failed to reveal any major differences in the responses of the AR-A and AR-B isoforms to a variety of androgen agonists and antagonists, suggesting that the previously reported functional defect of the AR-A is due principally to its level of expression. When assays of AR function are performed under conditions in which levels of expression of the two isoforms are equivalent, the AR-A and AR-B possess similar functional activities (Gao and McPhaul, 1998). The ratio of AR-B:AR-A may vary among tissues and at different stages of development. However, it is unknown whether these isoforms have divergent biologic signal transduction capacities in humans, therefore we cannot predict what is the physiological correlate of a low AR-B:AR-A ratio as observed in sperm. By immunohistochemical assays, we have demonstrated that AR protein is detectable in the sperm head. Solakidi *et al*. (2005) reported AR prevalently localized in the midpiece region and the labelling pattern was similar to that of ERα. The apparent discrepancy between the latter finding and ours may be due to the different methods used to process samples.

An increasing body of evidence suggests that androgens and other steroid hormones can exert rapid, nongenomic effects (Peterziel *et al.*, 1999; Cato *et al.*, 2002). Different nuclear receptors such as progesterone receptor (Calogero *et al.*, 2000), estrogen receptors α and estrogen receptor β (Aquila *et al.*, 2004) were found to be present in human ejaculated spermatozoa, regulating cellular processes through nongenomic mechanisms. All these findings strengthen the importance of the nuclear receptors in nongenomic signalling (Cato *et al.*, 2002) which may represent their exclusive modality of action

p-AKT T308 (**C**), p-BCL2 (**D**) and p-PTEN (**E**). The autoradiographs presented are representative examples of experiments that were performed at least six times with repetitive results. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of six independent experiments. $^{\bullet\bullet}P < 0.01$ capacitated (CAP) or DHT-treated versus untreated cells, $^{\blacklozenge}P < 0.05$ T-treated versus untreated cells, $^{\blacklozenge\blacklozenge}P < 0.05$ 10 and ** $P < 0.01$ μM OH-Flut plus DHT or Cax plus DHT-treated versus DHT-treated cells. (E) pI3K activity of sperm incubated with E_2 and/or DHT at the indicated increasing concentrations. The negative controls were performed using a sperm lysate, where p110 catalyzing subunit of PI3K was previously removed by preincubation with the respective antibody (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose (N). The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. The histograms indicated on the bottom of the figure are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. $^{\bullet\bullet}P < 0.01$ DHT- and E_2-treated versus untreated cells, $^{\blacklozenge}P < 0.05$ 10, $^{\blacklozenge\blacklozenge}P < 0.01$ and *** $P < 0.001$ E_2 plus DHT-treated versus DHT-trated cells

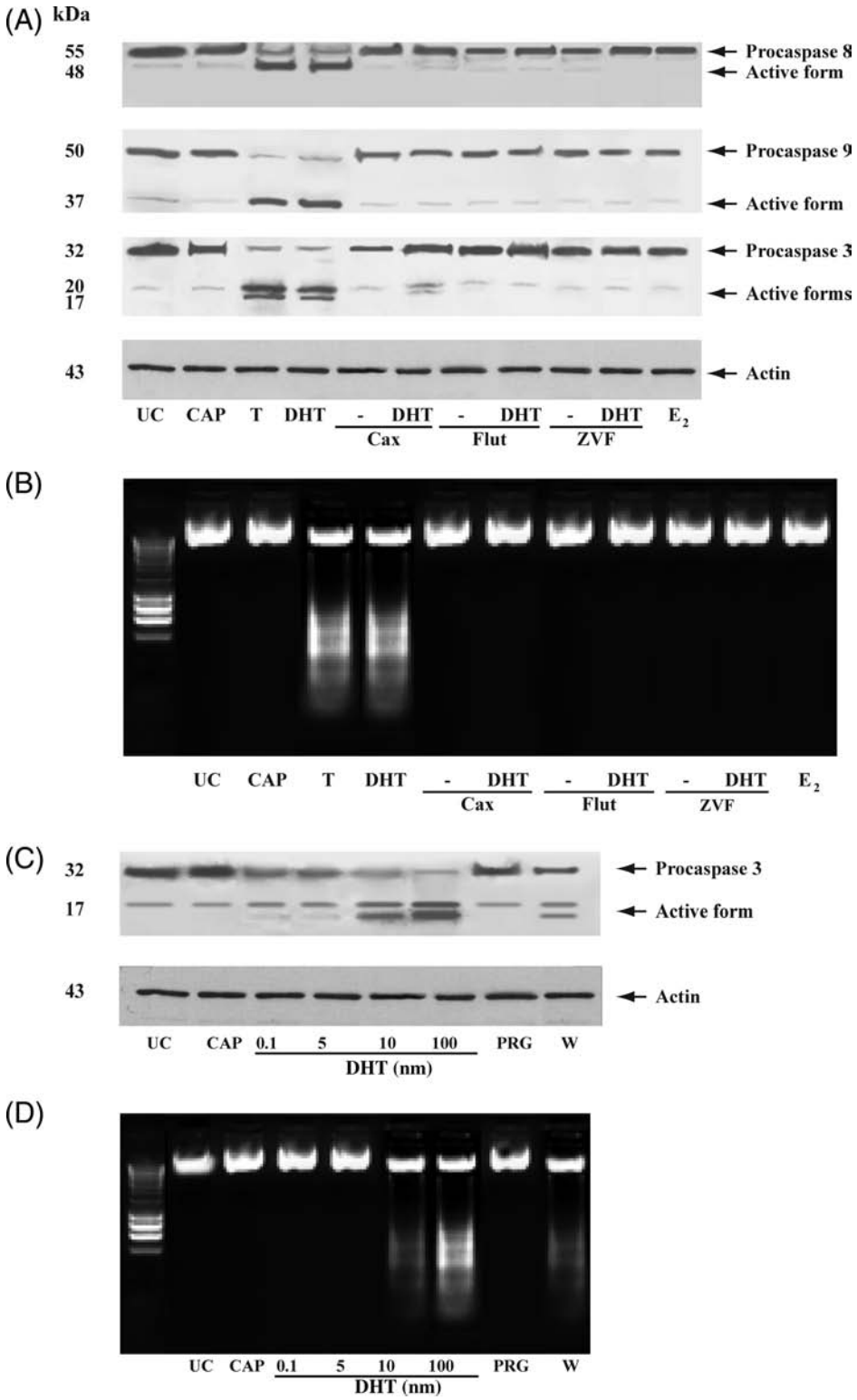

Figure 7: Androgens effects on caspases are mediated by AR
(**A**) washed pooled sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 (UC) in the presence of 10 nM T or 10 nM DHT or 100 nM E for 30 min. Some samples were washed with the unsupplemented Earle's medium and incubated in capacitating medium (CAP). Some samples were treated with Cax or Flut or ZVF each alone or combined with 10 nM DHT. The sperm were lysed and subjected to western blot analysis. 70 μg of sperm lysates were used for western blot analysis of caspase 8, caspase 9 and caspase 3. (**B**) DNA laddering was performed in sperm treated as indicated. (**C**) effect of increasing DHT concentrations (0.1–100 nM), 100 nM PRG and 10 μM wortmanninn (W) on caspase 3 cleavage. The experiments were repeated at least six times and the autoradiographs of the figure show the results of one representative experiment. (**D**) DNA laddering was performed in sperm treated as indicated

in spermatozoa since they are apparently transcriptionally inactive cells. Here, we have demonstrated that in human ejaculated sperm, short exposure to androgens produces an increase in AR phosphorylation in a dose-dependent manner, while the antagonist OH-Flut significantly reduces this effect. Furthermore, we observed the most prominent phosphorylation on the 110 kDa band which is the less expressed isoform in sperm. It is known that the function of nuclear receptors is strongly correlated with their phosphorylation status rather than the level of total receptor proteins. The 110 kDa isoform exhibits a major length of the N-terminal domain which is an important effector of the cell signalling (Wilson and McPhaul, 1994,1996) This may explain why the phosphorylated status of the 110 kDa appears much more pronounced than the smaller isoform. From these findings it emerges that in sperm the 110 kDa is the most involved isoform in mediating AR trafficking signals. Furthermore, in sperm, the phosphorylation of the less expressed isoform appears to occur, upon androgen binding, in tyrosine, threonine and to a greater extent with respect to the basal values, on serine aminoacidic residues. Therefore, we repeated the experiments with the LNCaP cells to see whether they show a similar result. In LNCaP cells, the major phosphorylation event appears to affect serine residues. In any case, we should take into account that even in uncapacitated sperm, the autocrine effect of a pool of cytokines, insulin-like growth factors, as we previously demonstrated (Aquila *et al.*, 2005a,b) may *per sè* influence the phosphorylation status of AR in addition to that determined by its natural ligand.

On the basis of our data androgens are able to modulate sperm survival depending on their concentration. To investigate the molecular mechanism involved in these effects we evaluated their action on the PI3K/AKT pathway, since it represents the main cell survival pathway and it was identified in sperm (Aquila *et al.*, 2004). The 0.1 and 1 nM androgens induced PI3K activity, which was reduced by higher concentrations (10 and 100 nM). The 10 nM DHT was able to reduce the PI3K downstream signalling, while phosphorylation of PTEN, a proapoptotic marker which inhibits the PI3K pathway, was enhanced. To gain further insight into the mechanism involved in the PI3K/AKT modulation by AR, we investigated the association between AR/PIK3R1/p-SRC tyrosine kinase since it was reported depending on androgen concentration in somatic cells (Castoria *et al.*, 2003; Sun *et al.*, 2003). In our study, high androgen concentrations (10 and 100 nM) produce a detachment of SRC tyrosine kinase from the PIK3R1/AR complex, confirming that the triple complex is needed for the PI3K pathway activation. Furthermore, wortmannin, a specific PI3K/AKT inhibitor, induced caspase 3 cleavage in sperm, showing that the PI3K/AKT pathway is involved in the modulation of the caspases activity. The sperm death under high androgens (10 nM T, 10 and 100 nM DHT) was confirmed both by DNA laddering and cleavage of caspases 8, 9 and 3. In addition, increasing androgen concentrations were able to counteract the E_2-induced PI3K activity previously documented (Aquila *et al.*, 2004).

It is well established that in men intratesticular T levels are ~800 nM (Coviello *et al.*, 2004), whereas they are ranging from 16 to 20 nM in serum (Luboshitzky *et al.*, 2002; Coviello *et al.*, 2004). The androgenic milieu in seminal plasma is dependent on circulating androgen levels and no longer intratesticular levels (Kuwahara, 1976; Andò *et al.*, 1983). The biologically active amount of T, represented by its free fraction, in the genital tract is mostly converted in DHT by 5 alpha-reductase which is particularly expressed in the epididymis and in the adnexal glands (Steers, 2001). A careful evaluation of the total androgenic milieu in seminal plasma, prevalently represented by the two most important androgens T and DHT, reveals the presence of about 1 nM of T and 2 nM of DHT and their ratio is about T/DHT 0.61 (Andò *et al.*, 1983). Therefore, the seminal androgenic milieu, prevalently represented by the total molar concentration of T plus DHT corresponds to ~3 nM. In our study, the effects induced by 10 nM DHT were opposite to those induced by the lower doses and the same opposite pattern of androgen effects on PI3K pathway was previously documented in other cell type (Castoria *et al.*, 2003).

In conclusion, the importance of androgen in the completion of male gamete maturation during the epididymal transit has been proved by the presence of AR in epididymal tissue (Zhou *et al.*, 2002). Now on the basis of our results we may speculate how the importance of androgen in the sperm maturative process goes beyond the length of their life in seminiferous tubules, and continues when they became transcriptionally silent, through the AR nongenomic signalling. Even though to date, we cannot establish the physiopathologic correlates of these findings, we observed that an excess of androgens in the local hormonal milieu inhibits PI3K activity and negatively interferes with sperm survival. Further work will be required to more fully elucidate the role that AR plays in this aspect of male fertility.

Acknowledgements

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Long-term delivery of superoxide dismutase and catalase entrapped in poly(lactide-co-glycolide) microspheres: In vitro effects on isolated neonatal porcine pancreatic cell clusters

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Abstract

To counterbalance the restricted availability of pancreatic islet tissue for transplant in Type 1 Diabetes Mellitus (T1DM), new methods to provide viable and functional islet cells need to be established. We report on our approach to enhance in vitro viability and function of isolated neonatal pancreatic porcine cell clusters (NPCCs) by co-culturing them with PLGA microsphere entrapped, slowly release superoxide dismutase and catalase. These powerful antioxidizing agents were shown to significantly improve morphology, viability and function, as assessed by microscopy, molecular, biochemical and functional studies, of the incubated NPCCs, as compared to control. Preliminarily, in vitro exposure of isolated NPCCs to slow release microsphere-embedded SOD and CAT could permit or contribute to overcome hurdles associated with scarcity in islet tissue procurement for transplant in T1DM.

Keywords: Diabetes; Superoxide dismutase; Catalase; Microspheres; Cell viability

1. Introduction

Correction of hyperglycemia by exogenous insulin administration may delay or attenuate, but not eliminate, the risk for developing long-term secondary

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complications associated with T1DM [1]. TX of insulin-producing tissue, whether be it comprised of whole pancreas or isolated islet cells, might fully restore normoglycemia, while replacing the need for daily exogenous insulin supplementation. Moreover, higher stability of metabolic control, as achieved by islet TX, could favorably impact both the onset and severity of the disease-related chronic complications themselves.

Of the 267 allografts transplanted since 1990, only 8.2% have resulted in insulin independence for periods of more than 1 year. In this regard, 100% remission of hyperglycemia, over a period of 1 year, with complete normalization of key metabolic parameters by application of human islet allografts, was first reported by the Edmonton group, University of Alberta, Canada [2]. The "Edmonton protocol" for islet TX is actually being tried by some of the Centers belonging to the Immunotolerance Transplant Network (ITN) [3]. Nevertheless, at least two major issues are still incumbent: (a) the restricted availability of cadaveric human donor pancreata; (b) the lack of safety reports on the effects of life-long recipient general immunosuppression.

With respect to the limited availability of donor pancreata, recent attention has been devoted to Neonatal Porcine Cell Clusters (NPCCs), harvested from neonatal piglet pancreata, for human substitutes [4], that, under appropriate conditions, can successfully acquire physiological competence in terms of insulin release in response to glucose stimulation [5]. Moreover, NPCCs (when compared with adult islets) hold additional advantages, in terms of reproducibility of the isolation, high yield and lack of a need for donor selection.

The cellular composition of NPCCs that at the moment of in vitro fresh isolation consists of 7% endocrine cells, 74% exocrine cells and 11% of duct precursor cells, is known to change, to 35% endocrine cells, 3% exocrine cells and 57% ductal precursor cells at day 9 of in vitro culture maintenance [6,7]. The relatively low β-cell mass (35%), at day 9, seems to be one of the causes of delayed normalization of blood glucose levels after transplantation; it takes 4–10 or more weeks for NPCCs to acquire adequate β-cell numbers and maturity so as to enable to reversal of hyperglycemia. The proportion of apoptotic cells in the NPCCs has been assessed on days 3, 6 and 9 of isolation in a previous paper [8]. In this paper it was demonstrated that apoptosis is highest on day 3 with an average of 52% positive cells, thereby decreasing gradually, on day 9 (30%). Furthermore, apoptotic cells were sorted out from necrotic cells (5–8%) by simultaneous double staining with FITC-Annexin V and propidium iodide (PI). From these studies, NPCC's heterogeneity can be ruled out upon differentiation. In fact, the processes occurring simultaneously during cell maturation can lead to formation of radicals and reactive species that may favor cell apoptosis and the reduction of β-cell population. As already reported by others [7], manipulation of culture conditions can accelerate maturation of β-cells and eventually improve this process. We had previously shown [9] that use of antioxidants, such as vitamins, was associated to prolongation β-cell viability, morphological integrity and physiological competence.

In particular, vitamins D3 and E were shown to exert beneficial effects on cell apoptosis and improve in vitro islet functional performance. In light of these results, use of even more potent antioxidants was considered to further improve β-cell function over 9 days of in vitro incubation. Natural occurring enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT) were looked at as potential useful adjuvants for NPCC's maturation. SOD and CAT are among the most potent antioxidants known in nature. SOD catalyzes dismutation of superoxide into oxygen and hydrogen peroxide and it is widespread in nature in eukaryotic and prokaryotic organisms [10]. CAT protects cells by catalyzing hydrogen peroxide decomposition into molecular oxygen and water with no free radical production. In addition, CAT acts on toxic compounds such as phenols, formic acid, formaldehyde and alcohols by peroxidative reaction. Sustained release of these enzymes from biodegradable polymeric matrices could be potentially useful to obtain a prolonged effect of such slowly release molecules that are made available to the islets over the investigated period of time. Poly(D,L-lactide-co-glycolide) (PLGA) was selected because of its wide use for microencapsulation of biological and non-biological molecules [11–13]. Recently, a W/O/W method was employed for SOD and CAT encapsulation in PLGA microspheres (MS) and the MS were characterized

in terms of drug content, size, in vitro release and activity retention of the entrapped enzyme [14]. The MS prepared using this method were incubated with NPCCs to assess the effects on the cells upon enzyme sustained release from the polymeric matrix. Aim of this work was to evaluate NPCC's viability, morphology and insulin secretion so as to establish, over 9 days of incubation, whether in vitro co-culture of NPCCs with SOD and CAT loaded PLGA MS would abbreviate the cellular maturation time-lag by accelerating the β-cell biological differentiation pathways.

2. Material and methods

2.1. NPCC's isolation

Neonatal "Large-White" pigs, aging 3 or 12 days, were used for pancreatic donors. NPCCs were isolated according to previously established methods modified in our laboratory [4,15]. Briefly, the piglets were anesthesized with 0.1 mg/kg azaperon (Stresnil® 40 mg/mL, Janssen, Bruxelles, Belgium) and 15 mg/kg ketamine (Imalgene® 100 mg/ml, Gellini Farmaceutici, Aprilia, Italy) co-administered intramuscularly. The piglets underwent total laparotomy, by midline incision, in order to carefully excise the pancreas. To prevent bacterial contamination, particular care was taken to avoid bowel nicking. Upon transportation to the laboratory in Eurocollins (SALF, Bergamo, Italy), the pancreas was cut into small pieces (1–3 mm^3) and washed in Hank's balanced salt solution (HBSS) (Sigma Chemical Co, St. Louis, MO). The tissue was then digested by gentle shaking in 2 mg/mL collagenase P (Roche Diagnostics, S.p.A., Monza, Italy) solution, for 5 min, at room temperature, and thereafter for additional 15 min, at 37 °C. The digest was centrifuged at 200 × g for 5 min, and subsequently washed twice in HBSS (Sigma) supplemented with 100 U/mL penicillin + 0.1 mg/mL streptomycin (Sigma). Finally, the tissue was resuspended in HAM-F-12 supplemented with 0.5% bovine serum albumin, fraction V (Sigma), 50 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), 10 mM nicotinamide (Sigma), 2 mM L-glutamine (Sigma), and penicillin + 0.1 mg/mL streptomycin (Sigma) and plated in 100 × 15 mm Petri dishes (Becton Dickinson Labware, Lincoln Park, NY) (10,000 NPCCs/plate). The culture medium was changed every 48 h.

2.2. SOD and CAT PLGA MS preparation and characterization

2.2.1. Microsphere preparation

Enzyme loaded poly(DL-lactide-co-glycolide) (PLGA, Sigma, Milan, Italy) MS were prepared by modification of a W/O/W double emulsion method as already reported in an our previous work [14]. Briefly, an amount of SOD and CAT (Sigma, Milan, Italy) corresponding to 10% theoretical loading was dissolved in 150 μL of 88 mM PEG400 (Sigma) aqueous solution. The protein solutions were added upon stirring to a small volume of 20% polymer-methylene chloride (J.T.Baker) solution to form a W/O emulsion. After proper emulsification, the mixture was injected into 50 mL of 6% PVA (Sigma) aqueous solution under stirring (1000 rpm, at 4 °C) to form a primary W/O/W double emulsion. The double emulsion was then poured in 500 mL of deionized water and kept at 4 °C. In order to evaporate the organic solvent, the temperature was slowly increased up to 15–20 °C along 2 h. The resulting MS were filtered by a Millipore 5 μm nitrocellulose filter (Millipore, Milan, Italy), washed with deionized water and vacuum-dried at room temperature overnight.

2.2.2. Assessment of enzyme encapsulation

In order to extract the proteins from the MS, enzyme loaded MS samples (5 mg) were dissolved in 0.5 mL 1 M NaOH by overnight rotation; the solution was then neutralized with 0.5 mL 1 M HCl. Protein content in the samples was determined by Micro-BCA protein assay (Sigma) [16] by using a UV/VIS Jasco N-520 spectrophotometer (Jasco Inc, Easton, MD, USA). All data are the result of three measurements and the error was calculated as S.D.

2.2.3. Size distribution and morphology

Size distributions of SOD and CAT containing PLA and PLGA MS were determined by Accusizer™ 770 Optical Particle Sizer (PSS Inc., Santa Barbara, CA, USA). Morphology was investigated by scanning electron microscopy (SEM) using a Philips XL30 microscope. The error was calculated as S.D. on triplicate samples.

2.2.4. In vitro release study

In vitro SOD and CAT release was determined by suspending PLGA MS in 10 ml of 0.1 M PBS (pH 7.4) and by incubating them at 37 °C. At predetermined intervals, samples were centrifuged (2000 rpm, 1 min, room temperature) and 1 ml of the supernatant was removed for Micro-BCA protein assay and replaced with an equal volume of fresh medium. The analysis was performed in triplicate. Enzyme content was established by Micro-BCA protein assay. Analyses were conducted in triplicate and the error was calculated as S.D.

2.2.5. In vitro activity

Entrapped SOD and CAT activity retention was evaluated in vitro according to previously reported methods [17,18]. Briefly, SOD activity was determined by reading the absorbance decrease at 340 nm during 10 min at 25 °C due to NADH (Sigma) oxidation induced by mercaptoethanol (Sigma). Slope increment due to SOD addition was calculated and the relative activity of the enzyme extracted from the MS determined using a standard curve created by plotting the logarithm of SOD amount vs. the percent slope increment. SOD activity retention was expressed as percent ratio between the active SOD amount resulting from the standard curve and the theoretical SOD amount added and measured by micro-BCA protein assay. All samples and standards were allowed to equilibrate at 25 °C before running each experiment. Similarly, CAT activity was measured at 25 °C by monitoring H_2O_2 (Sigma) absorbance decrease at 240 nm during 10 min after enzyme addition. In order to calculate the activity of the CAT extracted from the MS, a standard curve obtained by plotting H_2O_2 concentration vs. CAT amount was employed. CAT activity retention was expressed as percent ratio between the active CAT amount resulting from the standard curve and the theoretical CAT amount added measured by micro-BCA protein assay. All samples and standards were allowed to equilibrate at 25 °C before running each experiment. Enzyme extractions were carried out by suspending 10 mg of MS in 0.1 M PBS and the samples were incubated for 4 h at 37 °C. Analyses were performed in triplicate and the error was calculated as S.D.

2.3. In vitro assessment of isolated NPCCs

After 9 days of isolation, in vitro cultured NPCCs were examined either alone or in combination with SOD and CAT PLGA MS (100 mg/35 mL of medium), as far as viability, morphology and insulin secretion were concerned. The NPCCs/MS co-culture was performed in 100 × 20 mm Petri dishes where MS were added to the NPCCs in suspension.

2.3.1. Viability

At days 9 of culture, viability was assessed by staining the preparations with ethidium bromide (EB) (Sigma) and fluorescein-diacetate (FDA) (Sigma), as previously described [19]. Cells were visualized under fluorescence microscope (Nikon, Optiphot-2, Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein. Dead cells were stained in red, while viable cells appeared in green.

2.3.2. Function

At 9 days post-isolation, NPCCs were tested for insulin secretion capacity as following. Batches of 50 hand-picked NPCCs (from NPCC's cultures in 100 × 20 mm Petri dishes with or without SOD and CAT MS) were sequentially exposed to glucose at different concentrations (50–300–50 mg/dL) in a 2-h static incubation system. Briefly, the culture medium was replaced with low-glucose-containing (50 mg/dL) HAM F12 medium without serum for 2 h; then sequentially with high-glucose-containing (300 mg/dL) HAM F12 for another 2 h; and finally with HAM F12 with low glucose (50 mg/dL). After each treatment the cells were washed with Krebs solution supplemented with 1% albumin. The tissue culture medium was collected at the end of each treatment and stored at −20 °C; insulin content was determined by radioimmunoassay (intra-assay CV < 5.5%; inter-assay CV = 9.0%).

To assay DNA and insulin content, NPCC samples in microcentrifuge tubes were spun down, resuspended in 0.5 ml of high salt buffer (2.15 M NaCl, 10 mM NaH_2PO_4, 40 mM Na_2HPO_4, 2.3 mM EDTA, pH 7.4) and sonicated. The extracts were used to measure DNA content by fluorometry with a DyNa Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Fifty microliters of these extracts were mixed with 0.95 ml of acidic ethanol for

Table 1
SOD and CAT containing PLGA MS characterization

	Drug content (%) ± S.D.	Encapsulation efficiency (%) ± S.D.	Mean diameter (μm) ± S.D.
SOD PLGA MS	7.6 ± 0.9	76 ± 9	13 ± 1
CAT PLGA MS	7.2 ± 0.3	72 ± 3	14 ± 2

insulin extraction. Insulin was measured by radioimmunoassay as above reported.

2.3.3. RNA extraction, cDNA synthesis, and real time polymerase chain reaction (RT-PCR)

At day 9 of isolation, total cellular RNA was isolated from NPCCs by lysing the cells with TRIzol reagent (Invitrogen, London, UK). All RNA was treated with DNase I (Invitrogen), and purity and integrity of the RNA was confirmed spectroscopically and by gel electrophoresis before use. Four micrograms of total RNA were reverse transcribed in a final volume of 100 μL using the high capacity cDNA archive Kit (Applied Biosystems, Foster City, CA) and stored at −20 °C. Primers for the amplification were based on published sequences for the pig insulin gene (AF064555) and pig GLUT2 gene (AF054835). RT-PCR analyses were performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystem) in a total volume of 30 μL reaction mixture following the manufacturer's recommendations, using the SYBR Green Universal PCR Master Mix 2X (Applied Biosystems) and 0.150 μM of each primer using the dissociation protocol. Negative controls 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 TaqMan Ribosomal RNA Reagent Kit (Applied Biosystems) following the method provided in the TaqMan Ribosomal RNA Control Reagent kit (Applied Biosystems). The relative Ins and Glut-2 gene expression levels were normalized to a calibrator that was chosen to be the control sample (untreated). Final results, expressed as n-fold differences in Ins and Glut-2 gene expression relative to 18S rRNA and calibrator, were calculated following the ΔΔCt method, determined as follows: $n\text{-fold} = 2^{-(\Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator}))}$, where ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of the pig insulin and pig GLUT2 gene from the average Ct value of the 18S rRNA reference gene. Before using the ΔΔCt method for relative quantification, we perform validation experiments to demonstrate that efficiencies of target and reference are approximately equal, following instructions of Applied Biosystems [http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf (page 14)].

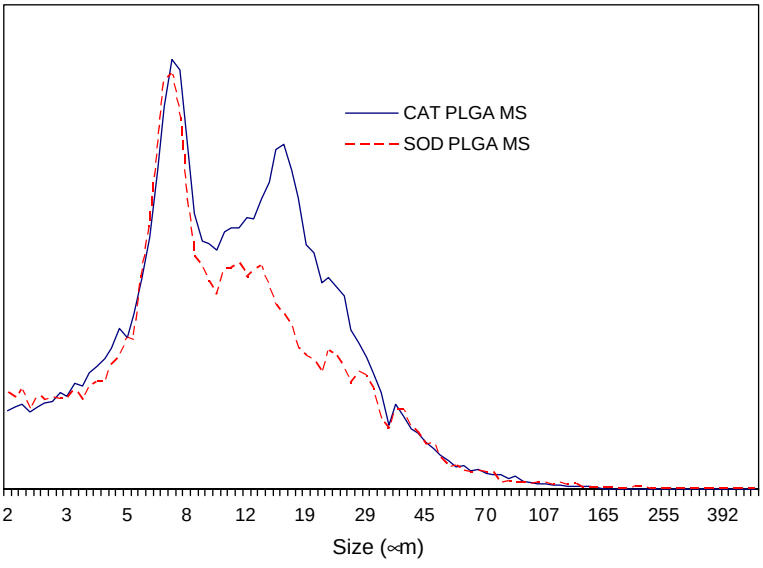

Fig. 1. SOD and CAT PLGA MS size distributions.

2.3.4. Morphological analysis

At day 9 of isolation, NPCCs were examined by optical microscopy in order to evaluate their morphology. Cells were fixed with 2.5% glutaraldehyde in PBS and stained with 1% osmium tetroxide (OsO_4) (Sigma, Milan) in PBS; the samples were dehydrated by gradient concentrations of ethanol and finally included in epon-araldite resins (Sigma). Semi-thin sections were stained with toluidine blue (Sigma) and examined under light microscopy (Nikon, Italy).

2.3.5. Statistical analysis

Results were expressed as means ± confidence at $p < 0.05$ significance level. As far as static incubation with glucose was concerned, the results were the mean of $n = 5$ independent experiments and each insulin determination was performed in triplicate ($n = 3$). Likewise, RT-PCR was performed in triplicate ($n = 3$). Two-way ANOVA and Holm-Sidak multi comparison tests were performed to analyze static incubation data.

3. Results and discussion

3.1. SOD and CAT PLGA MS

Encapsulation of the enzymes in PLGA MS was successfully achieved as reported in Table 1. Mean diameters of blank MS did not differ if compared to the loaded MS. These findings may indicate that protein entrapment does not modify MS morphology. Profiles of MS size distribution are shown in Fig. 1. Either SOD or CAT PLGA MS showed the presence of two main populations at around 7 μm and 15 μm. SEM of blank and loaded MS reported in Fig. 2 showed spherical morphology with a smooth surface and isolated small pores.

Enzyme in vitro release from PLGA MS is shown by Fig. 3 panel A. SOD release was sustained with 40–45% initial burst and the release was complete after 60 days, whilst CAT release was incomplete with 60–65% initial burst. Nevertheless, more than 80% of release was achieved after 25 days. Unfortunately, immediately after day 25, CAT concentration started to decrease slightly and reached about 60% at day 58. In our previous work, it was demonstrated that this trend was due to either CAT adsorption on the polymer or protein degradation

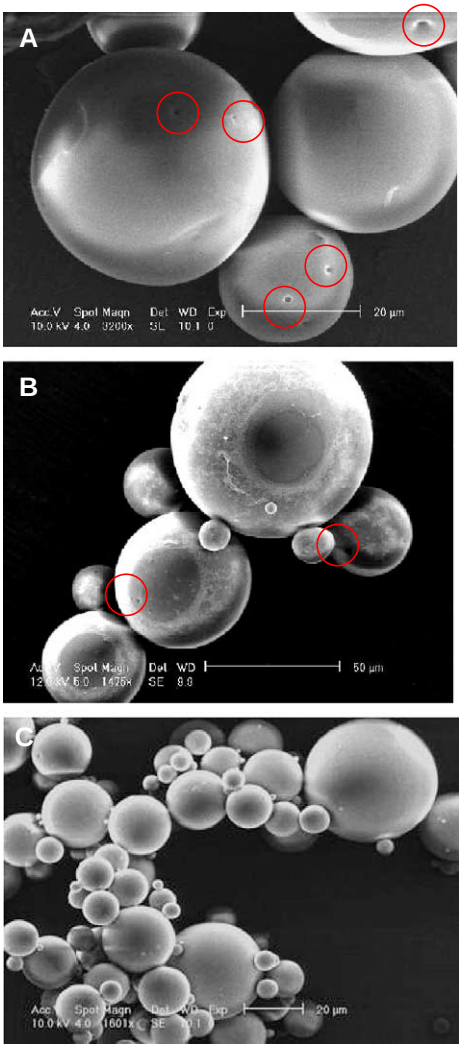

Fig. 2. SEM photomicrographs of (A) SOD and (B) CAT PLGA MS with PEG400. Blank MS are also reported (C). Visible pores are marked with red circles.

[14]. During the first 2 weeks, SOD and CAT release was equally sustained and in light of this observation, either SOD or CAT loaded MS could be used for modify NPCCs in vitro behaviour over 9 days of incubation.

In vitro activity tests performed on the encapsulated CAT and SOD are shown in Fig. 3 panel B. PEG400 seemed to successfully stabilize SOD and CAT during preparation, with an activity retention close to 90–100%. This particular strategy was useful to warrant the release of native proteins in the

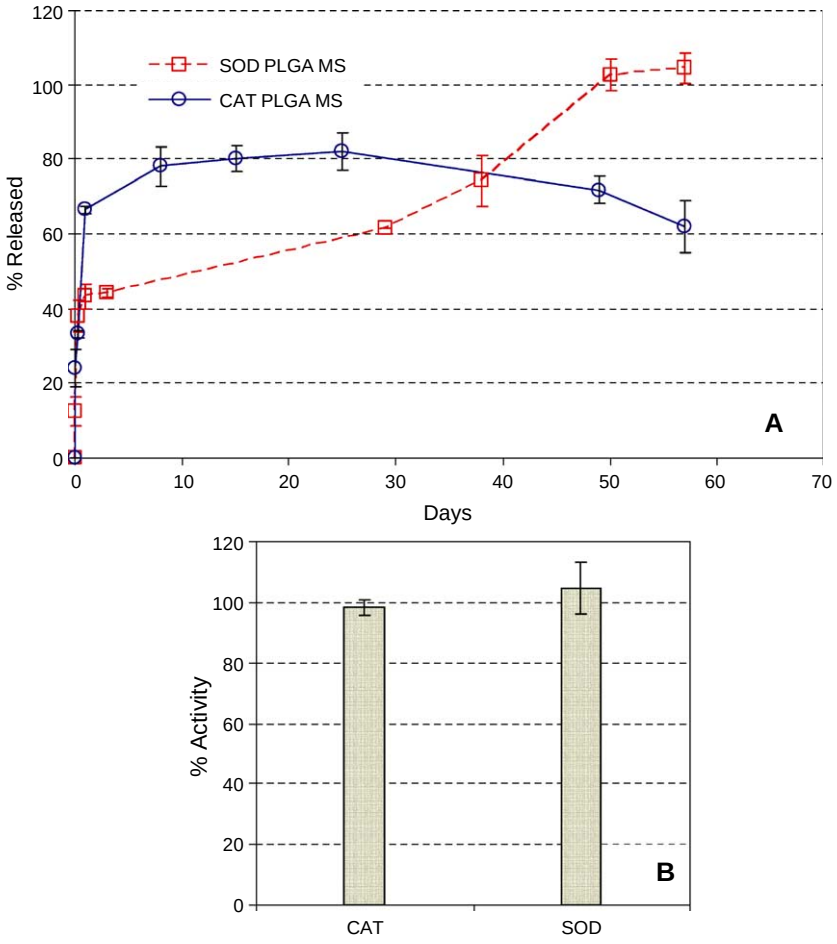

Fig. 3. (A) In vitro release of SOD and CAT from PLGA MS at 37 °C in 0.1 M PBS pH 7.4 in triplicate; (B) activity retention of CAT and SOD released from PLGA MS with PEG400 as stabilizer.

cell medium. The use of such formulations, able to release gradually the enzymes in their native forms, may be useful to prolong the protective effect of these potent antioxidants against radicals and other reactive species that may impair NPCC's function, with SOD and CAT continuously feeding the cells during incubation.

3.2. Effects of SOD and CAT loaded PLGA MS on incubated NPCCs

Photomicrographs of NPCCs co-incubated with CAT and SOD PLGA MS at 9 days of culture indicated (Fig. 4, panel B and C) a significantly improved viability as compared to NPCCs alone (panel A), as shown by the bright green fluorescence upon staining with EB+FDA.

Fig. 5 reports the optical microphotographs of NPCCs after fixation and staining with toluidine blue. The pictures illustrate morphology of the NPCCs maintained (for 9 days) in culture alone (panel A) and with CAT (Panel B) or SOD PLGA MS (Panel C). NPCCs alone showed irregular margins and loose cell texture with internal discontinuations. By sharp contrast, the NPCCs cultured in the presence of SOD were very viable, acquired more compact and spherical shape with an external, membrane-like structure surrounding the cell clusters. When NPCCs were maintained in co-culture with CAT (Panel B), the internal β-cells

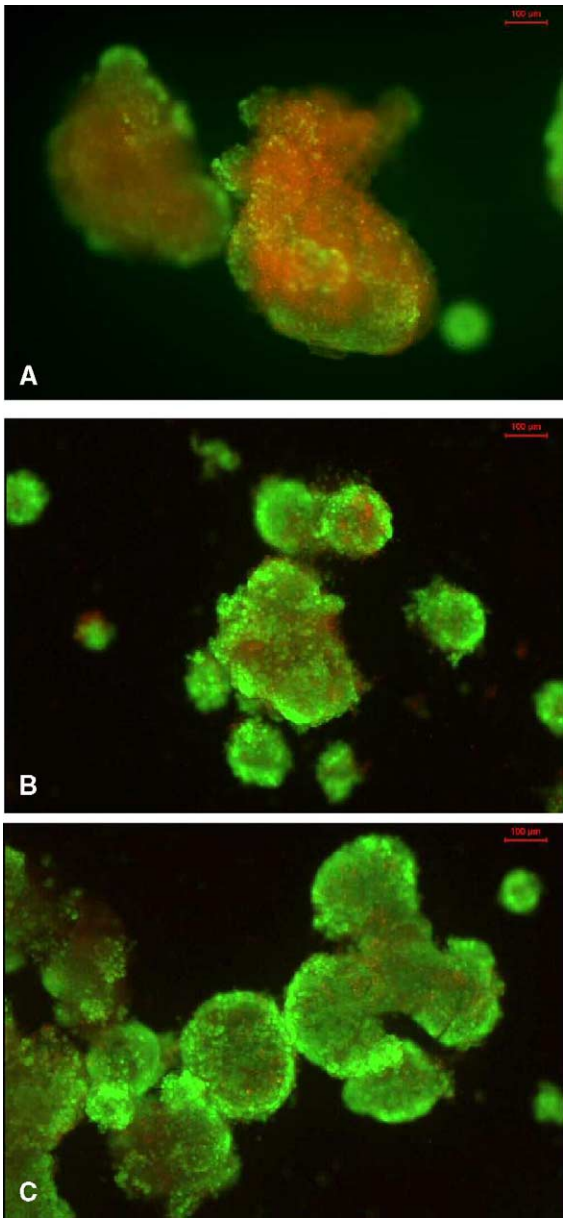

Fig. 4. Fluorescence microphotographs after viability staining with ethidium bromide (EB) and fluorescein-diacetate (FDA) of NPCCs maintained in conventional culture for 9 days. The pictures show not treated NPCCs (A), CAT (B) and SOD (C) treated NPCCs.

appeared to be less organized, possibly suggesting a certain reduction of cell viability as indicated by the less intense staining of the internal cells by toluidine blue in comparison to NPCC' co-cultured with SOD.

Presence of a β-cell population in the NPCCs co-cultured with SOD and CAT PLGA MS at 9 days was assessed by staining with DTZ (Fig. 6, panel C). In this regard, NPCCs in the presence of SOD and CAT PLGA MS showed a significant increase in the num-

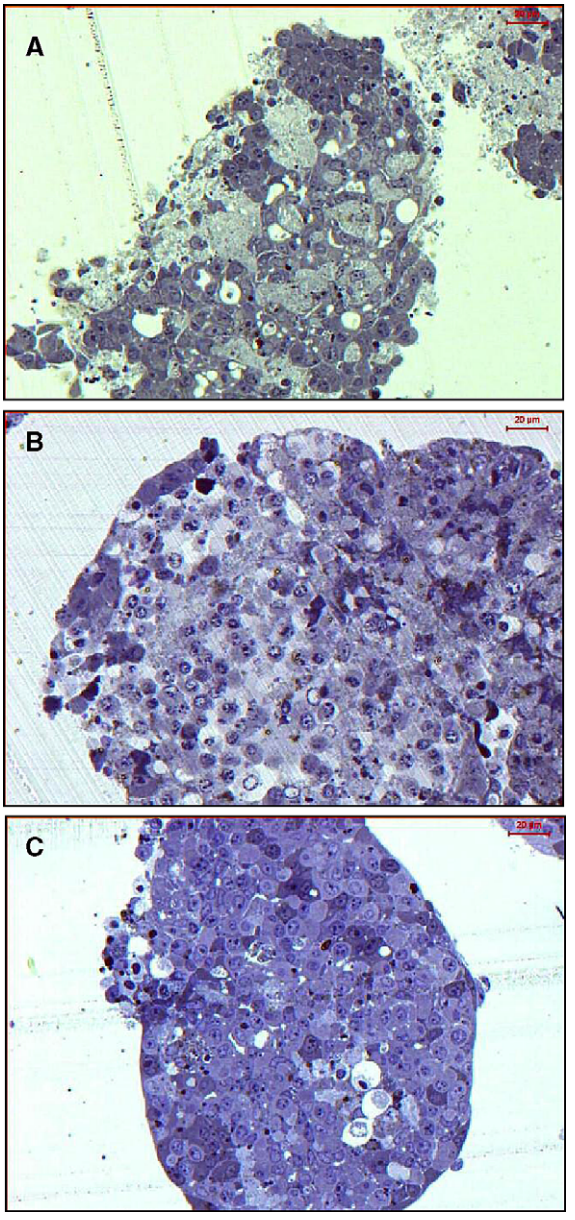

Fig. 5. Optical microphotographs of NPCCs after fixation and staining with toluidine blue. The pictures illustrate morphology of the NPCCs at day 9 in culture alone (A), with CAT (B) or SOD PLGA MS (C).

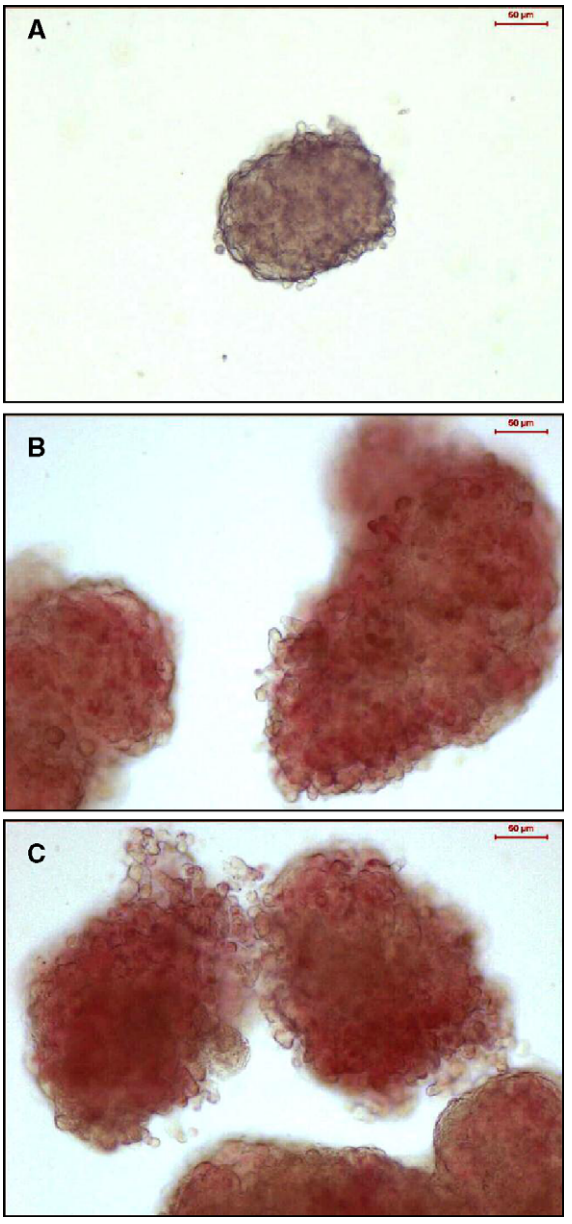

Fig. 6. Photomicrographs of NPCCs cultured for 9 days alone (panel A) or in combination with CAT PLGA MS (panel B) and SOD PLGA MS (panel C); staining with DTZ evidenced the presence of β-cell population. Bar = 100 μm.

ber of DTZ-positive cells with respect to NPCCs alone (Fig. 6, panel B and C). In fact, untreated NPCCs were almost colorless, revealing remarkable smaller β-cell population as compared to NPCCs incubated with either SOD or CAT PLGA MS (Fig.

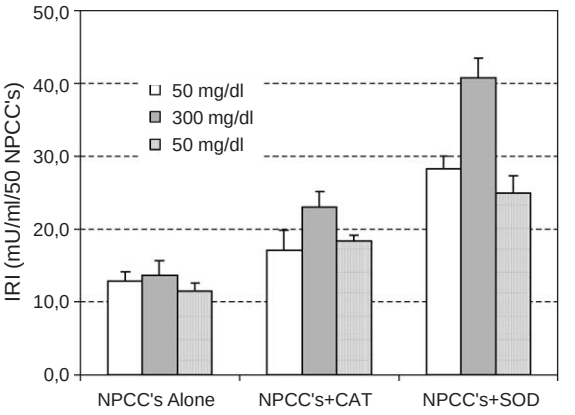

Fig. 7. Static incubation data of NPCCs alone and in the presence of CAT and SOD. Data represent the average of 5 independent experiments.

6, panel A). Moreover, we observed a significant increase in the endogenous insulin output, under glucose stimulation, from the NPCCs that were co-cultured with SOD, as compared to NPCCs alone and co-cultured with CAT, throughout in vitro culture maintenance (Fig. 7). We have reported insulin secretory patterns of 9-day NPCCs cultured alone, NPCCs cultured in the presence of CAT or in the presence of SOD MS, during static incubation with glucose. Insulin levels were determined after 9 days of cell culture (Table 2). In addition, DNA and insulin content of NPCCs cultured alone, in presence of CAT and in presence of SOD MS were determined: the DNA content reflecting the tissue cell mass (representing the average of proliferating, surviving and dead cells) and insulin content indicating NPCCs physiologic competence. NPCCs cultured in the presence of SOD MS exhibited an increase of DNA (124 ± 13%) and insulin (501 ± 4%) (data not shown) (data reported as percentage of control NPCCs alone for 3 independent experiments). The insulin/DNA ratio in the NPCCs co-cultured with SOD was significantly higher

Table 2
Static incubation data ($n = 5$, 0.05 significance level)

	Substrate	Glucose level (mg/dL)		
		50	300	50
Insulin levels (μU/mL ± S.D.)	NPCCs alone	12.8 ± 1.6	13.6 ± 2.4	11.4 ± 1.3
	NPCCs + CAT	17.2 ± 3.2	23.0 ± 2.3	18.3 ± 1.0
	NPCCs + SOD	28.3 ± 1.9	40.7 ± 3.0	25.0 ± 2.6

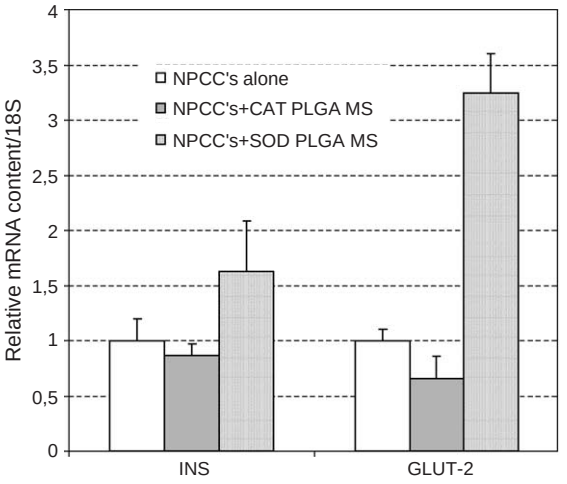

Fig. 8. Nine days NPCCs insulin secretory patterns cultured alone or in combination with CAT PLGA MS and SOD PLGA MS during static incubation with glucose. Insulin concentrations were determined after 9 days of cell culture. Data represent the average of 4 independent experiments; each insulin determination was performed in triplicate ± S.D.

(0.42 ± 0.1) as compared to NPCCs alone or co-cultured with CAT; the ratios for control NPCCs and NPCCs co-cultured with CAT were 0.06 ± 0.2 and 0.08 ± 0.6, respectively (data not shown). Moreover, RT-PCR analysis showed that the relative insulin mRNA and Glut-2 levels of NPCCs after 9 days of incubation with SOD PLGA MS were 1.62 ± 0.46 and 3.25 ± 0.35 times higher as compared to the control ($p < 0.05$) (Fig. 8). On the contrary, NPCCs incubated with CAT PLGA MS showed a moderately lower mRNA and Glut-2 expression 0.87 ± 0.11 and 0.66 ± 0.21 respectively, than NPCCs alone. These findings highlighted the larger effect of SOD with respect to CAT on NPCC's functionalities as reported in Figs. 7 and 8. In fact, although enhanced cell performances were observed when both CAT and SOD were fed to cells, either static incubation or RT-PCR data revealed a much higher SOD effect as compared to CAT. This observation might convey a greater effectiveness of SOD in protecting cells from radicals and other reactive species that may be produced during incubation and that may affect β-cell maturation.

In general, however, these results indicated an overall remarkable improvement of NPCC's functional performances upon SOD and CAT sustained release from PLGA MS. These data may be of great interest as the NPCC's enhanced performances may be interpreted as an improvement of β-cell maturation. In fact, mRNA expression of insulin and Glut-2 was much higher in the presence of SOD and insulin output upon glucose stimulation was considerably higher as a consequence of SOD and CAT effect. These findings may correlate with an increased number of mature β-cells.

3.3. Statistical analysis of static incubation data

Statistics was used to compare static incubation data. ANOVA analysis showed highly significant differences for either substrates or treatments (Table 3). The difference in the mean values among the different levels of substrate was greater than would be expected by chance, after allowing for effects of differences in treatment ($p < 0.001$). The same result was found for the mean values among the different treatment levels ($p < 0.001$). Moreover, the effects of different levels of substrate depended on what level of treatment was present. In other words, a significant interaction between substrate and treatment seemed to exist ($p < 0.001$). Holm-Sidak multi comparison procedure was carried out in order to discern the most significant differences among the groups investigated and to point out the most relevant gap among the responses of substrates upon glucose stimulus. The results of such a test (Table 4) showed that substrate differences were highly significant. On the other hand, treatment means A and C were basically not different showing that the cells were generally able to respond when glucose levels backed from 300 to 50 mg/dL. The

Table 3
ANOVA statistical comparison of static incubation data

Source of variation	df[a]	SS[b]	MS[c]	F[d]	p[e]
Substrate	2	2686.755	1343.378	262.846	<0.001
Treatment	2	495.789	247.895	48.503	<0.001
Substrate × Treatment	4	301.108	75.277	14.729	<0.001
Residual error	36	183.992	5.111		
Total	44	3667.644	83.356		

[a] Degrees of freedom.
[b] Sum of squares.
[c] Mean squares.
[d] F statistics: $F = (\mathrm{MS})/(df_{\text{Residual Error}})$.
[e] Probability of acceptance of the null hypothesis at 0.05 significance level.

Table 4
All pairwise multiple comparison procedures (Holm-Sidak method): overall significance level=0.05

Comparison	Difference of means	t[a]	Unadjusted p[b]	Critical level	
Substrate[c]					
3 vs. 1	18.712	22.667	6.672E−23	0.017	significant
3 vs. 2	11.820	14.319	1.951E−16	0.025	significant
2 vs. 1	6.892	8.349	6.10E−10	0.050	significant
Treatment[d]					
B vs. C	7.559	9.156	6.196E−11	0.017	significant
B vs. A	6.373	7.721	3.81E−09	0.025	significant
A vs. C	1.185	1.436	0.160	0.050	not significant
Treatment within 1					
B vs. C	2.216	1.550	0.130	0.017	not significant
B vs. A	1.396	0.976	0.335	0.025	not significant
A vs. C	0.820	0.574	0.570	0.050	not significant
Treatment within 2					
B vs. C	5.880	4.112	0.000	0.017	significant
B vs. A	4.740	3.315	0.002	0.025	significant
A vs. C	1.140	0.797	0.430	0.050	not significant
Treatment within 3					
B vs. C	15.720	10.994	0.000	0.017	significant
B vs. A	12.420	8.686	0.000	0.025	significant
A vs. C	3.300	2.308	0.027	0.050	significant
Substrate within A					
3 vs. 1	15.480	10.827	0.000	0.017	significant
3 vs. 2	11.120	7.777	0.000	0.025	significant
2 vs. 1	4.360	3.049	0.004	0.050	significant
Substrate within B					
3 vs. 1	27.080	18.940	0.000	0.017	significant
3 vs. 2	17.660	12.351	0.000	0.025	significant
2 vs. 1	9.420	6.588	0.000	0.050	significant
Substrate within C					
3 vs. 1	13.576	9.495	0.000	0.017	significant
3 vs. 2	6.680	4.672	0.000	0.050	significant
2 vs. 1	6.896	4.823	0.000	0.025	significant

[a] Calculated t value.
[b] p value to be compared to the critical level value.
[c] Substrate 1=control; Substrate 2=CAT; Substrate 3=SOD.
[d] Treatment A=50 mg/dL; Treatment B=300 mg/dL; Treatment C=50 mg/dL.

comparison of treatments within each substrate group highlighted the main effect of CAT and SOD as A–B and B–C differences were significant at 0.05 level, meanwhile A and C were significant only when SOD was employed. Moreover, A and C difference was less significant for CAT (substrate 2) than SOD (substrate 3), t values were much higher for SOD substrate than CAT and control (substrate 1). Comparison of substrate means within treatments showed all significant differences as expected when glucose levels are raised forth and back from 50 to 300 to 50 mg/dL. This effect could be ascribed to improved NPCC's performance owing to CAT and SOD supplementation. In particular, the higher significance of SOD difference

from control with respect to CAT was of great importance. This aspect is more evident if looking at the calculated t value of SOD substrate compared to CAT. In fact, all SOD *t* values were almost 3 fold higher than CAT's. This feature correlates well with a higher insulin output in the presence of SOD. Moreover, CAT and control were less significantly different as compared to SOD and control in treatment A group, showing that even at low glucose levels CAT was less effective than SOD.

4. Conclusions

In vitro supplementation of antioxidizing agent (SOD and CAT) containing PLGA MS was shown to significantly enhance cultured NPCC's viability and function. In light of the lack of fresh and viable pancreatic cell tissue for transplant purposes, the obtained results might provide access to a new approach to grow and maintain islet cells.

While issues on the potential human use of xenogeneic porcine islets are still being examined, methods to enhance in vitro functional performance of these islets could provide new alleys to bulk availability of transplant suitable pancreatic cell tissue for the treatment of T1DM.

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ATTI DI CONVEGNO

Ines Barone, Stefania Catalano, Cinzia Giordano, **Ivan Casaburi**, Guowei Gu, Pietro Rizza, Daniela Bonofiglio, Sebastiano Andò. EVIDENCE THAT ESTRADIOL, TROUGH A SHORT NON GENOMIC LOOP, DOWNREGULATES PTP1B AND ENHANCES AROMATASE ACTIVITY IN MCF-7 CELLS. 89th Endo 07 Annual Meeting, Toronto June 2-5.

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