Università degli Studi della Calabria

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Dipartimento Farmaco-Biologico (MED/04 PATOLOGIA GENERALE)

Dottorato di Ricerca in "Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XIX ciclo)

PI3-kinase/Akt mediates the early increase of aromatase activity induced by estradiol in MCF-7 cells

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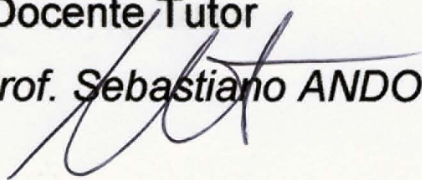

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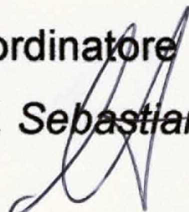

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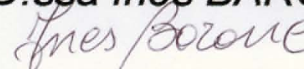

Anno Accademico 2005-2006

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SUMMARY

Estrogens are strongly associated with breast cancer development and progression. The intratumoral conversion of androgens to estrogens by aromatase within the breast may be an important mechanism of autocrine stimulation in hormone-dependent breast cancer.

The aim of this study was to investigate if E_2/ER can modulate aromatase activity in human breast cancer cells. In MCF-7 cells we examined, by tritiated water release assay, aromatase activity. Immunoprecipitation studies, using a vector containing aromatase gene with polyhistidine tags, were performed to evaluate phosphorylation status of aromatase protein.

Our results demonstrated that 17-β estradiol is able to enhance aromatase activity, at short time, through PI3K/Akt pathway in MCF-7 breast cancer cells. These up-regulatory effects involved rapid changes in tyrosine phosphorylation status of aromatase purified protein. Indeed, a tyrosine phosphatases inhibitor enhanced basal and E_2-induced aromatase activity. The overexpression of the tyrosine phosphatase PTP1B reduced enzymatic activity while E_2-induction was completely reversed in MCF-7 cells cotransfected with PTP1B and dominant negative of Akt supporting the involvement of PI3K/Akt pathway. It should be taken into account that a cross-talk between tyrosine kinase growth factor receptors and estrogen receptor signaling may influence aromatase activity.

Our data indicate the existence of a short non genomic autocrine loop between E_2 and aromatase in MCF-7 cells, giving a great emphasis to the role of aromatase in promoting breast cancer cell growth.

INTRODUCTION

Estrogens play a crucial role in the development and progression of breast cancer. Approximately 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent carcinomas (Chen, 1998). The biosynthesis of estrogens from androgens is catalyzed by the enzyme complex termed aromatase (also estrogen synthase or P450 AROM), which is composed of two polypeptides, an ubiquitous nonspecific flavoprotein, NADPH-cytochrome P450 reductase and a specific microsomial form of cytochrome P450arom encoded by the CYP19 gene (Fig. 1)(Simpson *et al*, 1994).

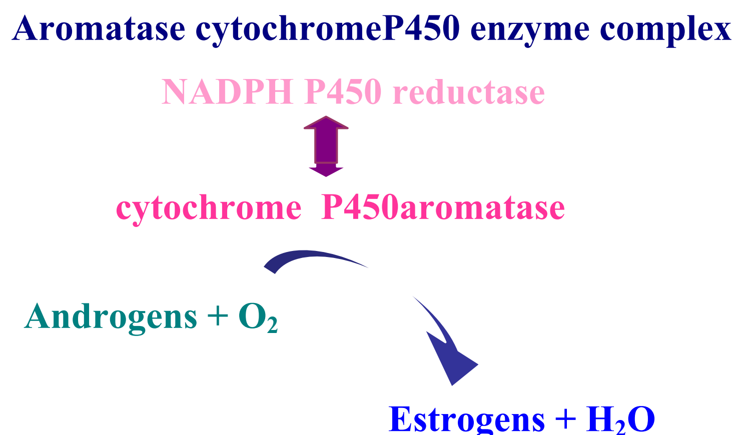

Fig. 1 Biosynthesis of estrogens from androgens by aromatase.

Aromatase is mainly expressed in the ovaries of premenopausal women. A very high level of aromatase is found in placenta in pregnant women. In postmenopausal women and men, adipose tissue and skin cells are the major sources of estrogen production, but the aromatase activity in these tissues is significantly lower than that

in ovaries and the level of circulating estrogen is much lower in postmenopausal women and men than in premenopausal and pregnant women (Chen, 1998).

Interestingly, P450arom is found to be expressed at higher levels in breast cancer tissue than normal breast tissue (Bulun *et al*, 1993; Miller, 1997; Harada, 1997). Aromatase presence in breast cancer tissue as well as in breast cancer cell lines has been shown by enzyme activity measurement, immunocytochemistry and RT-PCR analysis (Sasano *et al*, 1994; Sourdaine *et al*, 1996; Maggiolini *et al*, 2001). Cell culture (Santner *et al*, 1993; Sun *et al*, 1997) and nude mouse experiments (Yue *et al*, 1994) using aromatase-transfected MCF-7 cells have shown that aromatase expressed in breast cancer cells can promote breast cancer growth in both an autocrine and a paracrine manner. In addition, overexpression of aromatase in mammary gland of transgenic mice causes premalignant lesions, such as atypical ductal hyperplasia (Tekmal *et al*, 1996; Gill *et al*, 2001). These results indicate that *in situ* estrogen production, due to overexpressed aromatase in breast cancer cells, plays a more important role than circulating estradiol in breast tumor promotion. Indeed, intratumoral aromatase of breast carcinoma has been extensively studied for its potential clinical significance as a target for endocrine therapy using aromatase inhibitors (Brodie, 1991; Santen *et al*, 1999; Altundag & Ibrahim, 2006).

A complex mechanism is involved in the control of human aromatase expression. The human aromatase gene contains nine translated exons (II-X) and at least ten untranslated exon I's (Fig. 2).

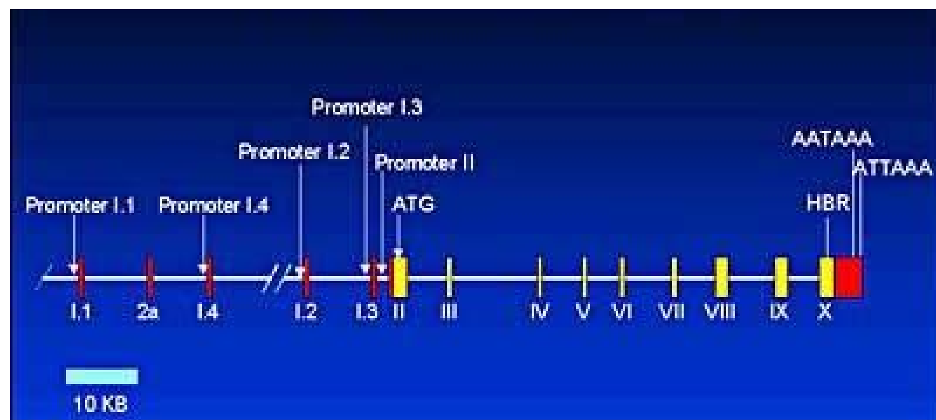

Fig. 2 Scheme of alternative utilization of tissue-specific exon Is and promoters of the human aromatase gene.

The translation start site is positioned in exon II and one of the 5'-untranslated exon I's of aromatase mRNA is spliced onto a common splicing junction of exon II, upstream of the translation start site (Chen *et al*, 1986; Means *et al*, 1991). It has been found that the various untranslated exon I's are present at different levels in different aromatase-expressing tissues and cells, providing tissue-specific controls of aromatase protein expression (Harada *et al*, 1993; Zhou *et al*, 1996; Simpson *et al*, 1997). In particular, it has been revealed that exons I.3 and PII are the major exon I's in aromatase mRNA isolated from breast cancer cells, indicating that promoters I.3 and II are the major promoters driving aromatase expression in breast cancer (Harada *et al*,1993; Zhou *et al*, 1996). Promoter I.4 is the major promoter expressed in breast stromal cells (Harada *et al*, 1993; Zhou D *et al*, 1996). On the other hand, there are only few studies regarding post-transcriptional regulation of aromatase protein. For instance, it has been documented for different members of the P450 enzyme family in vertebrates and insects post-transcriptional modulation of enzymatic activity. In

human P450c17 (CYP17), phosphorylation of serine and threonine residues by a cAMP-dependent protein kinase is essential to acquire 17,20-lyase activity (Zhang *et al*, 1995; Miller *et al*, 1997; Biason-Lauber *et al*, 2000). Bovine P450scc (CYP11A1) has been identified as an active form phosphorylated by a protein kinase C (Defaye *et al*, 1982; Vilgrain *et al*, 1984). Similar activation of P450s through phosphorylation has been found in human liver enzymes such as CYP2E1 and CYP2B1 (Oesch-Bartlomowicz & Oesch, 1990; Oesch-Bartlomowicz *et al*, 1998). The steroid metabolizing enzymes of insects, ecdysone 20-monooxygenase (Hoggard & Rees, 1988) and ecdysone 26-hydroxylase (Williams *et al*, 2000) are also activated by phosphorylation.

Bellino and Holben reported that aromatase activity in placental microsomes was more stable in phosphate buffer than in either Tris–HCl or Hepes buffer. In addition, stability of the enzyme was enhanced by either of the phosphatase inhibitors, tartaric acid and EDTA (Bellino & Holben, 1989). Besides, Balthazart et al. (2001, 2003) have demonstrated that phosphatases modulate, in a significant manner, the activity of brain aromatase and hence bio-availability of estrogens in quail. Notably, they have provided several evidences that the phosphorylation status of the enzyme is critical for its activity, identifying 15 potential consensus phosphorylation sites on aromatase sequence. They have shown in hypothalamic homogenates two different modes of regulation of aromatase activity that result from changes in the concentration of the enzyme or from changes in its conformation and phosphorylation status. The involvement of protein phosphorylation in the regulation of aromatase activity was suggested by other authors in long-term estrogen deprived MCF-7 cells (Yue *et al*, 2003) and in bone-derived cells (Shouzu *et al*, 2001). All these reports suggests that aromatase P450 activity is acutely regulated by phosphorylation–dephosphorylation, the process of which is mediated by a

serine/threonine/tyrosine kinase and the corresponding phosphatase (or serine/threonine/tyrosine phosphatase).

It has been reported that estradiol is able to modulate aromatase expression in other vertebrates (Tsai *et al*, 2001) and a recent report evidenced that estradiol up-regulates aromatase expression by a nongenomic action of ERα via cross-talk with growth factor-mediated pathways in breast cancer cells (Kinoshita & Chen, 2003).

The actions of E_2 are traditionally thought to be mediated by the nuclear estrogen receptor (ER), through the regulation of target gene transcription (Budhram-Mahadeo *et al*, 1998). This occurs when ER either binds estrogen response elements on the promoters of target genes, or acts through protein-protein interactions involving a variety of coactivators, corepressors and the basal transcriptional machinery protein complex. Emerging evidence, however, has implicated a second distinct mechanism of E_2 action, where this steroid binds a putative plasma membrane ER (Pietras R & Szego, 1980) and it elicits rapid non transcriptional effects involving activation of signal transducing pathways, such as protein kinase A, MAPK signaling and phosphotidylinositol 3-kinase/Akt pathway (Fig. 3) (Migliaccio *et al*, 1996; Simoncini *et al*, 2000; Hall *et al*, 2001; Simoncini *et al*, 2003).

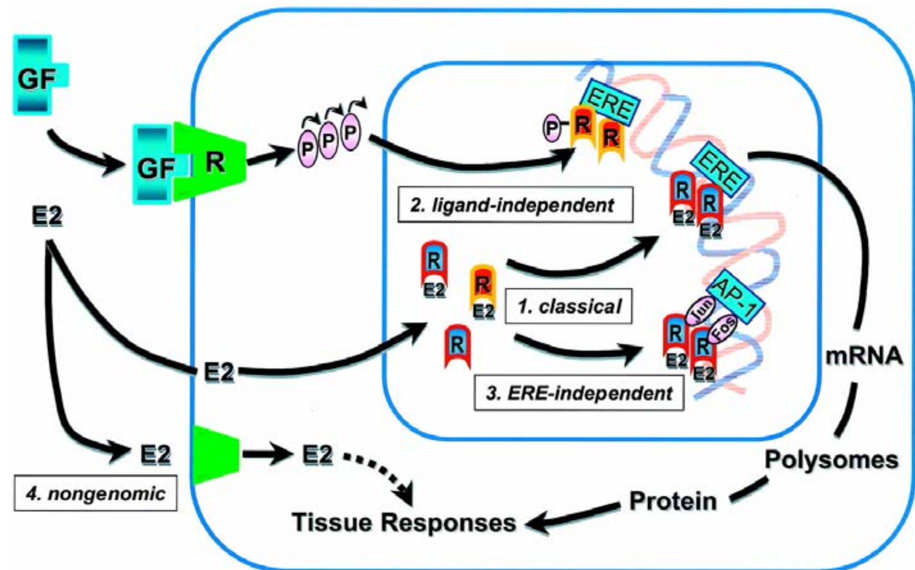

Fig. 3 The multifaceted mechanisms of estradiol and estrogen receptor signaling.

Akt (protein kinase B or PKB) has been identified as a downstream target of growth factor receptor (GF-R) activation, including insulin like growth factor-1 (IGF-1), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) (Datta *et al*, 1999; Downward, 1998). In addition, ERα has been also shown to interact with GF and induce GF-R and extracellular signal-regulated kinase activation, resulting in synergistic tumoral growth stimulation (Kahlert *et al*, 2000; Levin, 2005; Stoll, 2002; Surmacz & Bartucci, 2004).

In the present study, we investigate whether 17-β estradiol (E_2) is able to induce rapid effects on aromatase activity in estrogen dependent MCF-7 breast cancer epithelial cells. Our results, for the first time, demonstrate that E_2/ERα, through a non transcriptional event, can activate PI3K/Akt signaling pathway and then increase aromatase enzymatic activity. Besides, it emerges that aromatase is a target of a cross-talk between ERα and GF-R transductional pathways. Local estrogen production, through a short autocrine loop, sustains aromatase enzymatic activity in breast cancer cells and furthemore gives a great emphasis to the role of aromatase in promoting breast cancer cell growth.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, Tamoxifen, 17-β estradiol, LY294002, IGF-1, EGF, AG 1478 and AG1024 (Sigma, Milan, Italy).

L-Glutamine, penicillin, calf serum (CS), streptomycin, bovine serum albumine (BSA), phosphate-buffered saline (PBS) (Eurobio, Les Ullis Cedex, France).

Triazol Reagent, 100 bpDNA ladder, Ni-NTA Agarose, Lipofectamine 2000 Reagent (Invitrogen life technologies, Carlsbad, California).

FuGENE 6 (Roche, Indianapolis, Indiana).

GoTaq DNA polymerase (Promega, Madison, Wisconsin).

The RETROscript kit (Ambion, Austin, Texas).

Protein A/G-agarose plus (Santa Cruz Biotechnology , Santa Cruz, California) .

ECL System (Amersham Pharmacia, Buckinghamshire, UK).

[1β-^{3}H]androst-4-ene-3,17-dione (PerkinElmer Life Science, Wellesley, MA, USA).

Letrozole (Novartis).

ICI 182,780 (Tocris chemical, Bristol, UK).

Cell Cultures

Wild-type human breast cancer ERα-positive MCF-7 cells were gifts from E. Surmacz (Philadelphia, PA). This cell line was cultured in DMEM/F-12 medium

containing 5% calf serum, 1% L-glutamine and 1mg/ml penicillin-streptomycin. The cells were cultured in Phenol Red-free DMEM containing 0.5% bovine serum albumine and 2 mM L-glutamine for 24 h before each experiment.

His_6-arom plasmid construction

His_6-arom plasmid (Fig. 4) constructed was used to express the C-terminus 6xHis-tagged form of human aromatase. The plasmid pUC19-arom containing the complete coding sequence of human aromatase (CYP19), kindly provided by Dr. E. R. Simpson and Dr. C. D. Clyne (Prince Henry's Institute of Medical Research, Clayton, Australia), was used as template.

The 6xHis epitope tag was inserted by two PCR reactions using the following primers:

forward → 5'-ATATAAGCTTATGGTTTTGGAAATGCTGA-3'

reverse → 5'-ATGATGATGGTGTTCCAGACACCT-3'

5'-ATATTCTAGACTAATGATGATGATGATGATGGTGTTCCAGA-3'.

PCR product was sub-cloned into HindIII/XbaI sites of pcDNA3.1 and Hys_6-arom sequence was confirmed by nucleotide sequence analysis. We proved that the enzymatic activity of polyhistidine-containing recombinant protein was well preserved by measuring aromatase activity in MCF-7 cells transiently transfected with Hys_6-arom vector.

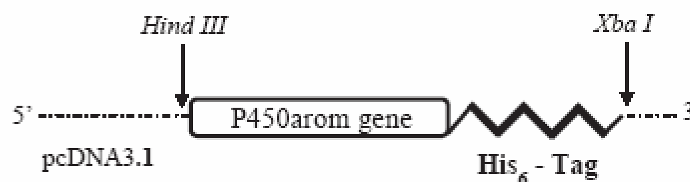

Fig. 4 Schematic map of the His_6-arom construct used in this study

Site-directed mutagenesis

This step was performed with the QuickChange™ Site-Directed Mutagenesis method from Stratagene. Briefly, this was based on a PCR reaction with two complementary oligonucleotide primers containing the mutation. The PCR was performed with the *Pfu* DNA polymerase during 16 cycles (30 s at 95 °C, 30 s at 55 °C and 7 min at 68 °C), using the templates and the specific oligonucleotides summarized in Table 1. The PCR products were then digested with *Dpn*I which only digests the parental methylated cDNA. Nicked vector DNA with the desired mutations was then transformed into *Escherichia coli* XL1-Blue supercompetent cells and the constructed mutated expression vector was confirmed by DNA sequencing.

Table 1

Mutants	Template	Primer	Sequence
YsbdF	CYP19	Forward	*5'-GGAAAACTTCATTtttGAGAGCATGCGGTACCAGCCTGTCG-3'*
		Reverse	*5'-CGACAGGCTGGTACCGCAGCTCTCaaaAATGAAGTTTTCC-3'*
YhemeF	CYP19	Forward	*5'-GGCTGTGCAGGAAAGttcATCGCCATGGTGATG-3'*
		Reverse	*5'-CATCACCATGGCGATgaaCTTTCCTGCACAGCC-3'*
Yheme/sbdF	YhemeF	Forward	*5'-GGAAAACTTCATTtttGAGAGCATGCGGTACCAGCCTGTCG-3'*
		Reverse	*5'-CGACAGGCTGGTACCGCAGCTCTCaaaAATGAAGTTTTCC-3'*

Transient transfection

Transient transfection was performed using the FuGENE 6 reagent as recommended by the manufacturer with the mixture containing 3 µg/well of CYP19 vector or YhemeF, YsbdF or Yheme/sbdF mutants. A set of experiments was performed cotransfecting 3 µg/well of CYP19 and 3 µg/well of the kinase-inactive dominant negative Akt mutant (AktK179M) a gift from Dr. T. Simoncini (University of Pisa, Italy). Another set of experiments was carried out using PTP1B-WT kindly provided by Dr MJ Quon (National Institute of Health, Bethesda, Meryland, USA). Empty vectors were used to ensure that DNA concentration was constant in each

transfection. 24 hours after transfection aromatase activity was determined under different treatments.

To perform immunoblotting assay, MCF-7 cells were plated in 10 cm dishes and then transfected with 10 µg of Hys_6-arom or CYP19 using FuGENE 6 reagent and then exposed to different treatment. For the siRNA studies, Stealth Select RNAi annealed duplexes (25pb double stranded RNA) against human PTP1B was obtained from Invitrogen and used according to the manifacturer's instructions.

Aromatase Activity Assay

The aromatase activity in subconfluent MCF-7 cells culture medium was measured by the tritiated water release assay using 0.5 µM [1β-^{3}H]androst-4-ene-3,17-dione as substrate (Lephart & Simpson, 1991). The incubations were performed at 37°C for 5 h under an air/CO_2 (5%) atmosphere. The results obtained were expressed as fmol/h and normalized to mg of protein (fmol/h/mg protein).

Total RNA Extraction and Reverse Transcription-PCR Assay

Total cellular RNA was extracted from MCF-7 cells using TRIzol reagent as suggested by the manufacturer. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures.

Aromatase mRNA was analyzed by the reverse transcription-PCR method. cDNA was synthesized by oligo(dT) using a RETROscript kit as suggested by the manufacturer. The cDNAs obtained were further amplified by a PCR using the following primers: 5'-CAAGGTTATTTTGATGCATGG-3' (forward, nucleotides 776–796) and 5'-TTCTAAGGTTTGCGCATGA-3' (reverse, nucleotides 1261–1241) for human P450arom and 5'-CTCAACATCTCCCCCTTCTC-3' and 5'-CAAATCCCATATCCTCGT-3' for the internal control gene 36B4. The PCR was performed for 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) for P450arom 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 5 µl and 1 µl of first strand cDNA, respectively, 1 µM each of the primers mentioned above, 0.5 mM dNTP, *Taq* DNA polymerase (2 units/tube) in a final volume of 25 µl. To check for the presence of DNA contamination, a reverse transcription-PCR was performed on 1µg of total RNA without Moloney murine leukemia virus reverse transcriptase (negative control). The PCR products were resolved on 2% agarose gels and bands were visualized by staining with ethidium bromide.

Standard DNA (100-bp DNA ladder) was run to provide the appropriate size marker.

Immunoblotting and Immunoprecipitation Analysis

For western blot analysis MCF7 cells, grown in 10 cm dishes to 70% to 80% confluence, were treated with 100nM of E_2 at different times before lysis in 500 µL of lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, and Na-orthovanadate)]. Equal amounts of

total protein were resolved on 11% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane, probed with rabbit polyclonal antiserum directed against the human placental P450arom (Hauptman-Woodward, Medical Research Institute, Inc, Buffalo, NY) or GAPDH (FL-335, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitation studies were carried out with cellular extracts from MCF-7 cells transiently transfected with empty vector or CPY19 plasmid for 24 hours or transfected with siRNA against PTP1B for 72 hours before lysis. The antibodies used for immunoprecipitation are rabbit polyclonal anti-PTP1B (H-135, Santa Cruz Biotechnology) or anti-Akt 1/2 (H-136, Santa Cruz Biotechnology). 600 µg of protein extracts were incubated at 4°C overnight under slow rotation with 1 µg of corresponding antibody and 20 µl of protein A/G. Immunoprecipitates were collected by centrifugation at 12,000 x g for 10 minutes, followed by washing three times with HNTG (immunoprecipitation) buffer [50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 2 µg/mL pepstatin]. Following the final wash, supernatant was removed. Samples were resuspended in the Laemmli sample buffer, subjected to SDS-polyacrylammide gel electrophoresis (11% gel [w/v]) and then transferred onto a nitrocellulose membrane. The immunoprecipitated proteins were detected by Western blot using a mouse monoclonal antibody against human cytochrome P450arom (Serotec, Oxford, UK), a rabbit polyclonal antibody against PTP1B and Akt 1/2. Microsomal extracts from placenta were used as positive control. Negative control was performed by incubation of MCF-7 lysates with protein A/G agarose and normal rabbit serum. The immunocomplexes were detected with an enhanced chemiluminescence detection kit.

Detection of His$_6$-tagged aromatase protein by Western Blotting

MCF7 cells were transiently transfected with His$_6$-arom vector and exposed to different treatments before lysis. 300µg of cellular proteins were incubated for two hours with Ni-NTA agarose beads a 4°C under slow rotation. Ni-NTA resin was used to isolate P450 aromatase tagged with six tandem histidine residues from cellular lysates. The beads containing bound proteins were washed thrice by centrifugation in PBS buffer added with a mixture of protease inhibitors, then denatured by boiling in Laemmli sample buffer and analyzed by Western blot to identify aromatase protein content and its phosphorylation status. Membranes were probed with a mouse monoclonal antibodies directed against P450 human aromatase or phosphotyrosine-containing proteins (pY99, Santa Cruz Biotechnology) and a rabbit polyclonal antiserum directed against phosphoserine-containing proteins (pSer, Bioreagents, Canada). Two set of controls were done in parallel: surnatant removed after the first centrifugation was added to one control and vector-transfected cell lysates plus Ni-NTA agarose beads was included in the other control. Microsomal extracts from placenta were used as positive control.

Statistical Analysis

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

RESULTS

Short time estradiol exposure increases aromatase activity in MCF-7 cells

We first aimed to evaluate the effects of estrogens on aromatase activity by tritiated water assay in MCF-7 cells incubated for 10, 30 and 120 minutes in the presence of 100nM of E_2. As reported in Fig. 5A, E_2 enhanced significantly enzymatic activity at all times investigated and a maximal stimulatory effect was achieved at 120 minutes. The same induction by E_2 was also observed in MCF-7 cells transiently transfected with the aromatase gene (CYP19 MCF-7), that displayed a 6-fold increase in enzymatic activity (95.36 ± 0.92 fmol/h/mg protein) compared to wild-type MCF-7 cells (15.16 ± 0.47 fmol/h/mg protein) (Fig. 5B).

The aromatase inhibitor letrozole and ER antagonists, ICI 182,780 and Tamoxifen (TAM) at a concentration of 1µM were able to abrogate the maximal enhancement induced by E_2 (Fig. 5C).

This latter result indicates that estrogen can increase aromatase activity by binding to estrogen receptors.

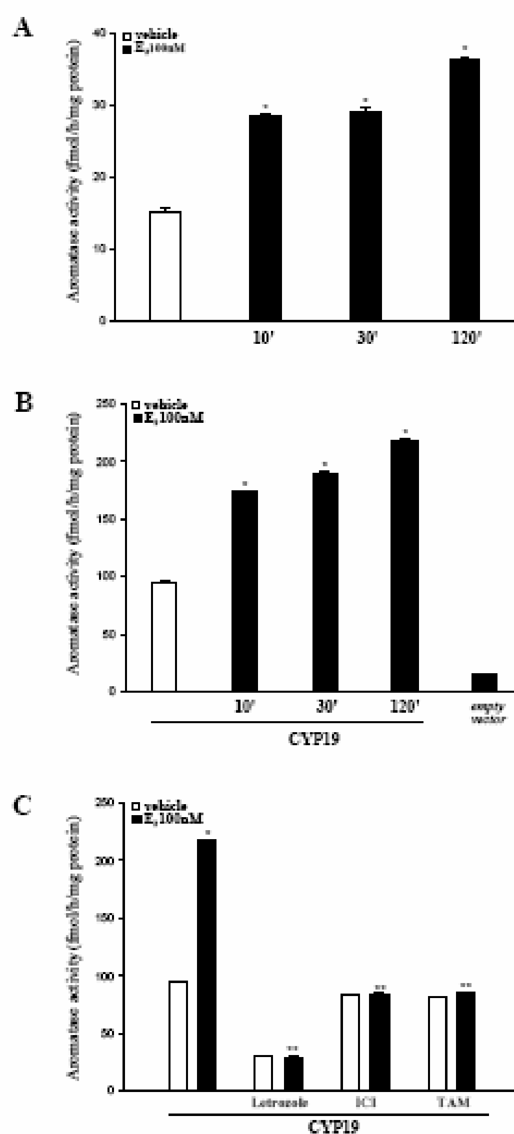

Fig. 5 Effects of E_2 on aromatase activity in cultured MCF-7 cells: Wild type (**A**) or CYP19 MCF-7 cells (**B**) were treated with vehicle or E_2 100nM for 10, 30 and 120 minutes. **C**, CYP19 MCF-7 cells were pretreated with 1µM letrozole, ICI 182,780 and Tamoxifene (TAM) for 30 minutes and then exposed to 100nM of E_2 for 120 minutes. Aromatase activity was evaluated by measuring the tritiated water released from MCF-7 cell cultures after incubation with 0.5 µM [1β-^{3}H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as fmol [^{3}H]/H_2O released and were normalized for mg of protein (fmol/h/mg protein). Empty vector: aromatase activity measured in cells transfected with pUC19 vector. The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. * $p<0.01$ compared to vehicle; ** $p<0.01$ compared to E_2-treated samples.

P450arom mRNA and protein expression in E_2-treated MCF-7 cells

In MCF-7 cell line we investigated the effects of E_2 on P450arom mRNA levels by reverse transcription-PCR. As shown in Fig. 6A, the expected transcript of 465 bp was clearly detected using primers designated to amplify the highly conserved sequence of P450arom, which includes the helical and aromatase regions. The treatment with E_2 100nM for 30 and 120 minutes did not induce any change on P450arom mRNA. mRNA expression of the aromatase gene was normalized by the human housekeeping gene 36B4 (Fig. 6A).

Next, we performed western blotting analysis using a rabbit polyclonal antiserum against human placental P450arom. A 55 kDa specific protein in MCF-7 cells comigrated with microsomal extracts from human placenta used as positive control. The intensity of the band in E_2-treated MCF-7 samples was not modified compared to the control (Fig. 6B).

These results evidence that the changes in E_2-induced aromatase enzymatic activity were not correlated with any variations in the transcription of P450arom mRNA and thus in concentration of the enzyme.

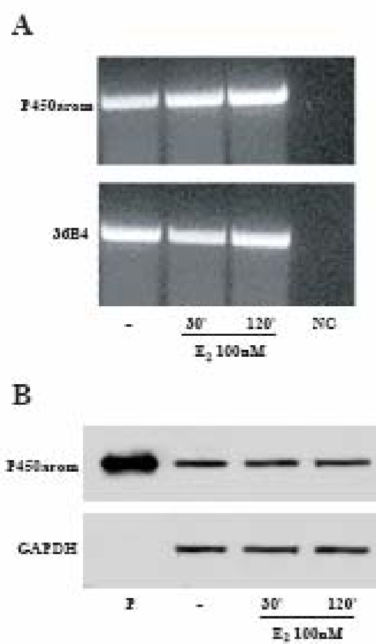

Fig. 6 P450arom mRNA and protein expression in E_2 treated MCF-7 cells: MCF-7 cells were treated with vehicle or E_2 100nM for 30 and 120 minutes. **A**, total RNA was isolated from MCF-7 cells and reverse transcribed. cDNA was subjected to PCR using specific primers for P450arom (35 cycles) or 36B4 (15 cycles). NC: negative control, RNA sample without the addition of reverse transcriptase. **B**, MCF-7 cells were immunoblotted with a rabbit polyclonal antiserum against human placental P450arom. Microsomal extracts from human placenta (P) were used as positive control. GAPDH served as loading control.

E_2 increases tyrosine phosphorylation levels of aromatase protein

It is well known that the activity of many enzymes can be modulated rapidly by phosphorylation processes inducing conformational changes in the enzyme molecule (Albert *et al*, 1984; Daubner *et al*, 1992). Previous analyses of the aromatase gene in a variety of mammalian and avian species had demonstrated several consensus sites of phosphorylation on aromatase cDNA and deduced aminoacid sequence (Harada, 1988; McPhaul *et al*, 1988; Shen *et al*, 1994).

To evaluate carefully the phosphorylation status of aromatase protein, we constructed a plasmid (His_6-arom) containing full length of human aromatase gene bearing six tandem histidine residues on the carboxyl terminus, as described in Materials and Methods (Fig. 7A). Indeed, this His_6-tagged protein had the advantage to allow a higher yield of purified aromatase due to the specificity of Ni-NTA agarose beads and avoid interference with the band of 55kDa from heavy chains of antibodies used for immunoprecipitation. Thus, MCF-7 cells were transiently transfected with His_6-arom and treated with E_2 100nM for 10, 30 and 120 minutes. Equal amounts of proteins were incubated with Ni-NTA agarose beads for isolation of recombinant P450arom. Western blotting with antibodies directed against phospho-tyrosine and phospho-serine residues showed that E_2 was able to increase phospho-tyrosine levels of purified aromatase protein, while no changes were detectable on serine phosphorylation status. P450 human aromatase antibody was used as loading control (Fig. 7B and C).

These data indicate, for the first time, how tyrosine phosphorylation processes may play a key role in the modulation of human aromatase enzymatic activity by E_2.

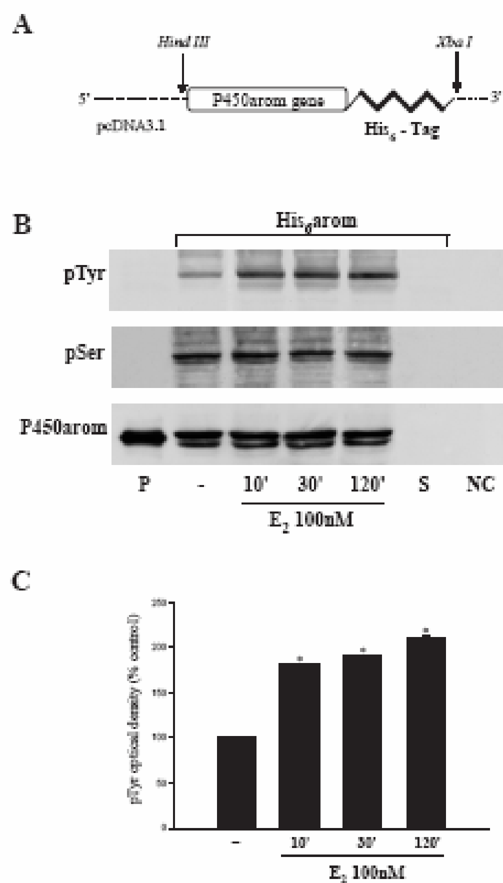

Fig. 7 Enhancement of tyrosine phosphorylation of aromatase protein induced by E_2 : A, Schematic map of the His_6-arom construct used in this study. **B**, MCF-7 cells transiently transfected with His_6-arom were treated with vehicle or $E_2$100nM for 10, 30, 120 minutes. Aromatase was purified using Ni-NTA agarose beads and then the complexes were resolved in SDS-PAGE. Immunoblotting was performed using the anti-phosphotyrosine (pTyr) and anti-phosphoserine (pSer) antibodies. To verify equal loading, the membrane was probed with an antibody against P450 human aromatase. Microsomal extracts from placenta (P) served as positive control. As negative controls we used the surnatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). **C**, The histograms represent the means ± S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.01$ compared to vehicle.

Aromatase activity is enhanced by tyrosine phosphatases inhibitor

To support the idea that aromatase activity may be modulated through changes in protein tyrosine phosphorylation status, we performed enzymatic assay and immunoblot analysis in the presence of a specific inhibitor of tyrosine phosphatases, sodium orthovanadate (Van).

Treatment of MCF-7 cells with Van (10μM) increased basal levels of aromatase activity compared to the control (vehicle) and potentiated the up-regulatory effects induced by E_2 (Fig. 8A). Both events are amplified in MCF-7 cells transiently transfected with CYP19 vector (Fig. 8B).

It is worth to underline that the enzymatic changes induced by Van well fit with a specific enhancement of the tyrosine phosphorylated status in the His_6-tagged purified aromatase protein (Fig. 8C and D).

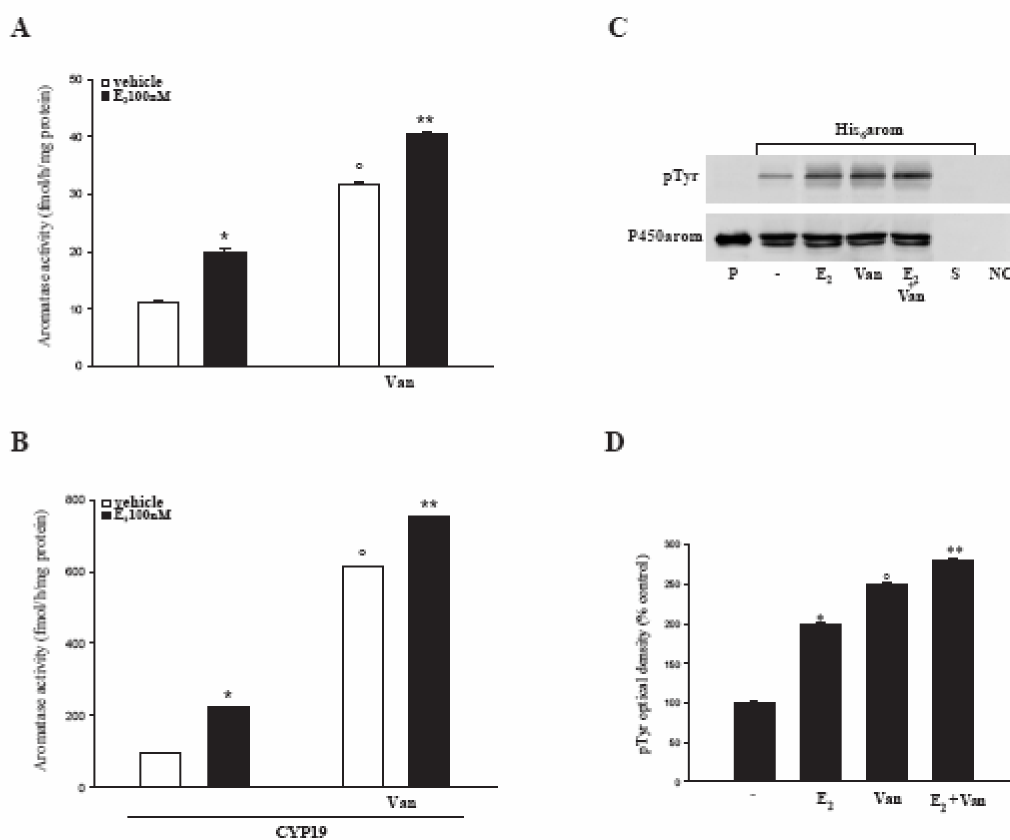

Fig. 8 Tyrosine phosphatase inhibitor, sodium orthovanadate, increases aromatase activity: Wild type (**A**) or CYP19 MCF-7 cells (**B**) were pretreated with 10µM sodium orthovanadate (Van) for 30 minutes and then exposed to E_2 100nM for 120 minutes. Aromatase activity was evaluated by measuring the tritiated water released from MCF-7 cell cultures after incubation with 0.5 µM [1β-^{3}H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as fmol [^{3}H]/H_2O released and were normalized for mg of protein (fmol/h/mg protein). The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. * $p<0.01$ E_2 vs vehicle; ° $p<0.01$ Van vs vehicle; ** $p<0.01$ E_2+Van vs Van. **C**, MCF-7 cells transiently transfected with His_6-arom were treated with vehicle or $E_2$100nM for 120 minutes, with or without Van. Aromatase was purified using Ni-NTA agarose beads and then the complexes were resolved in SDS-PAGE. The membrane was probed with anti-phosphotyrosine (pTyr) antibody. To verify equal loading, the membrane was probed with an antibody against P450 human aromatase. Microsomal extracts from placenta (P) served as positive control. As negative control we used the surnatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). **D**, The histograms represent the means ± S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. * $p<0.01$ E_2 vs vehicle; ° $p<0.01$ Van vs vehicle; ** $p<0.01$ E_2+Van vs Van.

Role of the protein tyrosine phosphatase, PTP1B, in E_2-stimulated aromatase activity

The phosphorylation of proteins on tyrosine residues is modulated by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs), a large family of enzymes that serves as key regulatory components in signal transduction pathways. Since PTP1B is the PTPase highly expressed in several human breast cancer cell lines (Wiener *et al*, 1994; Bjorge *et al*, 2000), we focused our attention on the potential role of this phosphatase in modulating aromatase activity in MCF-7 cells.

Here, by western blot analysis, we demonstrated the expression of PTP1B in MCF-7 cell extracts and the specific association between PTP1B and aromatase at protein-protein level using cell lysates from wild-type and CYP19 MCF-7 cells immunoprecipitated with anti-PTP1B (Fig. 9A).

To directly evaluate the role of PTP1B in E_2-stimulated aromatase activity, we performed tritiated water release assay in MCF-7 cells overexpressing wild type human PTP1B.

PTP1B overexpression reduced basal and E_2-induced levels of aromatase activity by about 50% compared to the control (empty vector), even though the estradiol stimulatory effects still persist (Fig. 9B).

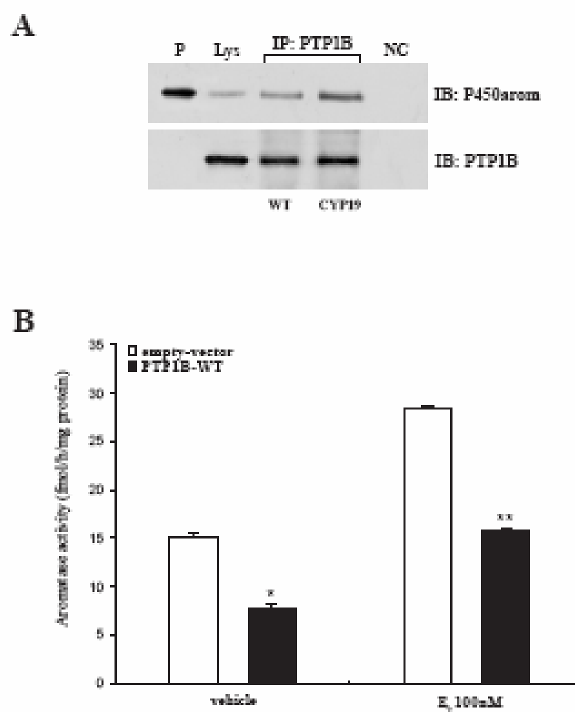

Fig. 9 Physical and functional interaction of P450arom and PTP1B: A, In vitro association of P450arom and PTP1B was revealed by co-immunoprecipitation analysis. Protein extracts (600µg) from MCF-7 cells transiently transfected with empty vector or CYP19 vector were immunoprecipitated with an antiserum against PTP1B (IP: anti-PTP1B) and then the immunocomplexes were resolved in SDS-PAGE. The membrane was blotted with P450arom antibody. To verify equal loading, the membrane was probed with an antibody against PTP1B. Lys: lysates from wild-type MCF-7 cells. NC: negative control was performed by incubation of MCF-7 cell lysates with protein A/G agarose and normal rabbit antiserum. **B,** MCF-7 cells were transiently transfected with 3 µg of empty-vector or PTP1BWT and treated with E_2 100nM. After 120 minutes the cells were assayed for aromatase activity. The values represent the means ± S.E. from triplicate assays. * $p<0.01$ compared to cells transfected with empty vector; ** $p<0.01$ compared to E_2 treated cells transfected with empty vector.

Aromatase activity is up-regulated by E_2 through PI3K/Akt pathway

It has been demonstrated that the tyrosine phosphatase PTP1B is a substrate for Akt and the phosphorylation at Ser^{50} by Akt impairs the ability of the enzyme to engage and dephosphorylate its substrates (Ravichandran *et al*, 2001). On the other hand, it is well known that PI3K/Akt pathway is activated by non genomic estradiol signal in MCF-7 cell line (Castoria *et al*, 2001; Stoica *et al*, 2004). Thus, it is reasonable to assume that the same pathway may be a crucial intermediate of E_2 induction on aromatase activity. To support this assumption, we performed enzymatic assay in MCF-7 cells treated with the PI3-kinase inhibitor LY294002 or transfected with a dominant negative of Akt-expressing construct (Akt K179M). As shown in Fig. 10A and B, our results revealed that either LY294002 (10μM) or Akt K179M were able to abrogate the E_2 stimulatory effects in wild-type as well as in CYP19 MCF-7 cells. In agreement with these results, LY294002 reduced the E_2-associated tyrosine phosphorylation level of the protein aromatase purified from MCF-7 cells transfected with Hys_6-arom construct, as revealed by western blot analysis (Fig. 10C and D).

These data lead us to hypothesize that E_2-activation of aromatase is a phosphorylation dependent event mediated by PI3K/Akt pathway.

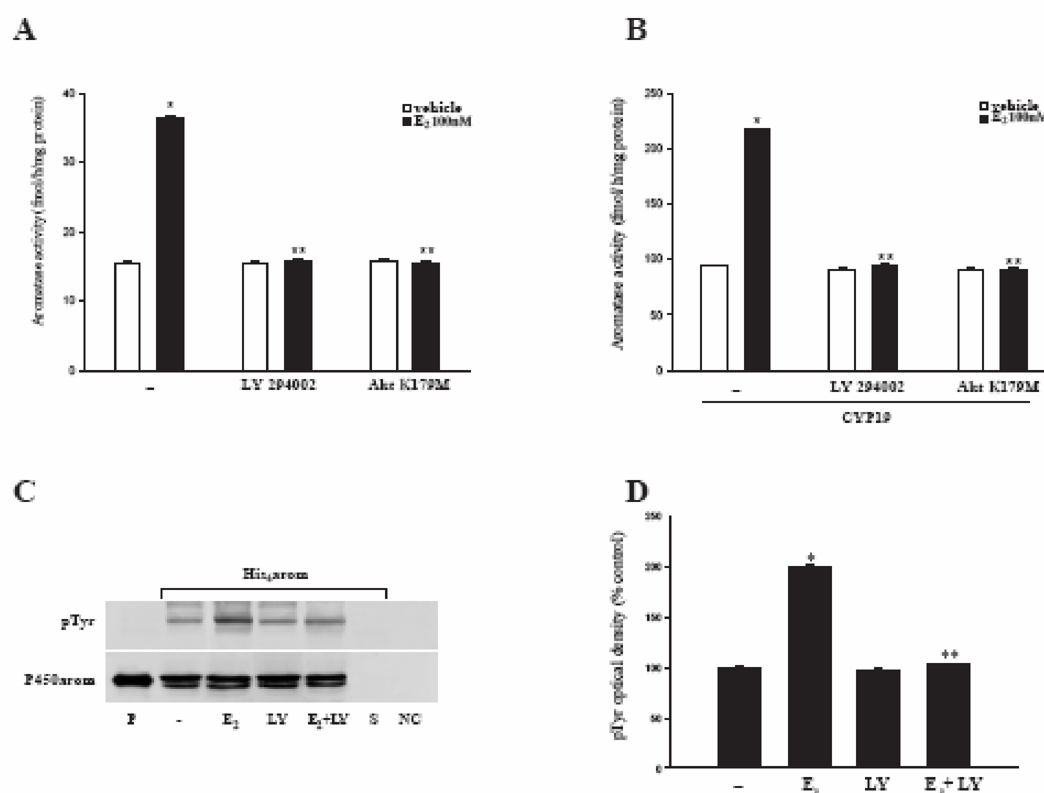

Fig. 10 PI3K/Akt pathway mediates E_2–induction on aromatase activity: Wild-type (**A**) or CYP19 MCF-7 cells (**B**) were transiently transfected with empty vector or AktK179M, or pretreated with LY294002 (10μM) 30 minutes before E_2 stimulation. After 120 minutes aromatase activity was performed. The values represent the means ± S.E. from triplicate assays. *p<0.01compared to vehicle; **p<0.01 compared to E_2 treated samples. **C,** MCF-7 cells transiently transfected with His_6-arom were treated with vehicle or E_2 100nM, LY294002 (10μM) or E_2 + LY. Aromatase recombinant protein was purified and then resolved in SDS-PAGE. The membrane was probed with anti-phosphotyrosine (pTyr) antibody. To verify equal loading, the membrane was probed with an antibody against P450 human aromatase. Microsomal extracts from placenta (P) served as positive control. As negative controls we used the surnatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). **D**, The histograms represent the means ± S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *p<0.01compared to vehicle; **p<0.01 compared to E_2 treated samples.

A protein complex involving Akt, P450arom and PTP1B activates aromatase

After previously demonstrating the physical association of PTP1B with P450arom (Fig. 9A), we identified a protein complex involving Akt, P450arom and PTP1B by immunoprecipitation of Akt and detection of coimmunoprecipitated PTP1B and P450arom under basal conditions in MCF-7 cell lysates (Fig. 11A). We confirmed the formation of the protein complex by using a reciprocal order of immunoprecipitation of PTP1B and then detection of both P450arom and Akt on Western blot (data not shown).

To test whether PTP1B is required for the physical interaction between P450arom and Akt, we transfected MCF-7 cells with a specific siRNA against human PTP1B and 72 hours after transfection we documented a marked decrease of PTP1B expression compared with levels of control proteins. Our results, using cell lysates immunoprecipitated with anti-Akt, revealed that down-regulation of PTP1B blocked P450arom/Akt interaction (Fig. 11A), suggesting that this tyrosine phosphatase may play an important role in MCF-7 breast cancer cells by linking the serine/threonine kinase Akt to P450 aromatase.

Moreover, to corroborate the involvement of PI3K/Akt pathway through PTP1B in E_2-activation of aromatase, we performed tritiated water release assay in MCF-7 cells cotransfected with PTP1B-WT and Akt K179M. The presence of the kinase-inactive dominant negative Akt mutant completely reversed the E_2-induction on enzymatic activity (Fig. 11B).

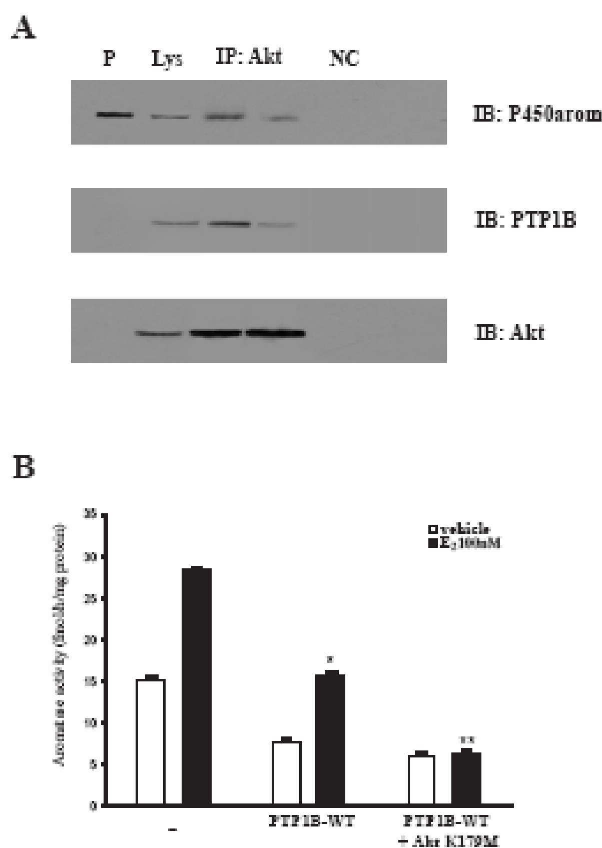

Fig. 11 Physical and functional interaction of P450arom, PTP1B and Akt: A, In vitro association of P450arom, PTP1B and Akt was revealed by co-immunoprecipitation analysis. Protein extracts (600µg) from MCF-7 cells untransfected or transfected with a specific siRNA against human PTP1B for 72 hours were immunoprecipitated with an antiserum against Akt (IP: anti-Akt) and then the immunocomplexes were resolved in SDS-PAGE. The membrane was blotted with P450arom and PTP1B antibody. To verify equal loading, the membrane was probed with an antibody against Akt. Lys: lysates from wild-type MCF-7 cells. NC: negative control was performed by incubation of MCF-7 cell lysates with protein A/G agarose and normal rabbit antiserum. **B,** Another set of experiments was carried out cotransfecting MCF-7 cells with PTP1BWT and AktK179M. 24h after transfection MCF-7 cells were treated with E_2 100nM for 120 minutes and then assayed for aromatase activity. The values represent the means ± S.E. from triplicate assays. *p<0.01compared to vehicle; **p<0.01 compared to E_2 treated samples.

In summary, the data obtained suggest that the rapid E_2 up-regulation of aromatase activity in MCF-7 cells occurs through the activation of PI3K/Akt pathway leading to a block of PTP1B and an enhanced tyrosine phosphorylation status of aromatase protein (Fig. 12)

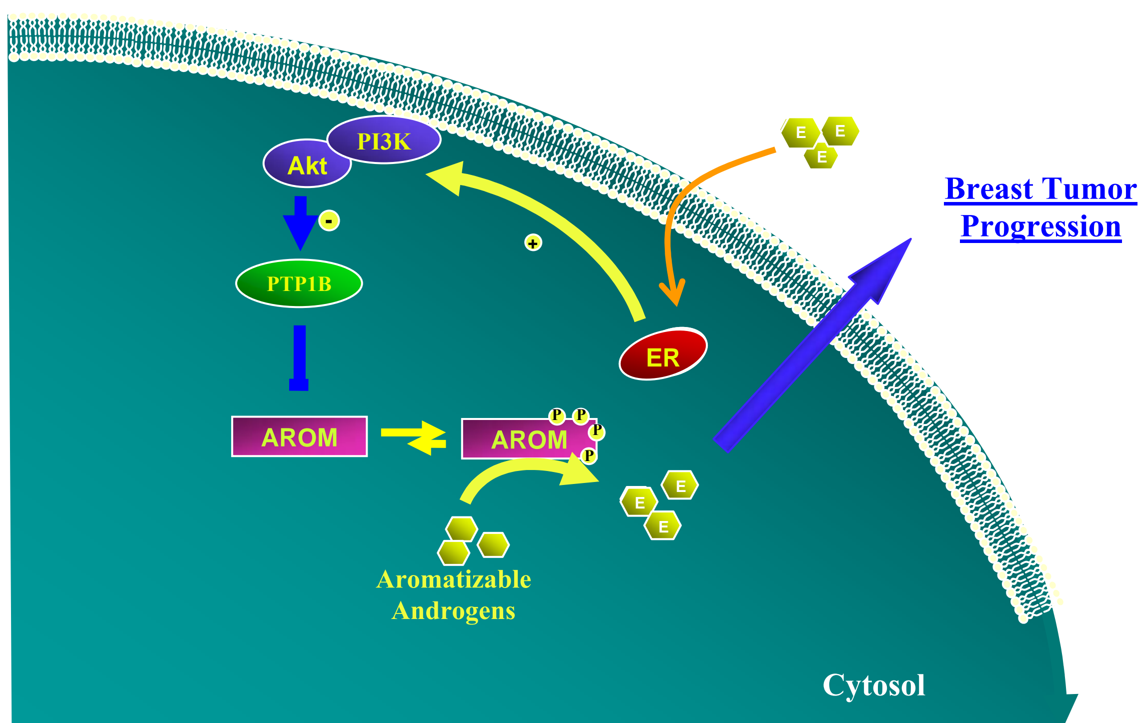

Fig. 12 Hypothesized model of estradiol signalling in modulating aromatase activity in breast cancer

Further investigations are necessary to asceirtain whether PTP1B/Arom complex may be the effector of other mitogenic-stimulated signaling, that contributes to the regulation of the intrinsic aromatase enzymatic activity in breast cancer cells.

Role of tyrosine residues in the E2-activation of aromatase

Site-directed mutagenesis experiments were performed to explore the role of the residues specifically involved in the E_2 induction. Consensus phosphorylation sites were analysed using the public domain software (NETPHOS 2.0 PREDICTION server) available on the web site of the Center for Biological Sequence Analysis at http://www.cbs.dtu.dk. Based on a deduced amino acid sequence (in the present case, the human aromatase) and on a previously encoded database of potential phosphorylation sites (PHOSPHOBASE 2.0), this program identified all serine, threonine and tyrosine residues in the protein that could potentially be phosphorylated (Kemp & Pearson, 1990; Kennelly & Krebs, 1991). The program also provided for each residue a phosphorylation score ranging from 0 to 1.0 whose value was proportional to the probability that the residue could in fact be phosphorylated *in vivo*. A score equal or larger to 0.5 was considered to predict a likely phosphorylation consensus site (see Blom *et al*, 1999 for more detail). The NETPHOS 2.0 program (http://www.cbs.dtu.dk/services/NetPhos/) identified four of the 17 tyrosine residues. These sites correspond to the Tyrosine residues located at positions 77, 184, 361 and 441 of the human aromatase sequence. Notably, the positions at 361 and 441 correspond to the two residues present in important functional domains of human aromatase (Fig. 13).

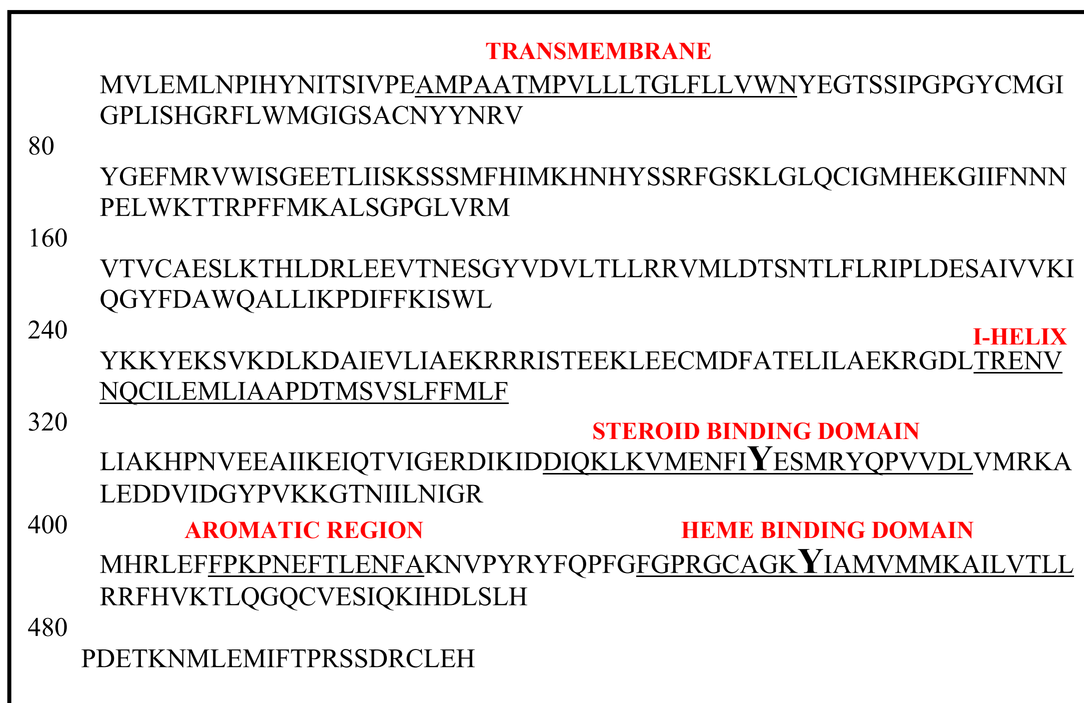

Fig. 13 Amino acid sequence of human aromatase: in red are indicated the different functional domains of the enzyme and in bold case Y the potential phosphorylation consensus site.

Therefore, we created three different constructs in which the tyrosine residues (Y) were mutated to phenylalanine (F):

1. YsbdF plasmid was mutated in Y361 in steroid-binding domain;
2. YhemeF plasmid was mutated in Y446 in heme-binding domain;
3. Yheme/sbdF plasmid was mutated in Y361 and Y446.

As shown in Fig. 14, E_2 as well as Van were not able to up-regulate aromatase activity in MCF-7 cells transfected with these mutant expression vector. These results further support that tyrosine phosphorylation may represent the molecular mechanism that rapidly changes aromatase activity following estradiol-treatment.

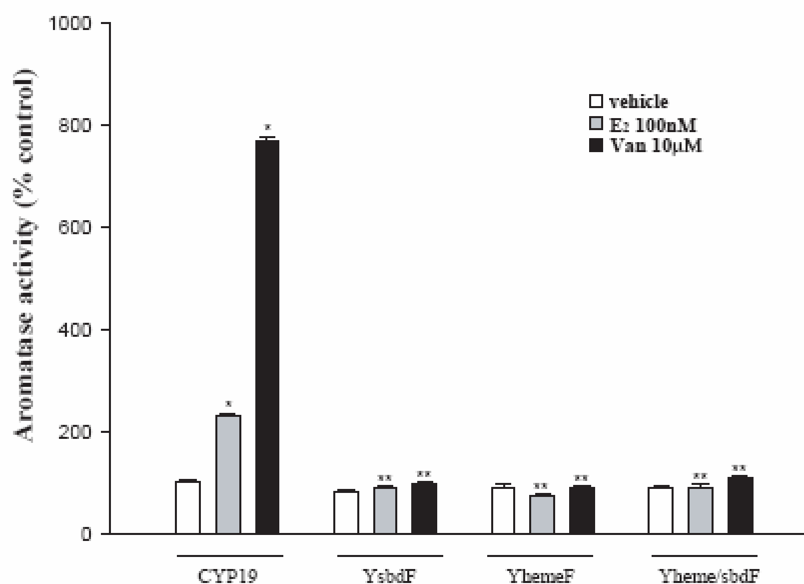

Fig. 14 Tyrosine residues involvement in aromatase activation: MCF-7 cells were transfected with CYP19 vector or YsbdF or YhemeF or Yheme/sbdF mutants and then treated with 100nM E_2 or 10µM Van for 120 minutes. Aromatase activity was evaluated by measuring the tritiated water released from MCF-7 cell cultures after incubation with 0.5 µM [1β-^{3}H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as percentages of the control, which was assumed to the 100%. The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. * $p<0.01$ compared with vehicle; ** $p<0.01$ compared with E_2 or Van treated CYP19 cells.

A cross-talk between GF receptors and ER-α is invoved in the increased aromatase activity in MCF-7 cells

PI3K/Akt pathway mediates the action of several growth factors (Toker & Cantley, 1997). In addition, it has been reported, in breast cancer, a cross-talk between endogenous membrane ER and growth-factor-signaling pathways, including EGF and IGF-I (Kahlert *et al*, 2000; Levin, 2005; Stoll, 2002; Surmacz & Bartucci, 2004).

In order to determine whether IGF-1R or EGF-R stimulation leads to an increased production of estrogen, both wild type and CYP19 MCF-7 cells were treated with IGF-1 and AG1024, a monoclonal antibody specific to IGF-1R or EGF and a selective inhibitor of the EGF-R kinase, AG1478. Our results demonstrated that IGF-1 or EGF 100ng/ml for 120 minutes significantly increased aromatase enzymatic activity reproducing the same up-regulatory effects induced by E_2 (Fig. 15A, B and C, D, respectively). Pretreatment with AG1024 or AG1478 10 μM for 30 minutes completely abrogated the growth-factor as well as the E_2 induction of aromatase activity (Fig. 15A, B and C, D). It is worth to evidence how in the presence of short exposure to ICI 182,780 or LY294002 , IGF-1 and EGF are not able to up-regulate aromatase activity. The latter result addresses how the induction of aromatase enzymatic activity may involve the cross-talk between estradiol/ERα and GF-R signaling, both converging on the activation of the downstream PI3K/Akt pathway.

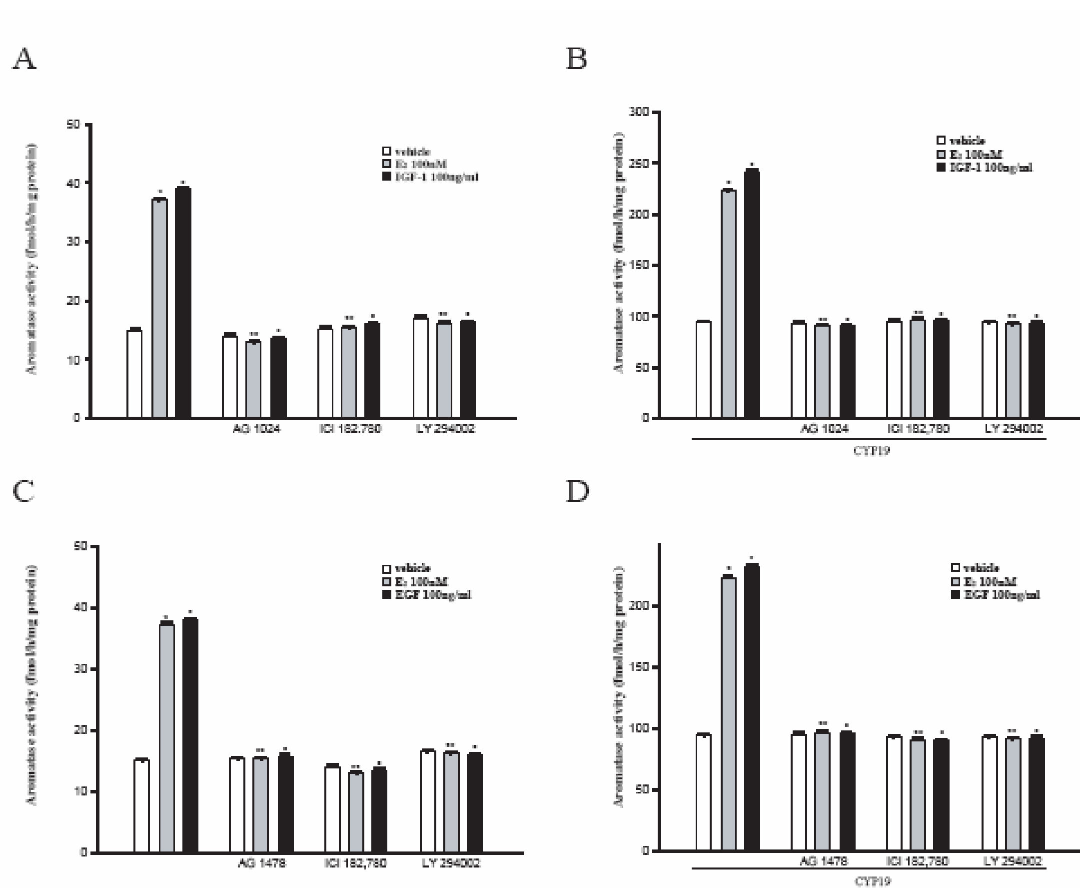

Fig. 15 Induction of aromatase activity by IGF-1 and EGF: Wild-type (**A, C**) or CYP19 MCF-7 (**B, D**) were pretreated with AG1024 or AG1478 or LY294002 10µM or ICI 182,780 1 µM for 30 minutes and then exposed to E2 100nM or IGF-1 or EGF 100 ng/ml. After 120 minutes aromatase activity was performed.

The values represent the means ± S.E. from triplicate assays. *p<0.01 compared to vehicle; **p<0.01 compared to E_2 treated samples; °p<0.01 compared to IGF-1/EGF treated samples.

DISCUSSION

Locally estrogen production plays a more important role than circulating estradiol in breast tumor promotion (Miller, 2006). Several experimental systems have been used to determine the biological significance of *in situ* estrogen production by breast tissue. For instance, in aromatase transgenic mice an increased risk of developing neoplasia and a major susceptibility to environmental carcinogens have been observed (Tekmal *et al*, 1996; Gill *et al*, 2001). On the other hand, aromatase inhibitors appears to enhance the survival outcome in breast cancer patients (Altundag & Ibrahim, 2006).

The present study shows, for the first time, that E_2 is able to modulate aromatase activity by PI3 kinase/Akt signaling cascade in estrogen dependent breast cancer epithelial cells. Of note, our results demonstrate that short exposure to E_2 induces an increase on aromatase enzymatic activity of the same extent in wild-type as well as in CYP19 MCF-7 cells. This up-regulatory effect was specifically mediated by the interaction of E_2 with ERs, since it was abrogated in the presence of ER antagonists, such as Tamoxifen and ICI 182,780.

Estrogen signaling has traditionally been identified with the transcriptional control of target genes via the binding of nuclear estrogen receptors to genomic consensus sequences (Green *et al*, 1990). Nonetheless, in the past few years, a large spectrum of biological actions of estrogen has been shown as too rapid to be compatible with transcriptional mechanisms (Hall *et al*, 2001). These “non classical” estrogen effects are attributed to the cell surface ERs which may activate several intracellular signal transduction pathways such as PI3 kinase/Akt, as reported in human vascular endothelial cells, human breast cancer cell lines (MCF-7) and rat

primary cortical neurons (Honda *et al*, 2000; Castoria *et al*, 2001; Simoncini *et al*, 2003). For instance, the most striking example is the direct association between the ER and PI3K in cells stimulated by estrogen (Simoncini *et al*, 2000).

Our findings demonstrate a direct involvement of PI3K/Akt activation in E_2-stimulated aromatase activity by the evidence that the PI3K/Akt inhibitor LY 294002 or a dominant negative of Akt completely reversed the increase of aromatase activity.

It is well known that PI3K/Akt pathway could be activated by several growth factor receptors (Toker & Cantley, 1997) and that ER and growth factor signaling pathways intersect and directly interact at many levels of signal transduction (Kahlert *et al*, 2000; Levin, 2005; Stoll, 2002; Surmacz & Bartucci, 2004). Our cell culture study shows that the treatment of ER positive MCF-7 cells with IGF-1 or EGF increased, at short time, aromatase activity. It is worth to evidence how the two GF-R tyrosine kinase inhibitor or the pure antiestrogen ICI 182,780 or LY 294002 were able to abrogate the GF as well as the E_2 induction of aromatase activity. These results suggest that the upregulation of aromatase activity in MCF-7 cells by E_2 may involve a cross-talk between ERα and the GF/ PI3K/Akt pathway.

The E_2-enhanced aromatase activity was not correlated with any increase in the level of aromatase mRNA and protein content, suggesting a post-transcriptional modulation of aromatase protein.

Post-translational modification of enzymatic activity has been demonstrated for different members of the P450 enzyme family in vertebrates. For instance, cAMP-dependent protein kinase was essential for the activation of human and rat cholesterol 7α-hydroxylase (CYP7A) (Nguyen *et al*, 1996) as well as for phosphorylation of serine and threonine residues in human P450c17 (CYP17) (Zhang

et al, 1995; Miller *et al*, 1997; Biason-Lauber *et al*, 2000). Bovine P450scc (CYP11A1) has been identified as an active form phosphorylated by a protein kinase C (Defaye *et al*, 1982; Vilgrain *et al*, 1984) and similar activation of P450s through phosphorylation has been found in human liver enzymes such as CYP2E1 and CYP2B1 (Oesch-Bartlomowicz *et al*, 1990; Oesch-Bartlomowicz *et al*, 1998). Besides, several studies uncovered evidences of a rapid regulation of P450 aromatase via processes such as protein phosphorylation in the brain (Balthazart *et al*, 2001; Balthazart *et al*, 2003). A rapid increase of aromatase activity was reported in human ejaculated spermatozoa through the activation of cAMP/PKA signaling pathway (Aquila *et al*, 2002).

In the current study, we demonstrate, in MCF-7 cells, that E_2 up-regulatory effects on aromatase activity involve rapid changes in phosphorylation status of this protein. Indeed, we observed after E_2 treatment a specific enhancement of tyrosine phosphorylation levels in recombinant aromatase protein purified by Ni-NTA agarose beads. Additional support to the idea that tyrosine phosphorylation processes may affect the enzymatic activity of aromatase protein was provided by experiments utilizing inhibitor of tyrosine phosphatases. Sodium orthovanadate increased basal and E_2 induced enzymatic activity as well as tyrosine phosphorylation of aromatase purified protein. In addition, site-directed mutagenesis experiments evidenced that phosphorylation of tyrosine residues present in important functional domains of human aromatase were involved in the up-regulation of enzymatic activity. Taken together, these data demonstrate that the rapid changes in aromatase activity result from a direct phosphorylation of the enzymatic protein itself.

Tyrosine phosphorylation is a reversible and dynamic process controlled by the activities of the protein tyrosine kinases (PTKs) and the competing actions of the

protein tyrosine phosphatases (PTPs). In this respect, our findings documented a protein-protein interaction between the protein tyrosine phosphatases, PTP1B, and aromatase. PTP1B, the primary phosphatase in human breast cancer cell lines, is an endoplasmic reticulum-associated protein tyrosine phosphatase (Wiener *et al*, 1994; Bjorge *et al*, 2000), implicated in the regulation of a variety of cellular processes such as cell growth, differentation and transformation (Dube & Tremblay, 2005). To better define the role of this phosphatase in modulating aromatase activity, we performed enzymatic assay in MCF-7 cells overexpressing PTP1B. In this condition, we observed a reduction of aromatase activity both in untreated as well as E_2-treated MCF-7 cells. It is worth to note that E_2-induction of enzymatic activity was completely reversed in MCF-7 cells cotransfected with PTP1B and the dominant negative of Akt. These data further support the involvement of PI3K/Akt pathway in E_2-enhanced aromatase activity through the regulation of PTP1B activity. Our findings well fit with previous results demonstrating that PTP1B is a substrate of the ser/thr kinase Akt (Ravichandran *et al*, 2001). Indeed, PTP1B contains the consensus phosphorylation motif ($RYRDVS^{50}$) in a region that forms important stabilizing contacts with aminoacids immediately upstream from the phosphotyrosine residue of PTP1B substrates. Thus, phosphorylation of Ser^{50} in this region by Akt negatevely modulates its tyrosine phosphatase activity. In the current work, we identified by immunoprecipitation studies a ternary protein complex involving Akt, P450arom and PTP1B. Using a specific siRNA against human PTP1B, we revealed that down-regulation of this phosphatase blocked P450arom/Akt interaction, suggesting that PTP1B may play an important role in MCF-7 breast cancer cells by linking the serine/threonine kinase Akt to P450 aromatase.

In summary, this study supports data suggesting that the rapid up-regulatory effects of E_2 on aromatase activity in MCF-7 cells involve phosphorylation of the aromatase protein. This occurs through the activation of PI3K/Akt pathway leading to a block of PTP1B and an enhanced tyrosine phosphorylation status of aromatase protein.

From the present findings, it emerges, for the first time, that aromatase is a target of a cross-talk between ERα and GF-R transductional pathways. Local estrogen production, through a short autocrine loop, further sustains aromatase enzymatic activity in breast cancer cells (Fig. 15). These data demonstrate how aromatase may be functionalized by different signaling involved in tumor cell growth and progression and provide rational to design novel combinatory therapeutic strategies for breast cancer treatment.

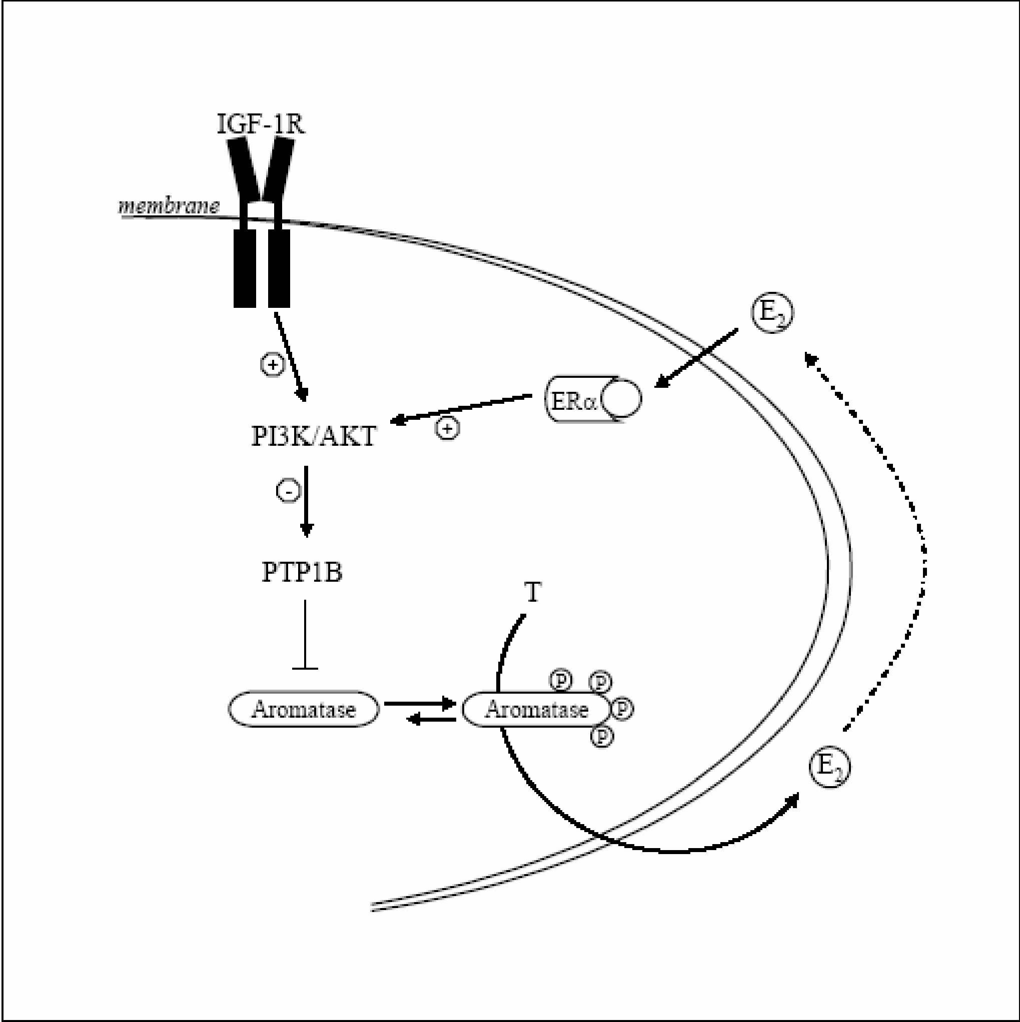

Fig. 16 Cross-talk between ERα and IGF-1R leads to downstream signaling and changes in aromatase activity in MCF-7 breast cancer cells.

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Evidences that leptin upregulates E-cadherin expression in breast cancer: effects on tumor growth and progression

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Running title: Leptin upregulates E-cadherin expression in breast cancer

Keywords: Leptin, E-cadherin, Breast cancer, Xenograft tumor, Three-dimensional cultures.

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Abstract

Leptin, a cytokine mainly produced by adipocytes, appears 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 days 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 appears to be dependent on E-cadherin, since 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 3D cultures as well as in xenografts. In MCF-7 cells both hormones were able to activate E-cadherin promoter. Mutagenesis studies, EMSA and ChIP assays revealed that CREB and Sp1 motifs, present on E-cadherin promoter, were important for the upregulatory effects induced by both hormones on E-cadherin expression in breast cancer MCF-7 cells. In conclusion, the present study demonstrates 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.

Introduction

Leptin is an adipocyte-derived hormone (1) which, 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, haematopoiesis, immune responses, cell differentiation and proliferation (4,5). The activities of leptin are mediated through the transmembrane leptin receptor (ObR) (6,7) by the activation of JAK/STAT (Janus-activated kinase/signal transducers and activators of transcription) and MAPK (mitogen-activated protein kinase) pathways (8,9).

Epidemiological studies demonstrate a positive association between obesity and an increased risk of developing cancer such as pancreatic, colon, prostate, ovarian, endometrial, lung, adrenal and breast cancer (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 might be expected, E-cadherin appears to have a major influence on primary cancer development and evolution. Alteration in the function of E-cadherin and the cadherin-catenin complex have 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 the 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 demonstrated 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 plasmid containing the human E-cadherin promoter was given by Dr. Y. S. Chang (Chang-Gung University, Republic of China). pHEGO plasmid, containing the full length of 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 base pairs located near the C terminus ($STAT^{-}$) was given by Dr. J. Turkson (University of South Florida, College of Medicine, Tampa). pCMV5myc vector containing the cDNA encoding dominant negative ERK2 K52R ($ERK2^{-}$) was provided by Dr. M. Cobb (Southwestern Medical Center, Dallas).

Cell Lines and Culture Conditions

MCF-7, HeLa and BT-20 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 containing 5% CS, and BT-20 cells were cultured in MEM supplemented with 10% FBS, 1% Eagle's non-essential amino acids and 1% sodium pyruvate (Sigma). Cells were cultured in phenol-red-free DMEM (SFM), containing 0.5% bovine serum albumin, 24h before each experiment. All media were supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma).

In vivo studies

The experiments *in vivo* were performed in 45 days old female nude mice (*nu/nu Swiss*; Charles River). At day 0, the animals were fully anaesthetized, by intramuscular injection of 1.0 mg/kg Zoletil (Virbac) and 0.12% Xylor (Xylazine), to allow the subcutaneous implantation of estradiol pellets (1.7 mg/pellet 60 days release, Innovative Research of America) into the intrascapular region of mice. The day after, exponentially growing MCF-7 cells (5.0×10^{6} cells/mouse) were inoculated

subcutaneously in 0.1ml of matrigel (BD biosciences). Leptin treatment was started 24h later, when animals were injected intraperitoneally with either solutions: -recombinant human leptin (230µg/kg) diluted in saline + 0.3% bovine serum albumin (BSA) or -saline + 0.3% BSA only, control. The treatment was performed for five days a week until the 13th. 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 formula: V = L x (W2)/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 grown to 70-80% confluence, trypsinized, and plated in single-cell suspension in 2%-agar-coated plates. Cells were untreated or treated with 1000 ng/ml leptin and/or 100 nM E_2 for 48h. To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; Chemicon International) or EGTA to a final concentration of 4 mM. To generate three-dimensional spheroids, the plates were rotated for 4h at 37°C. The three-dimensional cultures were photographed using a phase-contrast microscope (Olympus). 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 48h of treatments.

E-cadherin adhesion assay

MCF-7 cells were pretreated with leptin (1000 ng/ml) and/or E_2 (100 nM) for 48h and then plated on 6-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 3h at 37°C and then washed once with PBS. To test the effect of E-cadherin on cell binding, cells were incubated with E-cadherin-blocking antibody (1:100 dilution) or EGTA (4 mM) before adding to the wells.

After washing out nonadherent cells, adherent cells were incubated 3h in medium containing 500 μg/ml MTT 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). Reverse transcription was done using RETROscript kit (Ambion). The cDNAs obtained 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 performed for 30 cycles for cyclin D1 (94°C 1 min, 60°C 1 min, 72°C 2 min) and E-cadherin (94°C 1 min, 55°C 1 min, 72°C 2 min) and 15 cycles (94°C 1 min, 59°C 1 min, 72°C 2 min) to amplify 36B4, in the presence of 1 μl of first strand cDNA, 1 μM each of the primers mentioned above, dNTP (0.5 mM), Taq DNA polymerase (2 units/tube) (Promega) in final volume of 25 μl.

Western blot analysis

Protein lysates were obtained with a buffer containing 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 sodium orthovanadate). Equal amounts of total protein were resolved on an 8-10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, 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 ECL system (Amersham).

Site-directed mutagenesis

The E-cadherin promoter plasmid bearing CREB mutated site (CREB mut) was created by site-directed mutagenesis using Quick Change kit (Stratagene). We used as template human E-cadherin promoter and the mutagenic primers were the following: 5'-AGGGTGGATCACCTGAtacCAGGAGTTCCAGACCAGC-3' and 5'-GCTGGTCTGGAACTCCTGgtaTCAGGTGATCCACCCT-3'. The constructed reporter vector was confirmed by DNA sequencing.

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 or CREB mut construct. HeLa cells were transfected with E-cadherin promoter (0.5 µg/well) in the presence or absence of HEGO (0.2 µg/well). Another set of experiments was carried out cotransfecting HeLa cells with E-cadherin promoter construct and STAT3 or ERK2 dominant negative (0.5 µg/well). Twenty-four hours after transfection, the medium was changed and the cells were treated with 1000 ng/ml leptin and/or 100 nM E_2 for 48h. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK *Renilla* luciferase plasmid (5 ng/well) was used to normalize the efficiency of the transfection. Firefly and *Renilla* luciferase activities were measured by 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

Nuclear extracts were prepared from MCF-7 as previously described (24). Briefly, MCF-7 cells plated into 100-mm dishes were scraped into 1.5 ml of cold PBS. Cells were pelleted and resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 mM leupeptin). The cells were allowed to swell on ice for 10 minutes, samples were then centrifuged for 10 seconds 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 DTT, 0.2 mM PMSF, 1 mM leupeptin) and incubated on ice for 20 minutes for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at –70°C. The probe was generated by annealing single stranded oligonucleotides 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 competitors are the following: CRE: 5'-TGGATCACCTGAGGTCAGGAGTTCCAGACC-3',

Sp1: 5'-ATCAGCGGTACGGGGGGCGGTGCTCCGGGG-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 μl of buffer (20 mM HEPES pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glycerol, 1 mg/ml BSA, 50 μg/ml poli dI/dC) with 50000 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 minutes in the presence or absence of unlabeled competitor oligonucleotides. The specificity of the binding was tested by adding to the mixture reactions specific antibodies (anti-CREB and anti-Sp1). Mithramycin A 100 μM (ICN Biomedicals Inc.) was incubated with the labelled probe for 30 minutes a 4°C before the

addition of nuclear extracts. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 X Tris borate-EDTA for 3h at 150 V.

Chromatin immunoprecipitation assay

We followed ChIP methodology described by Shang et al. (25) with minor modifications. MCF-7 cells were grown in 150-mm plates and untreated or treated with 1000 ng/ml leptin and/or 100 nM E_2 for 1h. Thereafter, cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 minutes. 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-Cl pH 8.1) and left on ice for 10 minutes. Then, cells were sonicated four times for 10 seconds at 30% of maximal power (Fisher Sonic Dismembrator) and collected by centrifugation at 4°C for 10 minutes at 14,000 rpm. 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-Cl pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 µl of sonicated salmon sperm DNA/protein A agarose (UBI) for 1h at 4°C. The precleared chromatin was immunoprecipitated with anti-CREB or anti-Sp1 antibodies (Santa Cruz). Thereafter, 60 µl of salmon sperm DNA/protein A agarose were added and precipitation continued for 2h at 4°C. Pellets were washed sequentially for 5 minutes with the following buffers: Wash A (0.1% SDS, 1% Trition X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Trition X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl pH 8.1), and twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with Elution buffer (1% SDS, 0.1 M $NaHCO_3$). The eluates were reverse crosslinked by heating at 65°C for 12h and digested with proteinase K 0.5 mg/ml at 45°C for 1h. DNA was obtained by phenol/chloroform extractions. 2 µl of 10 mg/ml of yeast tRNA were added to each sample and DNA was precipitated with ethanol for 12h at 4°C and resuspended in 20 µl of TE buffer. 5 µl of each sample were used for PCR with the CREB primers: 5'-TGTAATCCAACACTTCAGGAGG-3', and 5'-

1 TTGAGACGGAGTCTCGCTCT-3', and Sp1 primers: 5'-TAGCAACTCCAGGCTAGAGG-3', and

2 5'-AACTGACTTCCGCAAGCTCACA-3'.

3 The PCR conditions were: 94°C 1 min, 56°C 2 min, 72°C 2 min for 30 cycles.

4

5 *Statistical Analysis*

6 Data were analyzed by analysis of variance using the STATPAC computer program. Statistical

7 comparisons for *in vivo* studies were made by Wilcoxon-Mann-Whitney test.

8

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 utilized 45 days 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. 1A). 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. 1B and C).

Leptin enhances cell-cell adhesion and cell proliferation

We performed three-dimensional MCF-7 cultures to evaluate *in vitro* the effects of leptin on cell aggregation. Multicellular spheroid culture was developed several years ago and has recently been shown to be of value as it can more closely mimic some *in vivo* biological features of tumors and improve the relevance of *in vitro* studies (26-29).

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

It is well known that 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. 2A, in the presence of the antibody MCF-7 cells formed small aggregates

1 demonstrating limited intercellular contact, while EGTA treatment prevented cell-cell adhesion and
2 cells remained rounded and singled suspended.
3 In addition, the role of E-cadherin was confirmed using an adhesion assay in which cells were
4 allowed to adhere to E-cadherin/Fc protein-coated dishes. This assay demonstrated a greater
5 binding of cells pretreated with leptin and/or E_2 for 48h with respect to untreated cells (Fig. 2B).
6 The adhesion was blocked using either a function-blocking E-cadherin antibody or EGTA (data not
7 shown). Thus, the increased aggregation observed in the presence of leptin and/or E_2 was dependent
8 on E-cadherin.
9 In three-dimensional cultures we also observed a significant increase of cell growth upon leptin
10 and/or E_2 treatment. The leptin-induced cell proliferation was completely abrogated when E-
11 cadherin function was blocked (Fig. 2C).
12 Since cyclin D1 is a regulator of cell cycle progression, we evaluated its expression either in MCF-7
13 spheroids or in xenografts. We observed an increase of cyclin D1 in terms of mRNA and protein
14 content in the presence of leptin and/or E_2 (Fig. 2D-G).
15
16 **Leptin upregulates E-cadherin expression**
17 To investigate if an enhanced expression of E-cadherin occurred in the above mentioned conditions,
18 we performed RT-PCR and western blotting analysis. Our results demonstrated that either leptin or
19 E_2 and in higher extent the exposure to both hormones increased expression of E-cadherin in terms
20 of mRNA (Fig 3A) and protein content (Fig 3B). The latter results were also evident in MCF-7
21 xenografts (Fig. 3C and D).
22 To evaluate whether both hormones were able to activate E-cadherin promoter, we transiently
23 transfected MCF-7 cells with a construct bearing the human E-cadherin promoter gene. A
24 significant increase of E-cadherin promoter activity was observed in the transfected cells exposed to
25 leptin and/or E_2 for 48h (Fig. 3E).
26

Leptin enhances CREB- and Sp1-DNA binding activity to E-cadherin promoter

The role of leptin and E_2 on the trascriptional activity of 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 Sp-1 (-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, while the activation of E_2 still persisted, even though in a lower extent with respect to the intact promoter (Fig. 3F). This well fits with the evidence that leptin enhanced CREB phophorylation (data not shown).

To characterize the role of these motifs in modulating E-cadherin promoter activity, we performed EMSA. Nuclear extracts from MCF-7 cells, using a DNA probe containing a CRE responsive element, showed two protein-DNA complexes (Fig. 4A lane 1), which were abolished by the addition of a 100-fold excess of non-radiolabeled probe (Fig. 4A lane 2). Leptin treatment induced a strong increase in CREB DNA-binding activity (Fig. 4A lane 3). The specificity of the binding was demonstrated by immunodepletion in the presence of CREB antibody (Fig. 4A 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. 4A lane 9), which was abrogated by the incubation of a 100-fold excess of unlabeled probe (Fig. 4A lane 10). In the presence of the MAPK inhibitor PD98059, the complex induced by leptin treatment was reduced (Fig. 4B lanes 5 and 9). These findings addressed a specific involvement of leptin signalling in the up-regulation of E-cadherin expression.

Using a DNA probe containing an Sp1 site, we observed in nuclear extracts of MCF-7 cells, a specific protein-DNA complex that was slightly enhanced by leptin, increased upon E_2 exposure and furthermore by the combined treatments (Fig. 4C lanes 1, 3, 5 and 7). Using the 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. 4C lane 10), which was abrogated by 100-fold excess of unlabeled Sp1 probe (Fig. 4C and D lanes 2 and 11). The addition of mithramycin A (100

1 μM), that binds to GC boxes and prevents sequential Sp1 binding to nuclear extracts treated with
2 leptin and E_2, blocked the formation of DNA-Sp1 complexes (Fig. 4C lane 9). In the presence of
3 Sp1 antibody the original band DNA-protein complex was supershifted (Fig. 4C lanes 4, 6 and 8).
4 In all hormonal treatments performed, the pure antiestrogen ICI 182,780 reduced the Sp1-DNA
5 binding complex (Fig. 4D lanes 5, 7 and 9) evidencing that leptin induced an activation of ERα, as
6 we previously demonstrated (30).
7
8 **Effects of leptin on CREB and Sp1 recruitment to the E-cadherin promoter**
9 To corroborate EMSA results, we performed ChIP assay. We found that the stimulation of MCF-7
10 cells with leptin increased the recruitment of CREB to E-cadherin gene promoter (Fig. 5A).
11 Furthermore, we observed that leptin or E_2 stimulated the recruitment of Sp1 to the E-cadherin
12 promoter and the combined treatment induced an additive effect (Fig. 5B). The latter event suggests
13 that leptin and E_2 may converge in activating ERα to recruit Sp1 on E-cadherin promoter.
14
15 **Involvement of ERα in the leptin-induced upregulation of E-cadherin expression**
16 Stemming from the data provided by EMSA and ChIP assays, we evaluated the involvement of
17 ERα in the enhanced E-cadherin expression induced by leptin. Our results demonstrated that, in
18 three-dimensional cultures, in the presence of the pure antiestrogen ICI 182,780 the upregulatory
19 effect of leptin on E-cadherin protein expression still persisted, while the stimulatory effects of E_2
20 was abrogated (Fig. 6A).
21 In addition, the specific role of leptin signalling in upregulating E-cadherin expression, was also
22 confirmed by transient transfection of E-cadherin promoter in ERα negative HeLa cells. We
23 evidenced that leptin was able to activate E-cadherin promoter (Fig. 6B) which was abrogated in the
24 presence of ERK2 and STAT3 dominant negative (Fig. 6C). The latter data sustain furthermore the
25 involvement of leptin signalling. It is worth to note how the ectopic expression of ERα in HeLa

1 cells was able to potentiate the effect of leptin (Fig. 6B). To test the activity of the transfected ERα
2 we performed western blotting analysis for phosphorylated ERα, while for dominant negative
3 ERK2 and STAT3 genes we evaluated the expression of c-fos, as target of both pathways (31-33).
4 Furthermore, in BT-20 cells lacking of ERα, we observed that the stimulatory effect of leptin on E-
5 cadherin protein content was reduced in the presence of either ERK2 or STAT3 dominant negative.
6 In the same cells, cotransfected with ERα and ERK2 or STAT3 dominant negative, E_2 alone or in
7 combination with leptin was unable to maintain the upregulatory effect on E-cadherin expression
8 (Fig. 6D).

Discussion

Leptin is a cytokine mainly produced by adipose tissue which 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 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, e.g. bladder, lung and pancreatic 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 demonstrated 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 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, appears to be dependent on E-cadherin molecule that have an indispensable role in this process. Indeed, the addition of a function-blocking E-cadherin antibody

1 or a calcium chelating agent, EGTA, blocked cell-cell adhesion induced by both hormones. Besides,
2 we demonstrated by adhesion assay a greater binding of cells pretreated with leptin and/or E_2 on E-
3 cadherin/Fc protein-coated dishes.
4 In the same experimental conditions an increased proliferative rate was observed upon leptin or E_2
5 exposure, which was completely abrogated when E-cadherin function was blocked.
6 An important cell cycle regulator, such as cyclin D1, resulted to be upregulated in three-
7 dimensional cultures as well as in xenografts.
8 Besides, in both models we demonstrated that leptin and/or E_2 enhanced E-cadherin expression in
9 terms of mRNA, protein content and promoter activity.
10 The analysis of E-cadherin promoter sequence, revealed the presence of CRE and Sp1 sites, as
11 potential target of leptin and E_2 signals. It is well documented how leptin and E_2 through non
12 genomic effects are able to activate MAPK pathway that induces activation of CREB kinase, a
13 member of the $p90^{RSK}$ family that corresponds to RSK2 and thereby phosphorylates CREB serine
14 133 (38-40). This well fits with our functional studies demonstrating that leptin was no longer able
15 to activate the E-cadherin gene promoter mutated in the CREB site, while E_2 maintained an
16 activatory effect even though in a lower extent with respect to the intact promoter. The latter data
17 suggest that the activatory effect of E_2 may persist through its binding to Sp1-DNA complex.
18 The important role of Sp1 responsive element in activating E-cadherin promoter was demonstrated
19 by EMSA and ChIP assays. Our results evidenced that E_2, as extensively documented, acts in a
20 nonclassic way through the interaction of ERα with Sp1 (41-45). It is worth to note that upon leptin
21 exposure we also observed an increase in Sp1-DNA binding activity, clearly reduced in the
22 presence of the pure antiestrogen ICI 182,780, as well as an enhanced recruitment of Sp1 to E-
23 cadherin promoter. These observations are supported by our previous findings reporting that leptin
24 is able to transactivate, in a unliganded-dependent manner, ERα through MAPK signal (30).
25 A cross-talk between leptin and E_2 has been well documented in neoplastic mammary tissues and
26 breast cancer cell lines (15,30,46,47). For instance, E_2 upregulates leptin expression in MCF-7 cells

1 (15), while leptin is an amplifier of E_2 signalling. The latter event occurs through a double
2 mechanism: an enhanced aromatase gene expression (46) and a direct transactivation of ERα (30).
3 Thus, we investigated whether the upregolatory effect induced by leptin on E-cadherin expression
4 can be modulated by ERα. We found that E-cadherin protein appears still upregulated by leptin in
5 the presence of the pure antiestrogen ICI 182,780. Moreover, in HeLa cells functional studies
6 showed that leptin was able to activate E-cadherin promoter even though the ectopic expression of
7 ERα potentiated the effect of the hormone. This stimulatory effect was abrogated in the presence of
8 ERK2 or STAT3 dominant negative, suggesting that leptin signalling is involved in enhancing E-
9 cadherin expression.These latter data are supported by western blotting analysis performed in BT-
10 20 breast cancer cells lacking of ERα in which ERK2 and STAT3 dominant negative reversed
11 leptin-enhanced E-cadherin protein content. The upregulatory effect induced by E_2 on E-cadherin
12 expression in the presence of ectopic ERα appeared inhibited in the presence of ERK2 and STAT3
13 dominant negative. The latter findings may be a consequence of the enhanced expression of leptin
14 receptor upon E_2 exposure (15), which may have an impaired signalling on E-cadherin expression.
15 An additional explanation, which may coexist with the previous one, may be that both ERK2 and
16 STAT3 dominant negative could interfere with ERα/Sp1 interaction at level of E-cadherin gene
17 transcription (48).
18 A hypothetical model of the possible mechanism through which leptin and E_2 may functionally
19 interact in modulating E-cadherin expression in breast cancer is shown in Scheme 1. Leptin through
20 MAPK activation may phosphorylate CREB and induce its transactivation. For instance, CREB
21 phosphorylated at serine 133 is often reported not only as an index of PKA but also as an effector of
22 MAPK activation (49). Concomitantly, leptin in the presence of E_2 may potentiate the
23 transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. On
24 the other hand it is well known that ERα, in the presence of its natural ligand, interacts with Sp1.
25 Our findings address how CREB is an effector of leptin action while Sp1 is a common target of

leptin and E_2 signalling in the upregulation of E-cadherin expression and in the enhanced homotypic adhesion in breast cancer.

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. Thus, 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 signalling may represent a target of combined pharmacological tools to be exploited in the novel therapeutical adjuvant strategies for breast cancer treatment particularly in obese women.

Acknowledgments

This work was supported by A.I.R.C. grants 2005 and 2006.

Dr. Sara Morales is supported by Spanish Ministry of Education Postdoctoral Research Grant.

We thank Dr. Domenico Sturino for the English revision of the manuscript and Dr. Pasquale Cicirelli for technical assistance.

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Figure Legends

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

MCF-7 cells were cultured as three-dimensional spheroids in SFM. 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 fiels under ×10 magnification. The results are mean ± S.E. from at least three experiments. Representative three-dimensional cultures are shown in Fig. 2A. * $p < 0.05$ versus leptin and E_2.

Fig. 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 estradiol 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. The histograms represent the mean ± S.E. 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%. * $p < 0.05$.

Fig. 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 mM). Cells were untreated (a) or treated with leptin (b), E_2 (c) and leptin plus E_2 (d) for 48h and then photographed under phase contrast microscopy. The bar in d equals 50 μm.

1 B. 6-well plates were coated with E-cadherin/Fc recombinant protein and binding of cells were
2 measured by the MTT assay. Each bar is the mean of five wells.
3 C. Proliferation of MCF-7 cells treated with leptin and/or E_2 for 48h in the absence or presence of
4 E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mM). The results are average of three
5 experiments.
6 Total RNA was isolated from MCF-7 three-dimensional cultures (D) or xenografts (F) and reversed
7 transcribed. cDNA was subjected to PCR using primers specific for cyclin D1 (30 cycles) or 36B4
8 (15 cycles). C^-, RNA sample without the addition of reverse transcriptase (negative control).
9 Protein extracts obtained from MCF-7 spheroids (E) and xenografts (G) were immunoblotted with a
10 specific antibody against human cylin D1.
11 Rapresentative results are shown. * $p < 0.05$
12
13 **Fig. 3 Leptin upregulates E-cadherin expression in MCF-7 spheroids and xenografts**
14 RT-PCR of E-cadherin mRNA was performed in MCF-7 three-dimensional cultures stimulated for
15 48h with 1000 ng/ml leptin and/or 100 nM E_2 (A) as well as in xenografts (C). 36B4 mRNA levels
16 were determined as a control. C^-, RNA sample without the addition of reverse transcriptase
17 (negative control).
18 Protein extracts obtained from MCF-7 spheroids (B) and xenografts (D) were immunoblotted with a
19 specific antibody against human E-cadherin.
20 Rapresentative results are shown. The histograms represent the mean ± S.E. of three separate
21 experiments in which the band intensities were evaluated in terms of optical density arbitrary units
22 and expressed as the percentage of the control assumed as 100%.
23 MCF-7 cells were transiently transfected with a luciferase reporter plasmid containing the human E-
24 cadherin promoter full length (E) or mutated in the CREB site (CREB mut) (F). The cells were left
25 untreated (control) or treated in the presence of 1000 ng/ml of leptin and/or 100 nM of E_2. The

values represent the means ± S.E. of three separate experiments. In each experiment, the activities of the transfected plasmid was assayed in triplicate transfections.

* $p < 0.05$, ** $p < 0.01$ compared with control.

Fig. 4 Effects of *in vitro* leptin treatment on CREB and Sp1 DNA-binding activity in MCF-7 cells.

Nuclear extracts from MCF-7 cells were incubated with a double-stranded CREB- (A and B) or Sp1- (C and D) specific consensus sequence probe labelled 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 performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 10). MCF-7 nuclear extracts treated with 1000 ng/ml of leptin and/or 100 nM of E_2 for 48h incubated with probe are shown in 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-starvated overnight with 10 μM 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 performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 11). MCF-7 nuclear extracts treated with 1000 ng/ml of leptin and/or 100 nM of E_2 for 48h incubated with probe are shown in 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 μM mithramycin A (lane 9). D. The pure antiestrogen ICI 182,780 (1 μM) was added in leptin- (lane 5) and/or E_2-treated (lanes 7 and 9) MCF-7 nuclear extracts. Lanes 12 contain probe alone.

1 **Fig. 5 Recruitment of CREB and Sp1 to the E-cadherin promoter in MCF-7 cells.**

2 The cells were treated for 1h with 1000 ng/ml leptin and/or 100 nM E_2 or left untreated. The
3 precleared chromatin was immunoprecipitated with specific antibodies, namely, anti-CREB for
4 CREB IPs (A) or anti-Sp1 for Sp1 IPs (B). E-cadherin promoter sequences containing CREB or
5 Sp1 sites were detected by PCR with specific primers, as detailed in Materials and Methods. To
6 determine input DNA, the E-cadherin promoter fragment was amplified from 5 μl purified soluble
7 chromatin before immunoprecipitation. PCR products obtained at 30 cycles are shown. ChIP with
8 non-immune IgG was used as negative control (C^-). This experiment was repeated three times with
9 similar results and the most representative experiment is shown. The histograms represent the mean
10 ± S.E. of three separate experiments in which the band intensities were evaluated in terms of optical
11 density arbitrary units and expressed as the percentage of the control assumed as 100%.

12

13 **Fig. 6 Influence of ERα on leptin-induced upregulation of E-cadherin expression.**

14 A. MCF-7 spheroids were preincubated with 1 μM ICI 182,780 for 1h and then treated with leptin
15 (1000 ng/ml) and/or E_2 (100 nM) for 48h. Total proteins (50 μg) were immunoblotted with a
16 specific antibody against human E-cadherin. GAPDH serves as loading control.

17 B. Estrogen receptor negative HeLa cells were transfected with a plasmid containing E-cadherin
18 promoter or cotransfected with E-cadherin promoter and pHEGO. Transfected cells were treated
19 with leptin (1000 ng/ml) and/or E_2 (100 nM) for 48h. The values represent the means ± S.E. of three
20 separate experiments. In each experiment, the activities of the transfected plasmids were assayed in
21 triplicate transfections. Small square shows western blot analysis for phosphorylated ERα using
22 anti-phospho-ERα (ser 118). * $p < 0.05$, ** $p < 0.01$ compared with control.

23 C. HeLa cells were transiently transfected with dominant negative ERK2 or STAT3 plasmid and
24 then treated for 48h with leptin. In each experiment, the activities of the transfected plasmids were
25 assayed in triplicate transfections. Small squares show western blot analysis for c-fos. * $p < 0.05$
26 compared with control.

D. BT-20 cells were transfected with ERK2 dominant negative or with STAT3 dominant negative in the presence or absence of ERα and then treated with leptin (1000 ng/ml) and/or E_2 (100 nM). Total proteins (50 μg) were immunoblotted with a specific antibody against human E-cadherin. GAPDH serves as loading control. Results are representative of three independent experiments.

Scheme 1 Hypothesized model of leptin signalling in modulating E-cadherin expression in breast cancer

Interaction of leptin 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.

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7 **Table 1**

MCF-7	Spheroids		
	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
Estradiol	7 ± 0.6	32± 2.1	78 ± 3.2
Leptin + Estradiol	3 ± 0.9	40.5 ± 2.3*	80.7 ± 2.9

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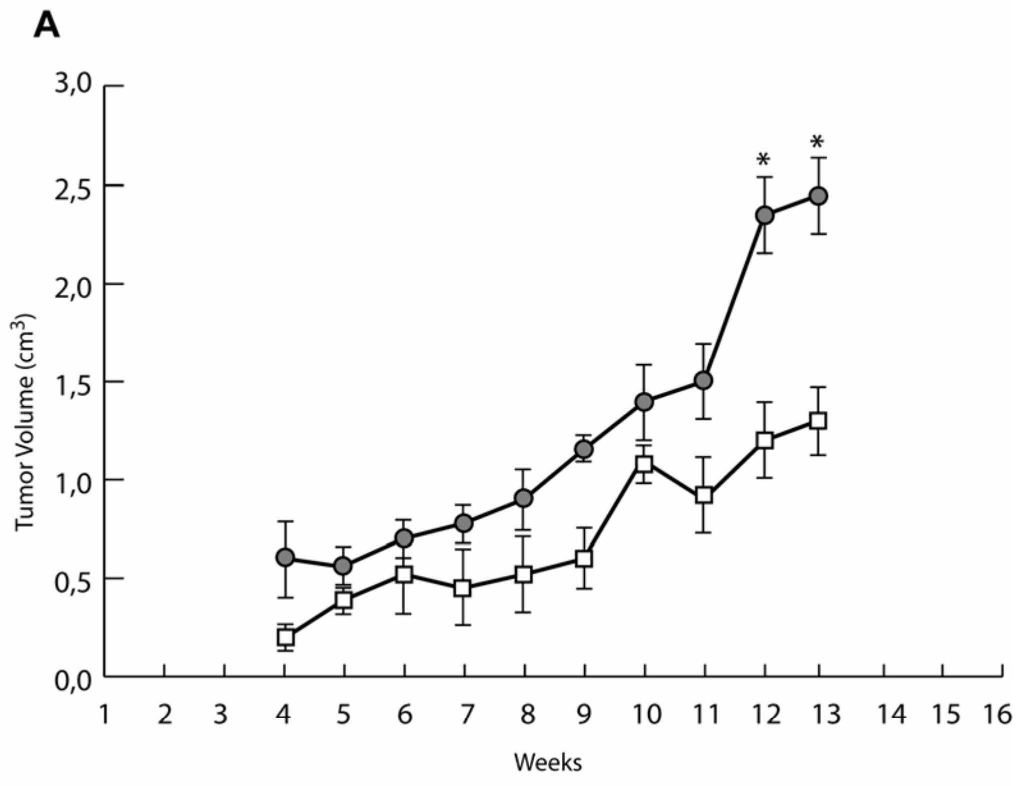

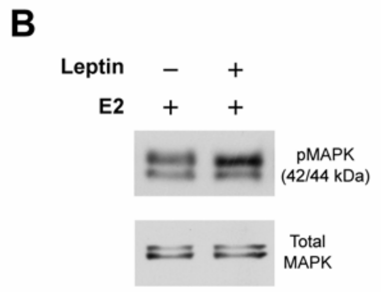

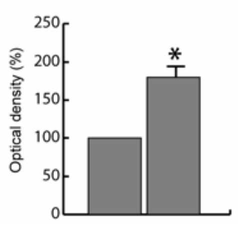

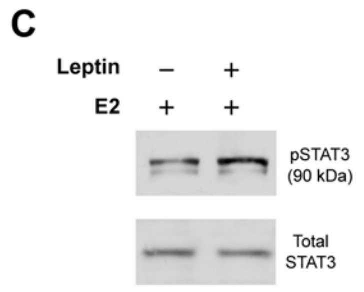

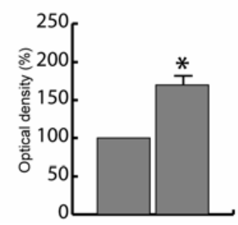

Fig. 1

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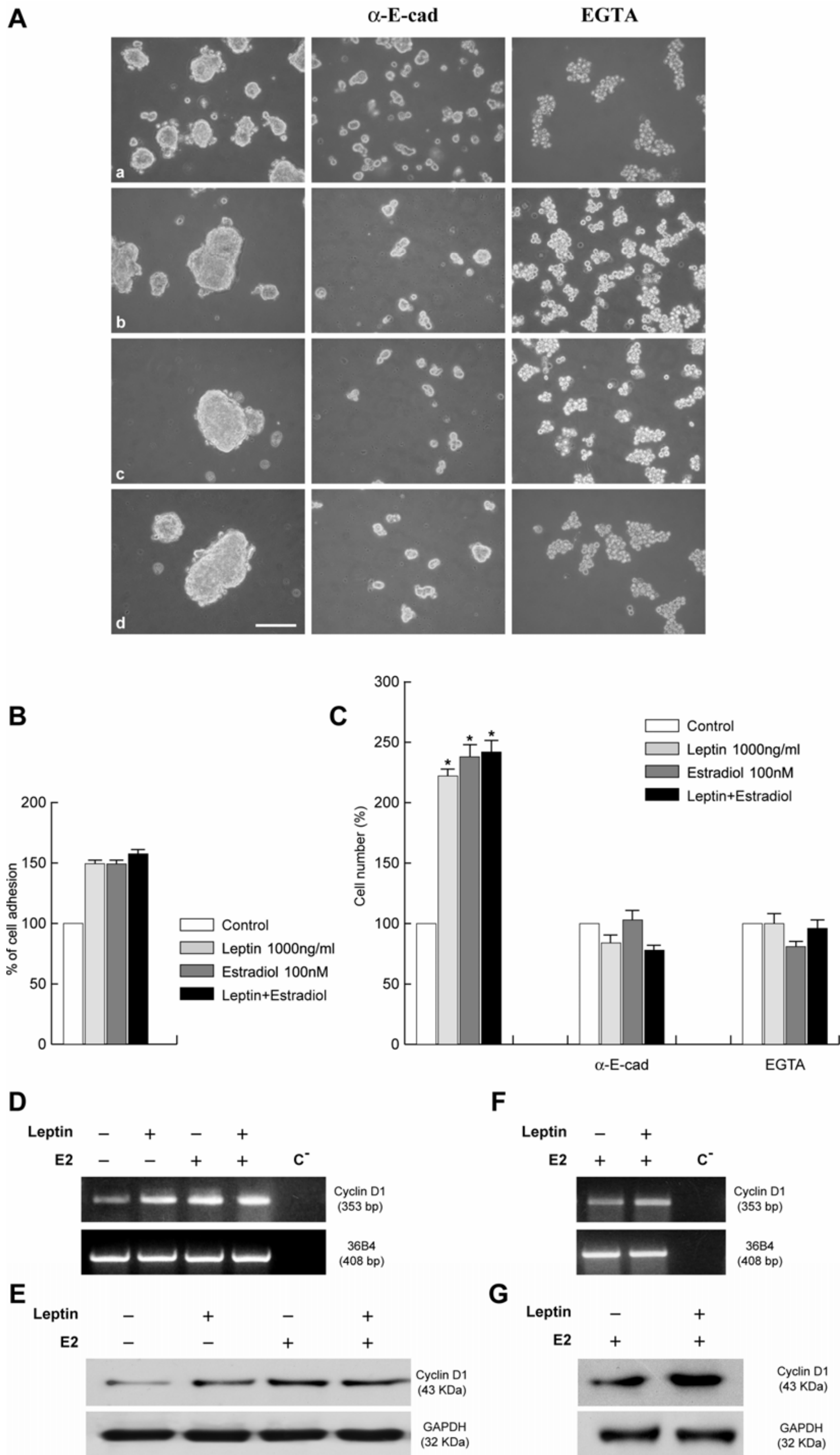

Fig. 2

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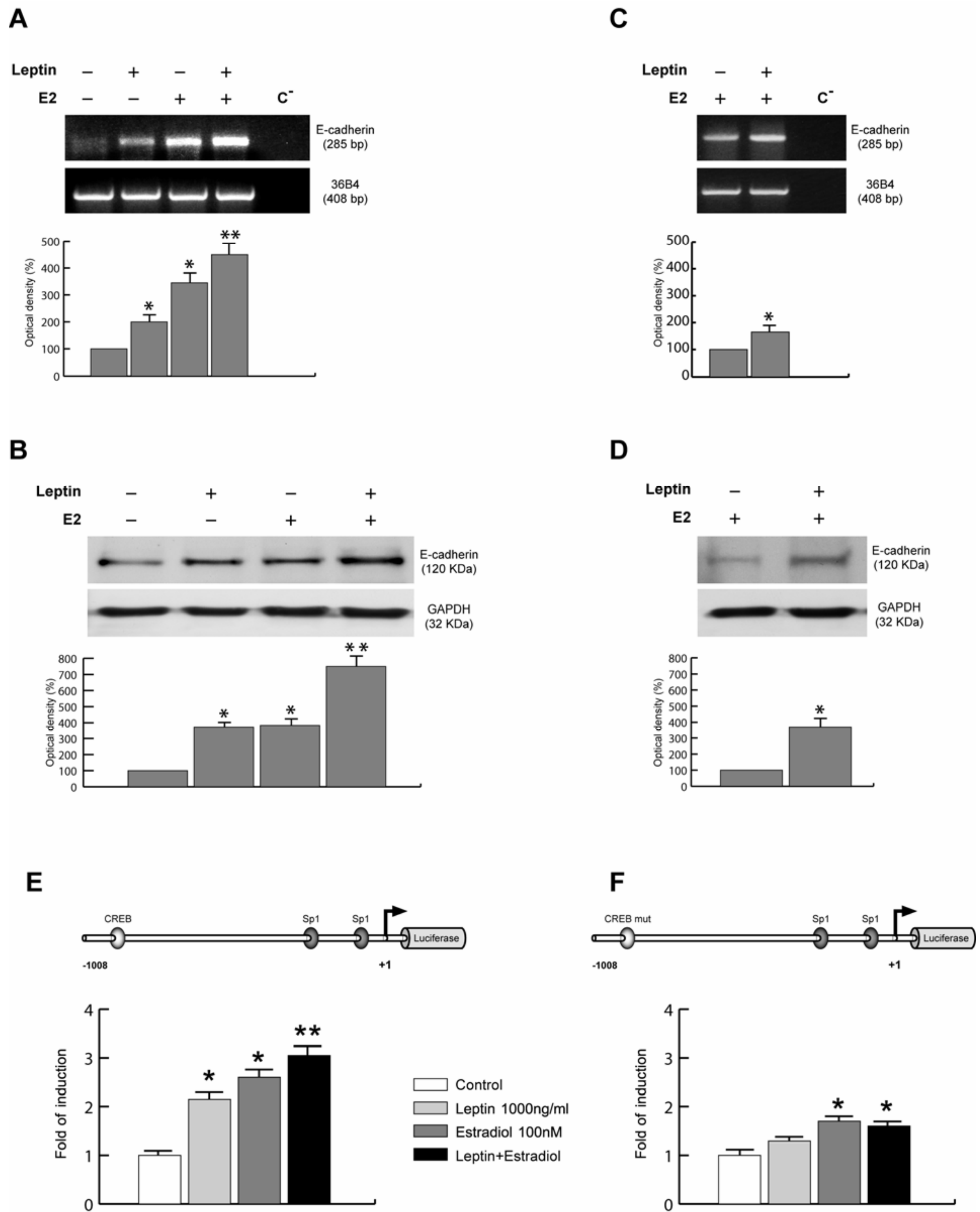
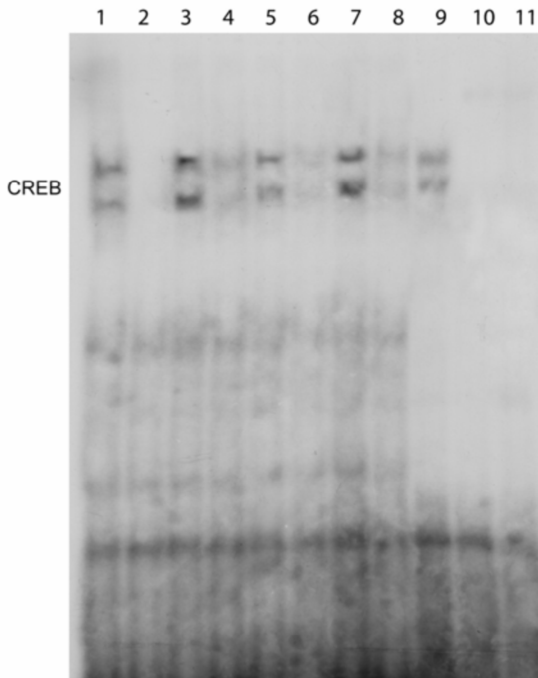
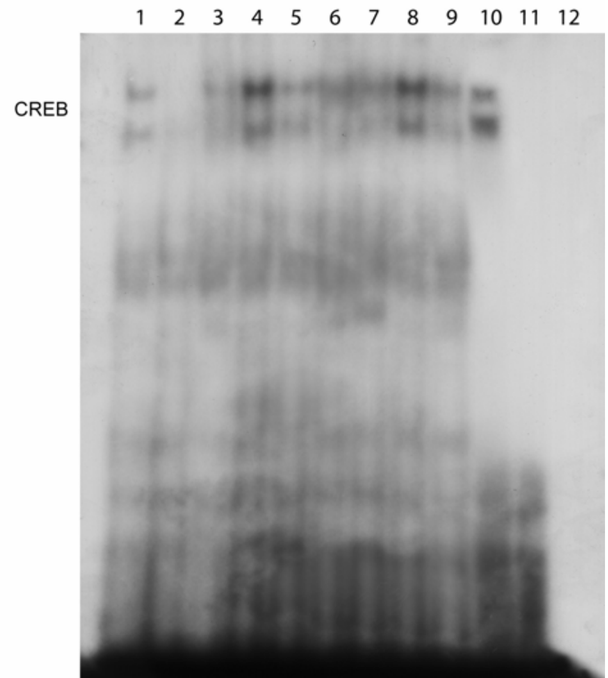
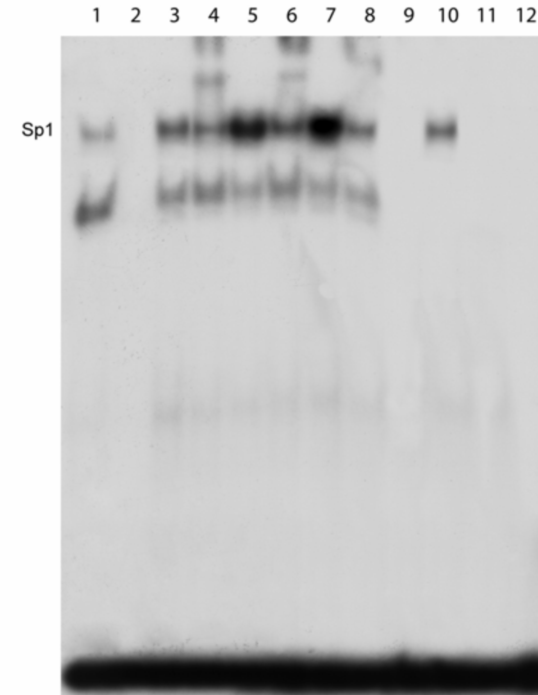


Fig. 3







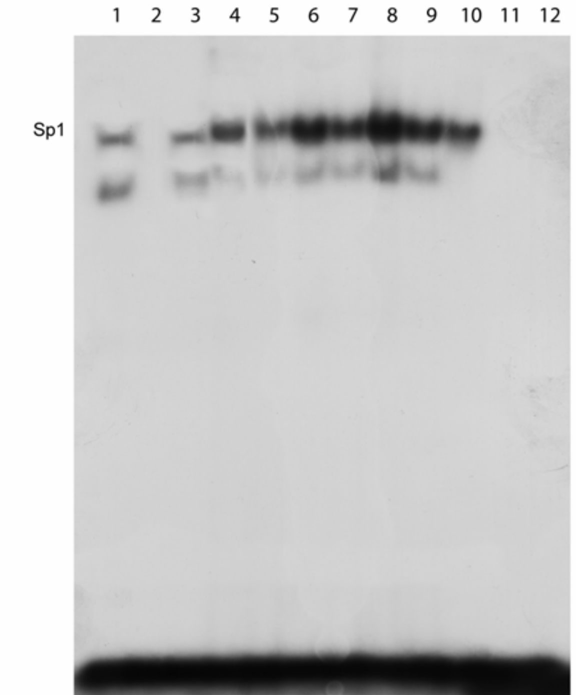


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Fig. 4

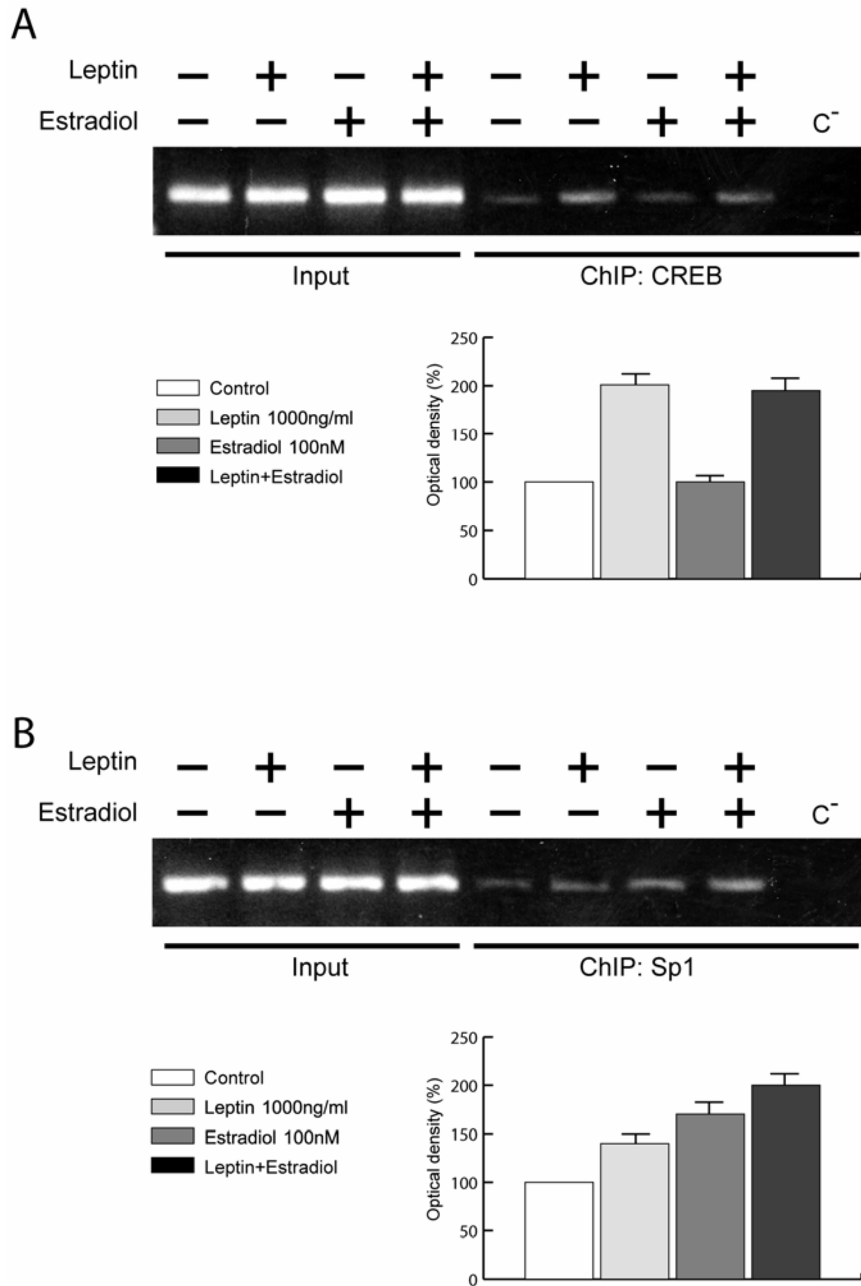

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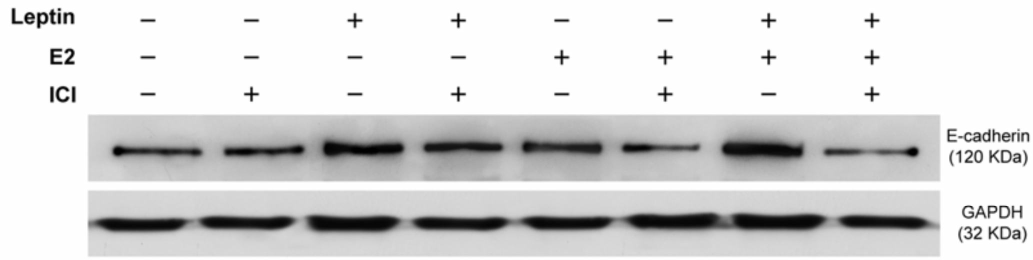
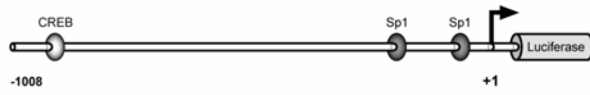
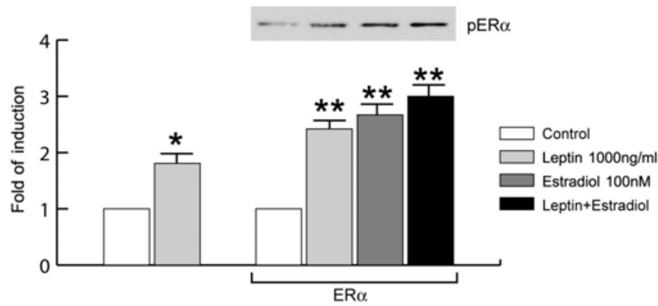
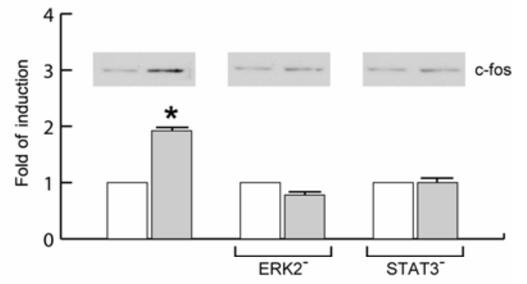
Fig. 5









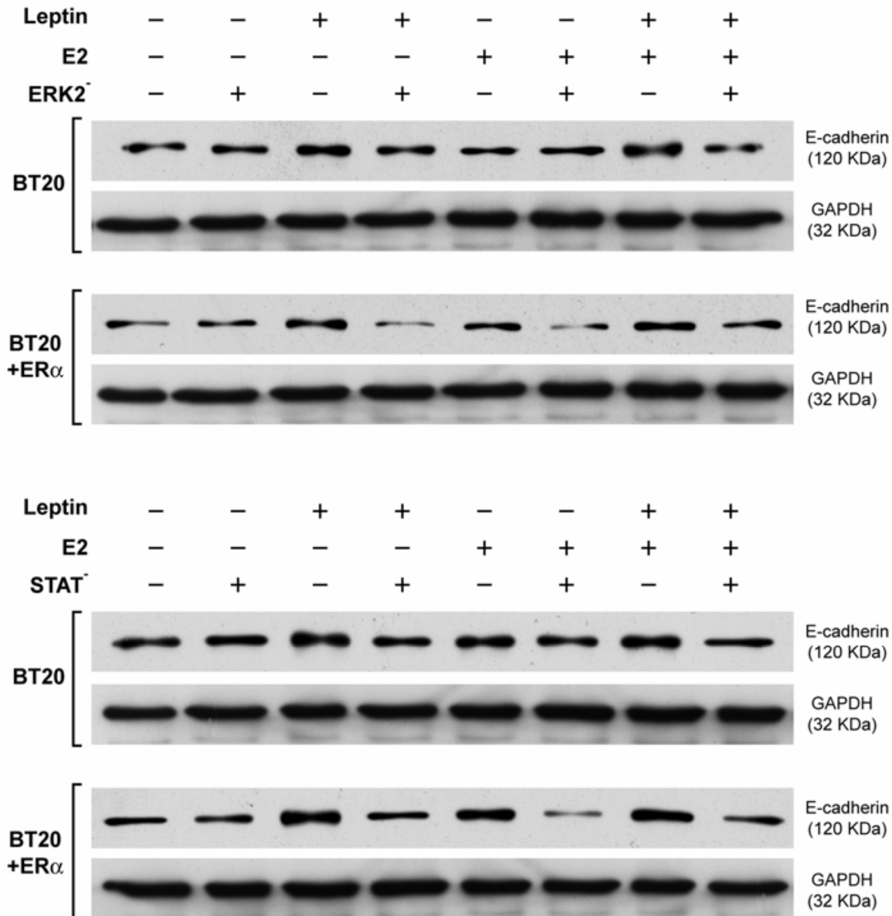


Fig. 6

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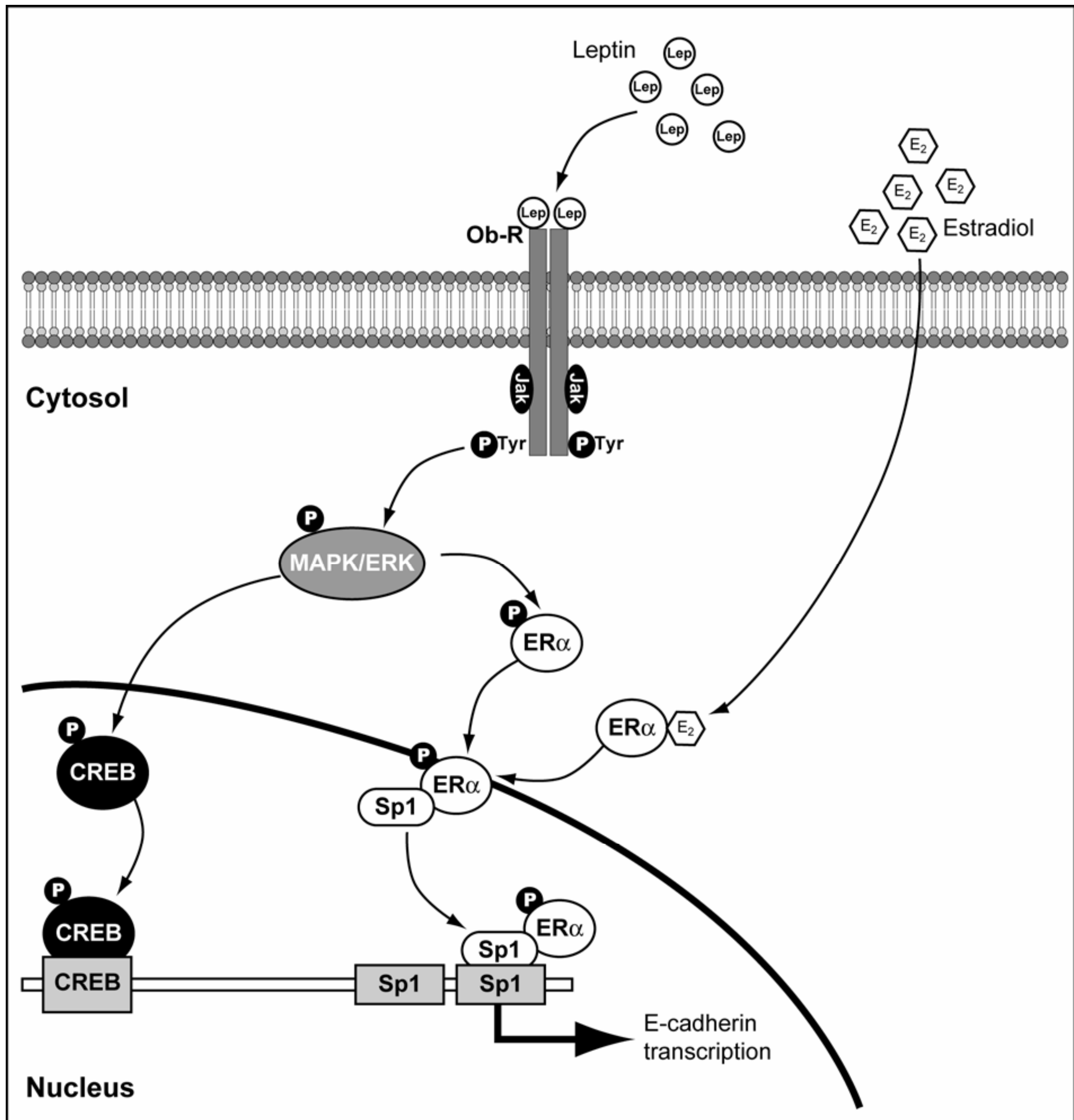

Scheme 1

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JOURNAL OF CELLULAR PHYSIOLOGY 9999:1–9 (2007)

Author Proof

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. J. Cell. Physiol. 9999: 1–9, 2007. © 2006 Wiley-Liss, Inc.

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

Stefania Catalano and Pietro Rizza contributed equally to this work.

Contract grant sponsor: PRIN-MIUR; Contract grant number: 2004067227.

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Received 22 June 2006; Accepted 24 October 2006

Published online in Wiley InterScience (www.interscience.wiley.com.), 00 Month 2006.
DOI: 10.1002/jcp.20952

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(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 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[Q2]). 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:

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

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.

Author Proof

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′-AAATTGT<u>GGGCG-G</u>AAACTTCCAGGGG-3′, mutated Sp-1 5′-AAATTGTGttCG-GAAACTTCCAGGGG-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′-GCAACT-GAGGCCTTGAAGGC-3′ (forward) and 5′-GCAGCTGGT-GAGTCAGGCCAG-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

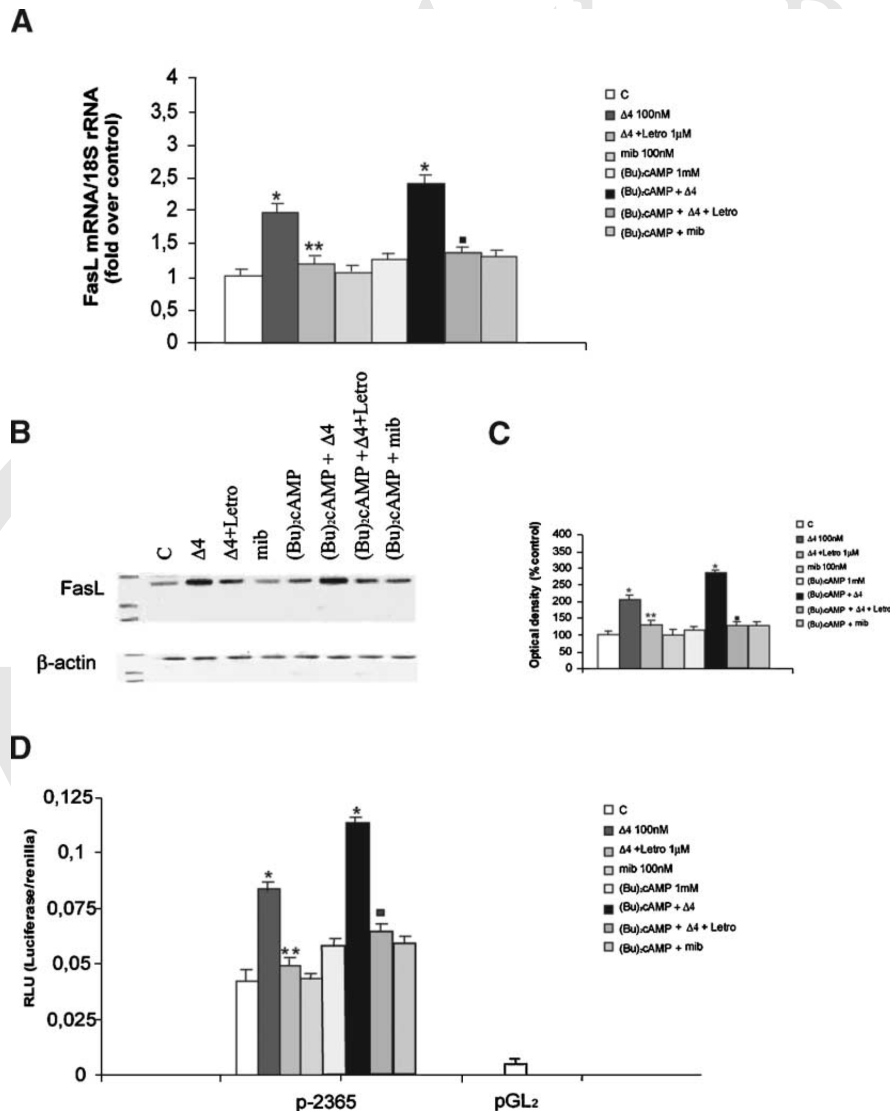

Fig. 1. Effects of Δ4 on FasL expression. **A**: Total RNA was obtained from TM4 cells untreated (control, C) or treated for 24 h with Δ4 (100 nM) mibolerone (mib 100 nM), $(Bu)_2$cAMP (1 mM), $(Bu)_2$cAMP + Δ4 and $(Bu)_2$cAMP + mib. One micromolar of aromatase inhibitor letrozole (Letro) was used.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

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.

Author Proof

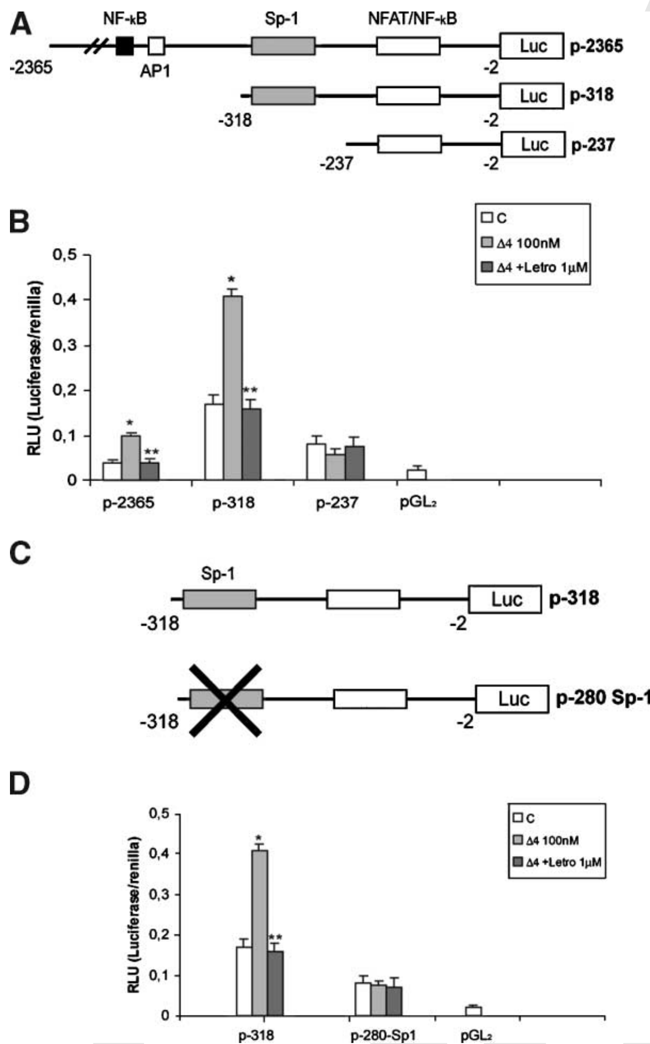

Fig. 2. Effects of estradiol "in situ" production on expression of human FasLpromoter/luciferase reporter gene construts in TM4 cells. **A**: Schematic map of the FasL promoter fragments used in this study. All of the promoter constructs contain the same 3′ boundary (−2). The 5′ boundaries of the promoter fragments varied from −237 to −2,365. 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 pGL2 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.

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 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 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

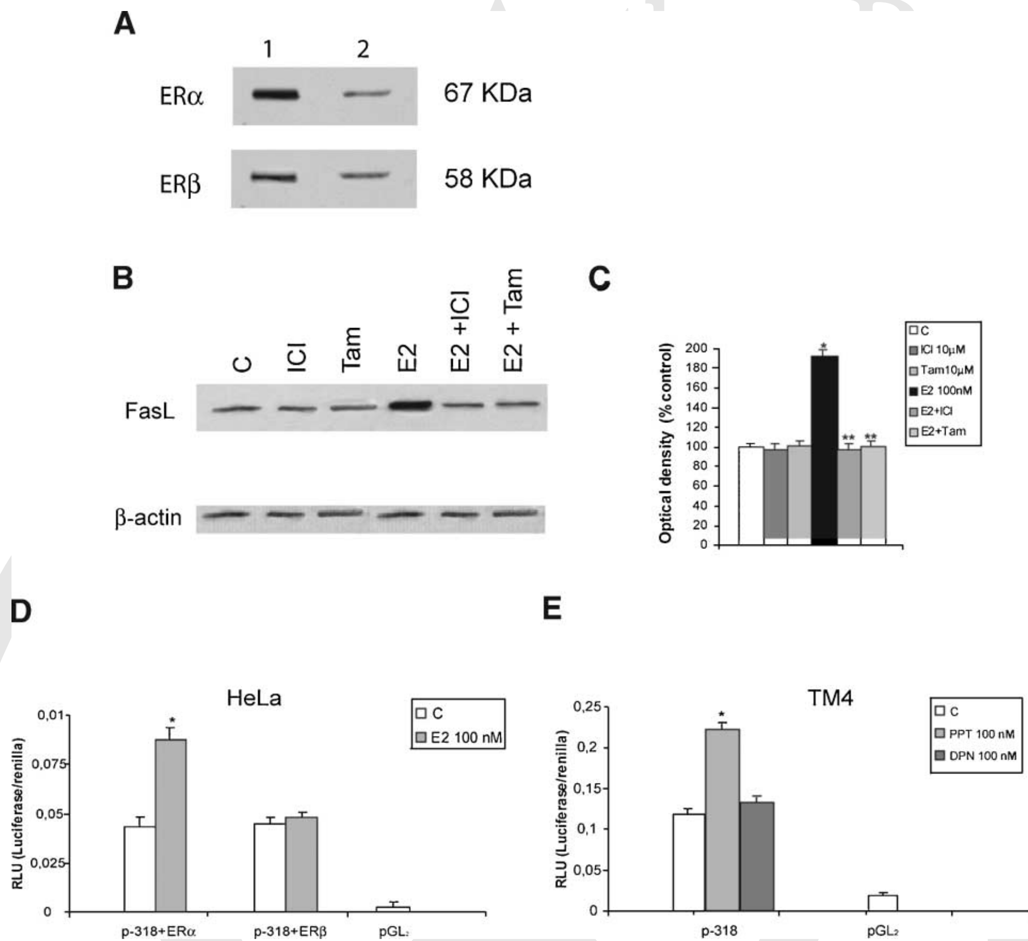

Fig. 3. 17β-Estradiol enhances FasL transcriptional activity through ERα. **A**: Lysates from TM4 cells were used to evaluate by Western blot analysis the expression of ERα and ERβ (lane 2). The human breast cancer cell line MCF-7 and human prostate cancer cell line LNCaP were used as positive control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

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 −317 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. 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

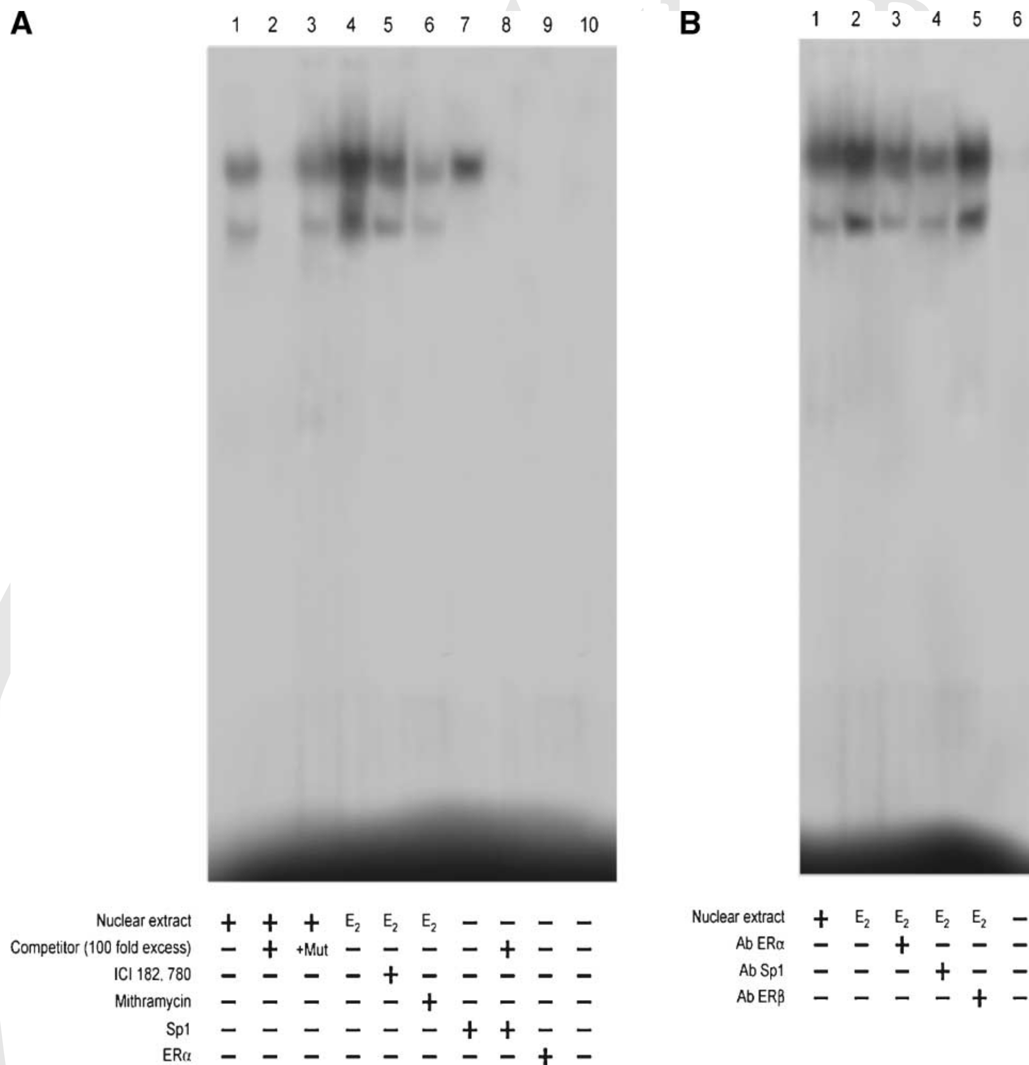

Fig. 4. Electrophoretic mobility shift assay of the Sp-1 binding site in the FasL promoter region. **A**: Nuclear extracts from TM4 cells were incubated with a double-stranded Sp1-specific consensus sequence probe labeled with [γ-^{32}P] ATP and subjected to electrophoresis in a 6% polyacrilamide gel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

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 induced by $(Bu)_2cAMP$ 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

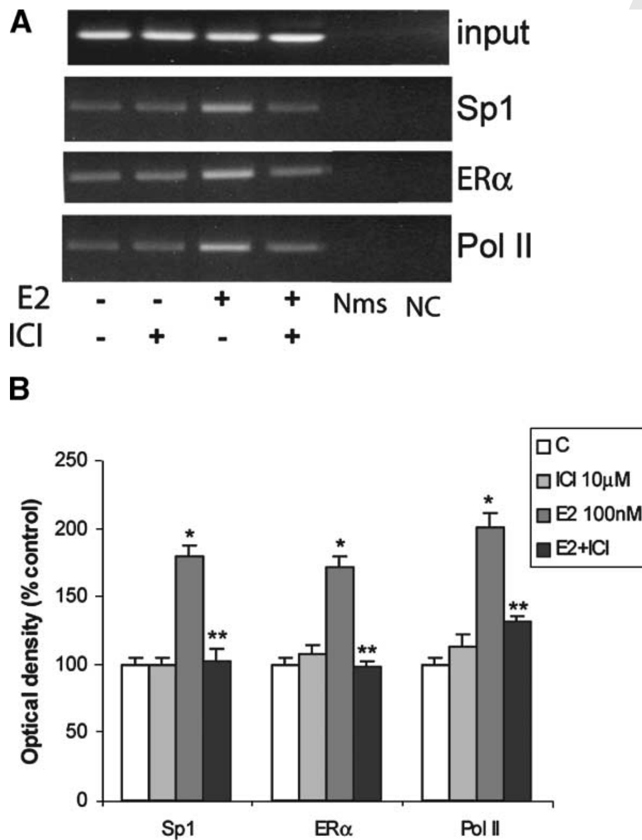

Fig. 5. 17β-Estradiol increases Sp-1/ERα recruitement to FasL promoter. **A**: Soluble precleared chromatin was obtained from TM4 cells treated for 1 h with 100 nM E2, 10 μM ICI and E2 + ICI or left untreated (C) and immunoprecipitated (IP) with an anti-Sp-1, anti ERα, anti polymerase II antibodies and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

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 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

We thank Dr C.V. Paya for providing us with the pGL2 promoter FasL (p-2365, p-318, p-237) and Dr Domenico Sturino for English revision of the manuscript.

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

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Short title: Androgen receptor in human sperm

Summary sentence: In human sperm androgen receptor is able to modulate PI3K/AKT pathway on the basis of the androgen levels.

Key words: androgen receptor, androgens, human sperm, male reproduction, PI3K/AKT.

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FOOTNOTES: This work was supported by PRIN 2004 Prot. N. 0067227, AIRC - 2003 and MURST and Ex 60% -2005. Saveria Aquila[1*] and Mariaelena Gentile[2*]contributed equally to this work.

1 **ABSTRACT**

2 Results from mice lacking the androgen receptor (AR) showed that it is critical for the proper
3 development and function of the testes. The aim of this study was to investigate whether a
4 functional AR is present in human sperm. By using RT-PCR and Western blot AR expression was
5 demonstrated. by immunocytochemistry the AR was located at the head region.
6 Dihydrotestosterone, in a dose-dependent manner, leads to the rapid phosphorylation of the AR on
7 tyrosine, serine and threonine residues which was reduced by the AR antagonist OH-Flutamide.
8 The effects of AR on PI3K/AKT pathway depend on androgen concentrations. Specifically, 0.1 and
9 1 nM dihydrotestosterone stimulated PI3K activity, while 10 nM dihydrotestosterone produced a
10 decrease of PI3K activity, p-AKT S473 and p-BCL2 in the presence of an enhanced PTEN
11 phosphorylation. In addition, 10 nM DHT was able to induce the cleavage of caspases 8, 9 and 3
12 which was reversed either by casodex or OH-Flutamide, confirming that the effect is mediated by
13 the AR. By using wortmannin, a specific PI3K inhibitor, the cleavage of caspase 3 was reproduced
14 confirming that in sperm the PI3K/AKT pathway is involved in caspase activation.

15 In conclusion, human sperm express a functional AR that has the ability to modulate the
16 PI3K/AKT pathway influencing sperm cell physiology.

17 **INTRODUCTION**

18 A functional androgen receptor (AR) is required for male embryonic sexual differentiation,
19 pubertal development and regulation of spermatogenesis in mammals. The role of AR during
20 spermatogenesis has been the subject of intense interest for many years (1). Several findings have
21 shown that AR function is required for the completion of meiosis and the transition of
22 spermatocytes to haploid round spermatids (2). Studies of androgen withdrawal and disruption of
23 AR activity, either by surgical, chemical or genetic means, have demonstrated that spermatogenesis
24 rarely proceeds beyond meiosis. In all of these model systems, very few round and even fewer

1 elongated spermatids are observed as clearly demonstrated in a previous study (3). However, the
2 mechanisms by which androgens regulate male fertility are not fully understood and the sites of
3 androgen action within the male reproductive system are not yet resolved. Whereas few studies
4 have raised the intriguing possibility that some germ cells may exhibit immunoreactive AR (4, 5),
5 other reports point to Sertoli cells or Leydig cells or peritubular/myoid cells as the exclusive
6 androgen target cells in the testis (6, 7, 8, 9). Recently, the presence of the AR in human sperm was
7 demonstrated by Western blot and by immunofluorescence assay (10).

8 It is generally accepted that androgens bind to intracellular androgen receptors resulting in
9 mRNA and protein synthesis (11). Nevertheless, rapid responses to androgens have been observed
10 in different tissues that cannot be explained by involvement of mRNA and protein synthesis (12,
11 13). These rapid, nongenomic effects are also seen for other steroid hormones (14) and their
12 importance as a complementary route for cell regulation has recently become evident. Different
13 nuclear receptors (15, 16) were found to be present in human spermatozoa, regulating cellular
14 processes through nongenomic mechanisms. This may represent the exclusive modality of action in
15 spermatozoa since they are apparently transcriptionally inactive cells.

16 In addition to stimulating cell growth, androgens and/or AR play important roles in the
17 promotion of cell apoptosis (17, 18, 19, 20). The term *apoptosis* defines programmed cell death,
18 which is executed by the activation of caspases, a family of cytoplasmic cysteine proteases (21)
19 through two major pathways: the intrinsic and the extrinsic. The intrinsic pathway involves the cell
20 sensing stress that triggers mitochondria-dependent processes, resulting in cytochrome *c* release and
21 activation of caspase 9 (22). The extrinsic pathway involves the final cleavage of caspase 8 (23).
22 Both caspases 8 and 9 can be directly regulated through protein phosphorylation from AKT (24,
23 25). The PI3K signaling pathway is an important intracellular mediator of cell survival and
24 antiapoptotic signals (26). PI3K activation leads to production of 3'-phosphoinositide second
25 messengers, such as phosphatidylinositol 3,4,5-trisphosphate, which activate a variety of

1 downstream cell survival signals. Accumulation of phosphatidylinositol 3,4,5-trisphosphate at the
2 membrane recruits a number of signaling proteins containing pleckstrin homology domains,
3 including protein kinase B (also known as AKT). On recruitment, AKT becomes phosphorylated
4 and activated and exerts its antiapoptotic activity through inactivation of proapoptotic proteins. In
5 addition, the PI3K pathway has also been shown to be negatively regulated by Phosphatase and
6 tensin homologue (PTEN), a lipid phosphatase that cleaves the D3 phosphate of the second
7 messenger phosphatidylinositol 3,4,5-trisphosphate (27, 28). Recently in fibroblasts, it has been
8 demonstrated that AR mediates androgen nongenomic function and that androgen activates
9 PI3K/AKT through the formation of a triple complex between AR, the regulatory subunit p85 of
10 PI3K (PIK3R1) and SRC tyrosine kinase. Indeed, this interaction is dependent on androgen
11 concentration, particularly high androgen concentration which dissociates the AR-SRC tyrosine
12 kinase-PI3K complex (29).

13 The functional impact of programmed cell death in human sperm is poorly understood (30). Up
14 to now it has been unclear whether apoptosis in ejaculated spermatozoa takes place in a similar way
15 as in somatic cells or whether spermatozoa, which are thought to have a transcriptionally inactive
16 nucleus, undergo abortive forms of this process (30). However, sperm constitutively express
17 proteins required to execute apoptosis. Active caspases were observed predominantly in the
18 postacrosomal region (caspases 8, 1, and 3) and caspase 9 was particularly localized in the
19 midpiece, associated with mitochondria (31). Moreover, a wide spectrum of cell cytoskeletal
20 proteins and membrane components are also targets of caspase 3 (31) and the proper regulation of
21 the caspase cascade plays an important role both in sperm differentiation and testicular maturity
22 (32). In addition, caspases have been implicated in the pathogenesis of multiple andrological
23 pathologies such as impaired spermatogenesis, decreased sperm motility, increased levels of sperm
24 DNA fragmentation, testicular torsion, varicocele and immunological infertility (32). 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 AR in sperm. It emerges from our data that low androgen concentrations stimulate PI3K activity that is inhibited at higher levels. It should be mentioned that in the latter circumstance increases in PTEN phosphorylation and caspases 8, 9 and 3 cleavages 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 (DMSO), anti-rabbit IgG FITC conjugated, Earle's balanced salt solution, Hoechst 33342, steroids and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond™ ECL™, [γ-^{32}P]ATP, 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, 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

1 anti-p-serine, anti-p-threonine, anti-p-AKT1/AKT2/AKT3 S473, anti-p-BCL2, anti-p-PTEN, anti-
2 caspase 8, anti-caspase 9 and anti-caspase 3 antibodies were from Cell Signaling (Milan, Italy).
3 Casodex (Cax) was from Astra Zeneca (Milan, Italy) and Hydroxy-flutamide (OH-Flut) was from
4 Schering (Milan, Italy). The specific caspases inhibitor Z-VAD-FMK (ZVF) was from R&D
5 Systems (Milan, Italy). PCR 2.1 vector was from Promega (Milan, Italy) and the sequencing was by
6 MWG AG Biotech (Ebersberg, Germany).

7 ***Semen samples and spermatozoa preparations***

8 Semen specimens from normozoospermic men were obtained after 3 days of sexual
9 abstinence. The samples were ejaculated into sterile containers and left for at least 30 minutes (min)
10 in order to completely liquefy before being processed. Sperm from ejaculates with normal
11 parameters of semen volume, sperm count, motility, vitality and morphology, according to the
12 WHO Laboratory Manual (33), were included in this study. In each experiment, three normal
13 samples were pooled. Spermatozoa preparation was performed as previously described (34).
14 Briefly, after liquefaction, normal semen samples were pooled and subjected to centrifugation (800
15 *g*) on a discontinuous Percoll density gradient (80:40 % v:v) (33). The 80 % Percoll fraction was
16 examined using an optical microscope equipped with a x100 oil objective to ensure that a pure
17 sample of sperm was obtained. An independent observer, who observed several fields for each slide,
18 inspected the cells. Percoll-purified sperm were washed with unsupplemented Earle's medium and
19 were incubated in the same medium (uncapacitating medium) for 30 min at 37 °C and 5 % CO_2,
20 without (control) or with treatments (experimental). Some samples were incubated in capacitating
21 medium (Earle's balanced salt solution medium supplemented with 600 mg BSA /100 ml and 200
22 mg sodium bicarbonate /100 ml). When the cells were treated with the inhibitors Cax, OH-Flut and
23 ZVF, a pre-treatment of 15 min was performed. The study was approved by the local medical-
24 ethical committees and all participants gave their informed consent.

25 ***RNA isolation and Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)***

1 Total RNA was isolated from human ejaculated spermatozoa and purified as previously
2 described (34). Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease
3 (DNAse) I in single-strength reaction buffer at 37 °C for 15 min. This was followed by heat
4 inactivation of DNAse I at 65 °C for 10 min. Five micrograms of DNAse-treated RNA samples
5 were reverse transcribed by 200 IU M-MLV reverse transcriptase in a reaction vol of 20 µl (0.4 µg
6 oligo-dT, 0.5 mM deoxy-NTP and 24 IU RNAsin) for 30 min at 37 °C, followed by heat
7 denaturation for 5 min at 95 °C. PCR amplification of cDNA was performed with 2 U of Taq DNA
8 polymerase, 50 pmol primer pair for *AR*. These primers were chosen to amplify the region of the
9 DNA binding domain plus the hinge region of the *AR*. Contamination by leucocytes and germ cells
10 in the sperm preparations was assessed by amplifying *PTPRC* and *KIT* transcripts respectively. The
11 applied PCR primers and the expected lengths of the resulting PCR products are shown in Table 1.
12 PCR was carried for 40 cycles using the following parameters: 95°C/1 min, 55°C/1 min, 72°C/2
13 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
14 *PTPRC*. For all PCR amplifications, negative (reverse transcription-PCR performed without M-
15 MLV reverse transcriptase) and positive controls (LnCap for *AR*, human testis for *KIT* and human
16 leucocytes for *PTPRC*) were included. The PCR-amplified products were subjected to
17 electrophoresis in 2 % agarose gels stained with ethidium bromide and visualised under UV
18 transillumination.
19 ***Gel extraction and DNA sequence analysis***
20 The *AR* RT-PCR product was extracted from the agarose gel by using a gel band purification
21 kit, the purified DNAs was subcloned into PCR 2.1 vector and then sequenced.
22 ***Western blot analysis of sperm proteins***
23 Sperm samples washed twice with Earle's balanced salt solution (uncapacitating medium),
24 were incubated for 30 min without or with the treatments indicated in the figures. During Western
25 blot analysis, sperm samples were processed as previously described (34). The negative control was
26 performed using a sperm lysate that was immunodepleted of AR (i.e. preincubation of lysates with

anti-AR antibody for 1 hour (h) at room temperature and immunoprecipitated with Protein A/G-agarose) (16). 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 (1:200) as primary antibody and an anti-mouse IgG FITC-conjugated (1:100) as secondary antibody. To stain DNA in living cells, Hoechst 33342 (Hoechst) was added at a final concentration of 10 µg/ml. The specificity of AR was tested by using normal mouse serum instead the primary antibody (Fig. 2 D); sperm cells incubated without the primary antibody were also used as negative controls (data not shown). The cellular localization of AR and Hoecst was studied with Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135 M inverted microscope with 600 magnification. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap, scoring a minimum of 200 spermatozoa per slide.

Immunoprecipitation of sperm proteins

Spermatozoa were washed in Earle's balanced salt solution and centrifuged at 800 x *g* for 20 min. Sperm were resuspended in the same uncapacitating medium and incubated without (control, UC) or in the presence of DHT at increasing concentrations (0.1 nM, 1 nM, 10 nM, 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 min, 30 min, 1 h). To avoid non-specific binding, sperm lysates

1 were incubated for 2 h with protein A/G-agarose beads at 4 °C and centrifuged at 12,000 x *g* for 5
2 min. The supernatants (each containing 600 µg total protein) were then incubated overnight with 10
3 µl anti-AR and 500 µl HNTG (IP) buffer (50 mM HEPES, pH 7.4; 50 mM NaCl; 0.1% Triton X-
4 100; 10% glycerol; 1 mM phenylmethylsulfonylfluoride; 10 µg/ml leupeptin; 10 µg/ml aprotinin; 2
5 µg/ml pepstatin). Immune complexes were recovered by incubation with protein A/G-agarose. The
6 beads containing bound proteins were washed three times by centrifugation in immunoprecipitation
7 buffer, then denaturated by boiling in Laemmli sample buffer and analyzed by Western blot to
8 identify the coprecipitating effector proteins. Immunoprecipitation using normal mouse serum was
9 used as negative control. Membranes were stripped of bound antibodies by incubation in glycine
10 (0.2 M, pH 2.6) for 30 min at room temperature. Before reprobing with the different indicated
11 antibodies, stripped membranes were washed extensively in TBS-T and placed in blocking buffer
12 (TBS-T containing 5% milk) overnight.

13 ***Evaluation of sperm viability***

14 Viability was assessed by using Eosin Y method. Spermatozoa were washed in
15 uncapacitating medium and centrifuged at 800 x *g* for 20 min. To test androgen effects on sperm
16 viability, spermatozoa were incubated in unsupplemented Earle's medium at 37 °C and 5 % CO_2
17 without (control, UC) or in the presence of dihydrotestosterone (DHT) at increasing concentrations
18 (0.1 nM, 10 nM, 100 nM) or 10 nM testosterone (T) for 2 h. In a different set of experiments, sperm
19 were incubated in unsupplemented Earle's medium at 37 °C and 5 % CO_2 without (UC) or in the
20 presence of 10 nM DHT or T at different times (0 min, 10 min, 30 min, 2 h, 6 h and 24 h). Some
21 samples were pre-treated for 15 min with 10 µM OH-Flut. 10 µl of Eosin Y (0.5 % in PBS) were
22 mixed with an equal volume of sperm sample on a microscope slide. The stained dead cells and live
23 cells that excluded the dye, were scored among a total of 200 cells and by an independent observer.
24 Further, viability was evaluated before and after pooling the samples.

1 ***PI3K activity***

2 Spermatozoa were washed in Earle's balanced salt solution and centrifuged at 800 x *g* for 20
3 min. Sperm were resuspended in the same uncapacitating medium and in different tubes containing
4 no androgens (control, UC), T or DHT at the indicated concentrations for 30 min. Some samples
5 were resuspended in capacitating medium (Earle's balanced salt solution medium supplemented
6 with 600 mg BSA / 100 ml and 200 mg sodium bicarbonate / 100 ml). Some samples were
7 pretreated for 15 min with 10 μM OH-Flut alone or each combined with increasing (0.1 nM, 1nM,
8 10 nM, 100 nM) DHT. The negative control was performed using a sperm lysate, where p110
9 catalyzing subunit of PI3K was previously removed by preincubation with the respective antibody
10 (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose. The
11 PIK3R1 was precipitated from 500 μg of sperm lysates. The immunoprecipitates were washed once
12 with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4) and finally with 10 mM Tris, 100 mM
13 NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by
14 incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50
15 μM ATP, 20 μCi [γ-^{32}P] ATP, and 10 μg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2) for
16 20 min at 37 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were
17 extracted with 200 μl $CHCl_3$/methanol. Phase separation was facilitated by centrifugation at 5000
18 rpm for 2 min in a tabletop centrifuge. The labelled products of the kinase reaction, the PI
19 phosphates, in the lower chloroform phase were spotted onto *trans*-1,2-diaminocyclohexane-
20 *N,N,N*',*N*'-tetraacetic acid–treated silica gel 60 thin-layer chromatography plates state running
21 solvent used for TLC. Radioactive spots were visualized by autoradiography.

22 ***DNA laddering***

23 DNA laddering was determined by gel electrophoresis. Spermatozoa were washed in Earle's
24 balanced salt solution and centrifuged at 800 x *g* for 20 min, then were resuspended in the same
25 uncapacitating medium and in different tubes containing no androgens (control, UC), T or DHT or

1 estrogen (E) or progesterone (PRG) or wortmannin (W) at the indicated concentrations for 30 min.
2 Some samples were resuspended in capacitating medium (CAP). Some samples were pretreated for
3 15 min with 10 μM Cax or 10 μM OH-Flut or ZVF alone or each combined with 10 nM DHT.
4 After incubation cells were pelletted at 800 x *g* for 10 minutes. The samples were resuspended in
5 0.5 ml of extraction buffer (50 mM Trios-HCl pH 8, 10mM EDTA, O.5% SDS) for 20 minutes in
6 rotation at 4 °C. DNA was extracted with phenol/chloroform for 3 times and once with chloroform.
7 The aqueous phase was used to precipitate acids nucleic with 0.1 volumes or of 3 M sodium acetate
8 and 2.5 volumes cold EtOH overnight at -20 °C. The DNA pellet was resuspended in 15 μl of H_2O
9 treated with RNAse A for 30 minutes at 37 °C. The absorbance of the DNA solution at 260 and 280
10 nm was determined by spectrophotometry. The extracted DNA (2 μg/lane) was subjected to
11 electrophoresis on 1.5 % agarose gels. The gels were stained with ethidium bromide and then
12 photographed.

13 STATISTICAL ANALYSIS

14 The experiments for RT-PCR, Immunofluorescence and Immunoprecipitation assays were
15 repeated on at least four independent occasions, Western blot analysis was performed in at least six
16 independent experiments, PI3K activity was performed in at least four independent experiments.
17 The data obtained from viability (six replicate experiments using duplicate determinations) were
18 presented as the mean ± SEM. Statistical analysis was performed using analysis of variance
19 (ANOVA) followed by Newman-Keuls testing to determine differences in means. $p<0.05$ was
20 considered as statistically significant.

21 **RESULTS**

22 ***AR mRNA and protein were detected in human sperm***

23 To determine whether mRNA for *AR* is present in human ejaculated spermatozoa, RNA
24 isolated from percoll-purified sperm samples from normal men was subjected to reverse PCR. The

1 nucleotide sequence of *AR* was deduced from the cDNA sequence of the human *AR* gene and our
2 primers amplified a region from 1648 to 2055 bp corresponding to the DNA binding domain plus
3 the hinge region of the AR. RT-PCR amplification of *AR* in human sperm revealed the expected
4 PCR product size of 400 bp. This product was sequenced and found identical to the classical human
5 *AR*. No detectable levels of mRNA coding either *PTPRC*, a specific marker of leucocytes, or *KIT*, a
6 specific marker of germ cells, were found in the same semen samples (Fig 1 A), thus ruling out any
7 potential contamination.
8 The presence of AR protein in human ejaculated spermatozoa was investigated by Western
9 blot using a monoclonal antibody raised against the epitope mapping at the 299-316 aa in the N-
10 terminus of AR from human origin (Fig. 1B). The antibody revealed in sperm two protein bands
11 with molecular weights of approximately 110 kDa and 85-87 kDa, the latter expressed to a greater
12 extent. The negative control (N) was performed as described in *Materials and method*.
13 ***Immunolocalization of AR in human sperm***
14 Using an immunofluorescence technique, we identified a positive signal for AR in human
15 spermatozoa (Fig. 2A). No immunoreaction was detected either by replacing the anti-AR antibody
16 by normal mouse serum (Fig. 2D) or when the primary antibody was omitted (data not shown),
17 demonstrating the immunostaining specificity. AR immunoreactivity was specifically
18 compartmentalized at the sperm head (Fig. 2A), where the DNA is packaged as it can be seen in
19 Fig. 2B in which the DNA is stained by Hoechst. Fig. 2C shows the merged images of A and B.
20 ***AR is phosphorylated in human sperm***
21 It was reported that the function of AR is strongly correlated with the phosphorylation status
22 (35) which is rapidly enhanced upon androgen exposure and it is able to activate signal transduction
23 pathways. AR immunoprecipitates were blotted with three different antibodies: anti-p-tyrosine, anti-
24 p-threonine and anti-p-serine. As shown in Fig. 3, two major AR antibody reactive proteins
25 corresponding to the 85-87 and 110 kDa were observed. To determine if the changes in
26 phosphorylation status of AR under androgen treatments may occur in ejaculated sperm, these were

1 exposed for 30 min to varying concentrations of DHT (0.1 nM to 10 nM). We observed that the AR
2 phosphorylation was enhanced in a dose related manner (Fig. 3 A) and was significantly reduced by
3 OH-Flut, an AR antagonist. To investigate if the enhanced phosphorylation status may represent an
4 early event we performed a time course study at the following times: 0 min, 5 min, 30 min, 1 h. The
5 time course revealed that AR phosphorylation occurred rapidly as they were observed from time 0
6 to 5 min and then dropped significantly after 1 h (Fig. 3 B). Moreover, all three phospho-antibodies
7 demonstrated a prevalence for phosphorylation of the 110 kDa isoform.

8 ***Androgens effect on sperm viability***

9 To evaluate sperm viability under androgen treatment we performed different sets of
10 experiments. Sperm were incubated in the presence of 10 nM T or 10 nM DHT at the indicated
11 times (Fig. 4 A). Other samples were incubated in uncapacitating medium for 2 hours in the absence
12 or presence of different T or DHT concentrations (0.1 nM to 100 nM). As shown in Fig. 4 B the
13 majority of cells remained viable in the control at 2 h. Cell viability significantly decreased with 10
14 and 100 nM T or DHT. Interestingly, the effect of androgen was reversed by using OH-Flut,
15 addressing an AR mediated effect. It deserves to be mentioned that 100 nM androgen concentration
16 is much higher than that commonly found circulating *in vivo* in man while about 3 nM is detected in
17 the seminal plasma (36).

18 ***Androgen action on PI3K activity, p-AKT, p-BCL2 and pPTEN is mediated by AR***

19 As shown in Fig. 5A low androgen concentration induced PI3K activity, while
20 concentrations from 10 to 100 nM reduced the enzymatic activity. Both 10 nM T and to a greater
21 extent 10 nM DHT treatments decreased PI3K activity and this effect was reversed by OH-Flut
22 (Fig. 5 A). Concomitantly, we obtained the reduction of the downstream p-AKT S473 (Fig. 5 B)
23 and of p-BCL2 (Fig. 5 C), a known antiapoptotic protein (37). Specifically, DHT and not
24 testosterone has a significant inhibitory effect on p-AKT S473 levels. Further, androgens

1 significantly increased the phosphorylation of PTEN, a specific inhibitor of PI3K (Fig. 5 D). All the
2 above mentioned effects were reversed by two known antiandrogens, Cax and OH-Flut, that were
3 added alone or combined with 10 nM DHT, indicating that the effects of androgens are mediated by
4 the classic AR in sperm. Recently, it was found that estradiol (E) enhances sperm survival signaling
5 (14). Therefore, we aimed to evaluate in sperm if a functional interaction exists between androgen
6 and estrogen on PI3K activity. In sperm samples incubated with 100 nM E combined with
7 increasing (0.1 nM, 1 nM and 10 nM) DHT concentrations, the estradiol-induced PI3K activity
8 progressively decreases (Fig. 5 E).

9 ***Androgens induce AR, PIK3R1 and phospho-SRC tyrosine kinase coimmunoprecipitation in***
10 ***human sperm***

11 It was reported that a triple complex between AR, PIK3R1, and SRC tyrosine kinase is
12 required for androgen-stimulated PI3K/AKT activation (38, 29), therefore we investigated if it also
13 occurs in sperm. At the 0.1 nM DHT concentration, phospho-SRC tyrosine kinase
14 coimmunoprecipitated with the two proteins immunodetected by the C-19 anti-AR antibody in
15 sperm that migrated at 110 and 85-87 kDa. Remarkably, no association of phospho-SRC tyrosine
16 kinase with AR occurred at the 100 nM DHT concentration. Fig. 6 shows immunocomplexes
17 blotted with anti-AR (A) or phospho-SRC tyrosine kinase (B) or anti- PIK3R1 (C) antibodies. The
18 possibility that androgen treatment could modify the AR level was excluded since the same amount
19 of AR was detected by immunoblot of sperm lysates, irrespective of DHT concentration.

20 Our results demonstrated that, in contrast to the 100 nM DHT concentration, the 0.1 nM
21 concentration increases coimmunoprecipitation of phospho-SRC tyrosine kinase and PI3-kinase
22 with AR. These data may explain the mechanism through which high DHT concentration reduced
23 PI3k activity.

24 ***Androgens effects on caspases are mediated by AR***

1 On the basis of the abovementioned results we sought to evaluate androgen action on the
2 caspases family (31), since these proteins are involved in cell death. Particularly, caspase 3 which is
3 the main effector of both caspases 8 and 9, executes the final disassembly of the cell by cleaving a
4 variety of cell structure proteins and generating DNA strand breaks. Our study revealed the
5 caspases 8, 9 and 3 activation upon 30 min of 10 nM T or 10 nM DHT treatments. The DHT effect
6 was reversed by both AR antagonists, 10 μM Cax or 10 μM OH-Flut (Fig. 7A). Notably, the effect
7 on caspases is specific for androgen as it was not observed with estradiol or progesterone
8 treatments. Particularly, progesterone treatment was performed because of the similarity in structure
9 between Progesterone Receptor and AR. The cleavage of caspase 3 was increased by androgens in a
10 dose-dependent manner (Fig. 7C). In the presence of wortmannin, a specific inhibitor of PI3K
11 activity, the cleavage of caspase 3 was also observed, addressing a regulatory role of PI3K in
12 caspase activation in sperm. Furthermore, in order to demonstrate a specific effect on caspase
13 activation an additional control experiment was included showing that activation of caspases by
14 androgens can be inhibited with a specific caspases inhibitor such as ZVK**.** All these data were
15 confirmed by DNA laddering assay (Fig. 7B and Fig. 7D).

16 **DISCUSSION**

17 Androgens and AR have been shown to play critical roles in testis function (39). AR has
18 been detected in Sertoli, Leydig, peritubular myoid, and spermatid cells (round and elongated) (4, 5,
19 6). The currently prevailing view is that sperm does not contain AR and this stems from previous
20 studies reporting that no AR immunostaining of germ cells was observed both in rat and in human
21 testis (6). However, several studies reported that in spermatozoa the binding capacity of androgens
22 was greater than that of estrogens or progesterone (40, 41) and recently AR was shown to be present
23 in sperm by Western Blot and Immunofluorescence assays (10).

24 In this study we have demonstrated the presence of AR in human sperm at different levels:
25 mRNA expression, protein expression and immunolocalization. By RT-PCR we amplified a gene

1 region corresponding to the DNA binding domain plus the hinge region of the human AR. This
2 product was sequenced and found to be identical to the classical human *AR*. As it concerns the
3 presence of mRNAs in mammalian ejaculated spermatozoa, originally it was hypothesized that
4 these transcripts were carried over from earlier stages of spermatogenesis, however new reports re-
5 evaluate the significance of mRNA in these cells (42, 43) and the issue is currently under
6 investigation.

7 To date, multiple isoforms of the AR have been described and among them two proteins
8 were well characterized: AR-B and AR-A (44). They are believed to be derived from the same gene
9 and differ only in the NH_2-terminal transactivation domain (44). Our antibody against an epitope
10 (aminoacids 299–311) that is common to both the AR-A and AR-B isoforms detected two protein
11 bands: one of 85-87 kDa and another one approximately of 110 kDa. Both, the AR-B and AR-A
12 isoforms are expressed in a variety of fetal and adult (male and female) human tissues and
13 especially in reproductive tissues (45). The B form migrates with an apparent mass of 110 kDa and
14 constitutes more than 80% of the immunoreactive receptor in most cell types. The A form of the AR
15 migrates with an apparent mass of 87 kDa. It was identified as an NH_2-terminally (from 1 to 187 aa)
16 truncated protein of AR-B and it was first described in human genital skin fibroblasts. The detection
17 of two distinct forms of the AR raised a number of issues. AR-A is expressed at low levels in many
18 androgen-responsive tissues; however, it appears to have functions similar to those of the full-length
19 AR-B isoform. Functional activities of cDNAs containing the two isoforms, were assessed using
20 cotransfection assays that employed two models of androgen-responsive genes (MMTV-luciferase
21 and PRE2-tk-luciferase) in response to mibolerone, a potent androgen agonist, in three different cell
22 lines (46). These studies demonstrated subtle differences in the activities of the A and B isoforms,
23 which depended on the promoter and cell context. Additional studies failed to reveal any major
24 differences in the responses of the AR-A and AR-B isoforms to a variety of androgen agonists and
25 antagonists, suggesting that the previously reported functional defect of the AR-A is due principally
26 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. 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.* (10) 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 to process samples.

An increasing body of evidence suggests that androgens and other steroid hormones can exert rapid, nongenomic effects (12, 14). Different nuclear receptors such as progesterone receptor (15), estrogen receptor α and estrogen receptor β (16) 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 (14) which may represent their exclusive modality of action 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 (44, 45) 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.

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 (16). 0.1 nM and 1 nM androgens induced PI3K activity which was reduced by higher concentrations (10 nM and 100 nM). 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 as it was reported depending on androgen concentration in somatic cells (38, 29). In our study high androgen concentrations (10 nM and 100 nM) produce a detachment of SRC tyrosine kinase from the PIK3R1 /AR complex, confirming that the triple complex is needed to the PI3K pathway activation. Furthermore, sperm treatment with wortmannin, a specific PI3K/AKT inhibitor, induced caspase 3 cleavage 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 nM 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 estradiol-induced PI3K activity already previously documented (16).

It is well established that in men intratesticular testosterone levels are approximately 800 nM (47), while they are ranging from 16 to 20 nM in serum (47, 36). The androgenic milieu in seminal plasma is dependent on circulating androgen levels and no longer intratesticular levels (48, 49). The biologically active amount of T, represented by its free fraction, is mostly converted in the genital tract in DHT by 5 alpha-reductase which is particularly expressed in the epydidimus and in the adnexal glands (50). 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 (49). Therefore, the seminal androgenic milieu, prevalently represented by the total molar concentration

1 of T plus DHT corresponds to about 3 nM. The effects induced by the dose of 10 nM DHT were
2 opposite to those induced by the lower dose of androgens. On the other hand, the same opposite
3 pattern of androgen effects according to the doses tested on PI3K pathway was previously
4 documented in other cellular type (29).

5

6 From all these findings, it emerges that an excess of androgens in the local hormonal
7 milieu through a PI3K activity inhibition, may negatively interfere in the sperm survival.
8 Concluding, in human sperm AR is able to modulate PI3K/AKT pathway on the basis of the
9 androgen levels. Further work will be required to more fully elucidate the role that AR plays in
10 male fertility.

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Acknowledgments

Our special thank to Domenico Sturino (Faculty of Pharmacy, University of Calabria – Italy) for the English review of the manuscript and to Dr. Vincenzo Cunsolo (Biogemina SAS, Catania – Italy).

FIGURE LEGENDS

FIG 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, S3). LNCap cells were used as positive control. (N), negative control performed as described in *Materials and method*. The experiments were repeated at least four times and the autoradiographs of the figure show the results of one representative experiment.

FIG 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/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.

FIG 3 AR is phosphorylated in human sperm

1 AR phosphorylation was determined by immunoprecipitation using an AR specific antibody. The
2 immunoprecipitates were blotted with three different antibody: anti-p-tyrosine (pTyrAR), anti-p-
3 threonine (pThrAR) and anti-p-serine (pSerAR). **A**: Sperm were incubated without (control, UC) or
4 in the presence of DHT at increasing concentrations (0.1 nM, 1 nM, 10 nM) for 30 min. Some
5 samples were pre-treated for 15 min with 10 μM OH-Flut. The autoradiographs presented are
6 representative examples of experiments that were performed at least four times with repetitive
7 results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on
8 the right of each blot are the quantitative representation after densitometry of data (mean ± S.D.) of
9 four independent experiments. •$P < 0.05$, ••$P < 0.01$ DHT-treated *vs* untreated cells and *$p<0.05$ 10
10 nM DHT *vs* 0.1 nM DHT. **B**: Time course of sperm incubated without (control, UC) or in the
11 presence of 10 nM DHT. Six hundred micrograms of sperm lysates were immunoprecipitated using
12 anti-AR and then blotted with specific antibodies raised to anti-p-tyrosine, anti-p-serine, anti-p-
13 threonine, anti-AR. Immunoprecipitation by using normal mouse serum was used as negative
14 control (N). The autoradiographs presented are representative examples of experiments that were
15 performed at least four times with repetitive results. Molecular weight markers are indicated on the
16 right of the blot. The histograms indicated on the right of each blot are the quantitative
17 representation after densitometry of data (mean ± S.D.) of four independent experiments. $P < 0.05$,
18 ••$P < 0.01$ DHT-treated *vs* untreated cells.

19 **FIG 4 Effect of androgen on sperm viability**

20 Viability was assessed by using Eosin Y as described in *Materials and Method*. **A**: Time course of
21 sperm incubated in the unsupplemented Earle's medium at 37 °C and 5 % CO_2 without (UC) or in
22 the presence of 10 nM T or 10 nM DHT. **B**: Sperm were incubated without (UC) or in the presence
23 of T or DHT at increasing concentrations (0.1 nM, 10 nM, 100 nM) for 2 h. Some samples were
24 pretreated for 15 min with 10 μM OH-Flut and then treated with 10 nM T or DHT. All experiments

1 were repeated at least six independent times with duplicate samples, and the values represent the

2 mean ± SEM. •P < 0.05, ••P < 0.01, •••P < 0.005 versus control.

3 **FIG 5 Androgens action on PI3K activity, p-AKT , p-BCL2 and pPTEN is mediated by AR**

4 Washed pooled sperm from normal samples were incubated in the unsupplemented Earle's medium

5 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

6 at increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM) for 30 min. 500 μg of sperm lysates

7 were used for PI3K activity in sperm incubated at the indicated DHT concentatrions in the absence

8 or in the presence of 10 μM OH-Flut (**A**). The autoradiograph presented is representative example

9 of experiments that were performed at least four times with repetitive results. The histograms

10 indicated on the bottom of the figure are the quantitative representation after densitometry of data

11 (mean ± S.D.) of four independent experiments. ••P < 0.01 T and DHT-treated *vs* untreated cells,

12 **p<0.01 10 nM and 100 nM DHT *vs* 0.1 nM and 1 nM DHT, ◆◆ p<0.01 and ◆ p<0.05 10 μM

13 OH-Flut plus DHT-treated *vs* DHT treated cells. 50 μg of sperm lysates were used for western blot

14 analysis of p-AKT S473 (**B**), p-BCL2 (**C**) and p-PTEN (**D**). The autoradiographs presented are

15 representative examples of experiments that were performed at least six times with repetitive

16 results. The histograms indicated on the right of each blot are the quantitative representation after

17 densitometry of data (mean ± S.D.) of six independent experiments. ••P < 0.01 Capacitated (CAP)

18 or DHT-treated *vs* untreated cells, •P < 0.05 T-treated *vs* untreated cells, ◆ p<0.05 10 and ◆◆

19 p<0.01 μM OH-Flut plus DHT or Cax plus DHT-treated *vs* DHT treated cells. **E**: PI3K activity of

20 sperm incubated with estradiol (E) and/or DHT at the indicated increasing concentrations. The

21 negative controls were performed using a sperm lysate, where p110 catalyzing subunit of PI3K was

22 previously removed by preincubation with the respective antibody (1 h at room temperature) and

23 subsequently immunoprecipitated with protein A/G-agarose (N). The autoradiographs presented are

24 representative examples of experiments that were performed at least four times with repetitive

1 results. The histograms indicated on the bottom of the figure are the quantitative representation after
2 densitometry of data (mean ± S.D.) of four independent experiments. ••P < 0.01 DHT- and E-
3 treated *vs* untreated cells, ◆ $p<0.05$ 10, ◆◆ $p<0.01$ and ◆◆◆ $p<0.001$ E plus DHT-treated vs DHT-
4 trated cells.

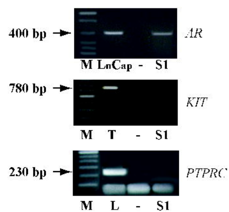
5 **Fig 6 AR, PIK3R1 and phospho-SRC tyrosine kinase coimmunoprecipitate in human sperm**.

6 Washed spermatozoa from normal samples were incubated in the unsupplemented Earle's medium
7 for 30 min at 37 °C and 5% CO_2, without (UC) or in the presence of DHT at increasing
8 concentrations (0.1 nM, 1 nM, 10 nM, 100 nM). 700 µg of sperm lysates were immunoprecipitated
9 using anti-AR antibody and then blotted with specific antibodies raised to AR (A), p-SRC tyrosine
10 kinase (B) and PIK3R1 (C). LnCap lysates were used as positive control (lane 1);
11 Immunoprecipitation by using normal mouse serum was used as negative control (N). The
12 autoradiographs presented are representative examples of experiments that were performed at least
13 four times with repetitive results. Molecular weight markers are indicated *on the left* of the blot.

14 **Fig 7 Androgens effects on caspases are mediated by AR**

15 **A:** Washed pooled sperm from normal samples were incubated in the unsupplemented Earle's
16 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
17 30 min. Some samples were washed with the unsupplemented Earle's medium and incubated in
18 capacitating medium (CAP). Some samples were treated with Cax or Flut or ZVF each alone or
19 combined with 10 nM DHT. The sperm were lysed and subjected to western blot analysis. 70 µg of
20 sperm lysates were used for western blot analysis of caspase 8, caspase 9 and caspase 3. **B:** DNA
21 laddering was performed in sperm treated as indicated. **C:** Effect of increasing DHT concentrations
22 (0.1 nM to 100 nM), 100 nM PRG and 10 µM wortmanninn (W) on caspase 3 cleavage. The
23 experiments were repeated at least six times and the autoradiographs of the figure show the results
24 of one representative experiment. **D:** DNA laddering was performed in sperm treated as indicated.

A



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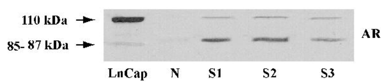
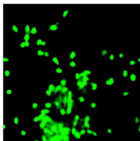


Fig. 1

A



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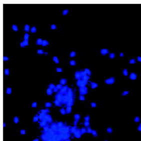

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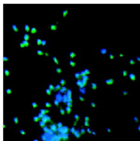

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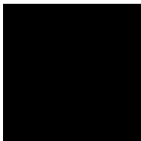
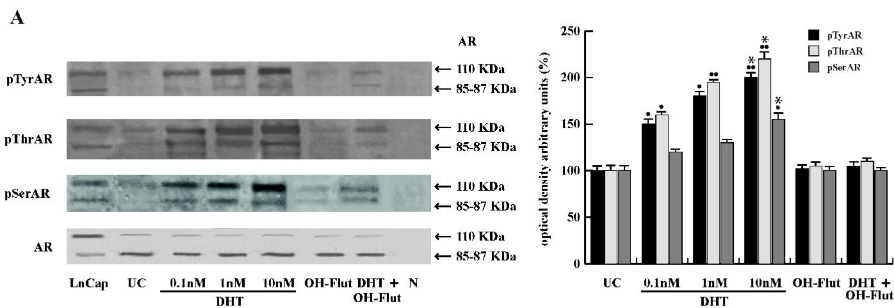
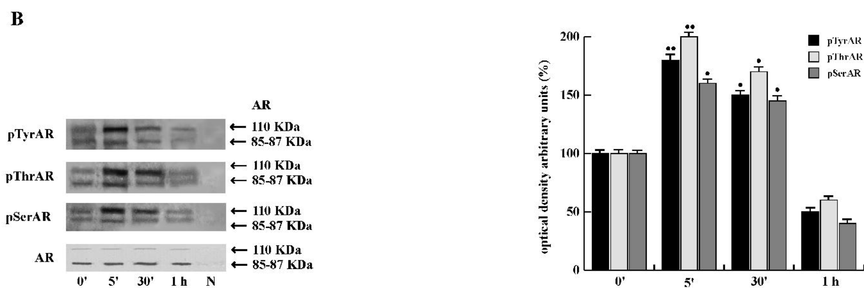


Fig. 2





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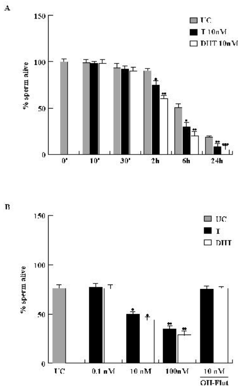

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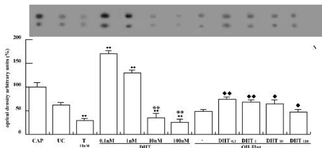


B p-AKTS473 →

actin →

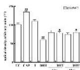
C p-BCL2 →

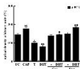
actin →

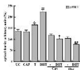
D p-PTEN →

actin →

UC CAP T 10nM DHT 10nM - DHT Cav - DHT OH-Flut







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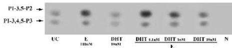


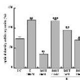
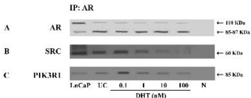


Fig. 5



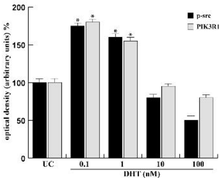


Fig. 6

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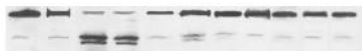

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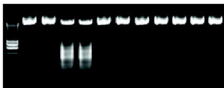

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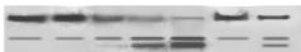

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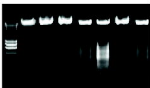

Fig. 7

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

Program & Abstracts

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Association analysis in a cohort of 710 breast cancer patients revealed a poor disease free survival (DFS) in insLQ carriers (HR=1.40; 95%CI (1.14-1.71); P=0.001). This negative effect of the insLQ-LHR on DFS was stronger in premenopausal women who have an active HPG axis (HR=1.59; 95%CI (1.15-2.20); P=0.005). Premenopausal carriers of the GnRH-16S-allele showed a borderline significant HR of 1.34 (95%CI: 0.97-1.85; P=0.08) for DFS. Finally, premenopausal patients that are carriers of both polymorphisms showed an increased HR of 2.1 (95%CI: 1.31-3.29; P=0.002). We conclude that the combined presence of the insLQ-LHR and GnRH-16S variants negatively affects DFS in breast cancer patients, and, when validated, these findings may lead to tailored treatment of breast cancer patients.

OR31-2

Molecular Mechanism through Which Leptin Upregulates E-Cadherin Expression in Breast Cancer. *In Vitro* and *In Vivo* Effects on Tumor Cell Growth and Progression.

Loredana Mauro[1], Michele Pellegrino[1], Stefania Catalano[1], Gianluca Bossi[3], Ines Barone[2], Sara Morales[1], Cinzia Giordano[1,2], Francesca Giordano[1], Viviana Bartella[1], Maria Luisa Panno[1], Sebastiano Andò*[1,2]. *[1]Cell Biol; [2]Ctr Sanitario, Univ of Calabria, Arcavacata, Cosenza, Italy; [3]Molec Oncol, Regina Elena Cancer Inst, Roma, Italy.*

Obesity is a risk factor for breast cancer in postmenopausal women. Leptin (lep) is an adipocyte-derived cytokine which can stimulates the proliferation of various cell lines, such as colon, renal, breast and prostate cancer cells. We have previously demonstrated that lep induces proliferation of the human breast cancer cell line MCF-7 in monolayer cultures. In the present study we investigated the effect of lep in three-dimensional cultures, which reproduce tumor *in vivo* situation as it concerns cell shape and environment. Under these conditions we observed a higher growth rate compared to monolayer cultures (monolayer cultures: lep=170%; 3-D cultures: lep=216%, *vs* C=100%). Thus, we focused our attention on the role of lep on homotypic cell-cell adhesion signaling in enhancing tumor cell growth. Our results demonstrated that lep was able to upregulate E-cadherin (E-cad) at mRNA and protein levels through a direct activation of E-cad promoter as it emerges from functional studies, using a plasmid containing human E-cad promoter (-1008/+49), conjugated with a luciferase reporter gene. Among the cis elements located on the regulatory region of E-cad gene, which may be crucial in modulating the upregulatory effect of lep, we identified a CREB site, located at the position -918. Indeed, when we used the deleted promoter construct (-83/+49), lacking the CREB site, the upregulatory effect induced by lep was no longer noticeable. Stemming from our recent data demonstrating that lep in breast cancer amplifies estradiol (E_2) signaling, we studied the combined effect of lep and E_2 on E-cad transcriptional activity. Our results revealed that the combined exposure to lep and E_2 display an additive effect in activating E-cad promoter respect the two separate treatments. Interestingly, the additive effect of lep and E_2 in enhancing the homotypic cell adhesion well fits with the stimulatory effect induced by both hormones in a xenograft model of MCF-7 cells in nude mice. Indeed, the combined treatment of the two hormones induces a higher tumor growth rate respect to the separate treatments after 8 weeks (C=0.1 cm^3, lep=0.3 cm^3, E_2=0.7 cm^3, lep+E_2=1.7 cm^3). The present study provides new insights into the mechanism through which leptin may affect tumor mass and tumor cell proliferation. This address to design novel pharmacological tools able to antagonize the stimulatory effect of leptin in breast cancer progression, particularly in postmenopausal obese women.

OR31-3

Dutasteride Affects Progesterone Metabolizing Enzyme Activity/Expression in Human Breast Cell Lines Resulting in Suppression of Cell Proliferation and Detachment.

John P Wiebe*[1], Lesley Souter[1], Guihua G Zhang[1]. *[1]Biol, Univ of Western Ontario, London, ON, Canada.*

Recent evidence indicates that progesterone (P) metabolites play important roles in regulating breast cancer. Previous studies showed that breast tumor tissue and tumorigenic cell lines have higher 5α-reductase (5α-R) and lower 3α-hydroxysteroid oxidoreductase (3α-HSO) and 20α-HSO activities and mRNA expression levels than normal tissue and non-tumorigenic cell lines [1-3]. The 5α-reduced P, 5α-dihydroprogesterone (5αP), promotes both mitogenic and metastatic activity, whereas the 4-pregnene metabolites, 3α-dihydroprogesterone (3αHP) and 20α-dihydroprogesterone (20αHP), have the opposite (anti-cancer-like) effects [1,4,5]. The type 1 and 2 5α-R inhibitor dutasteride (DU) has been shown to block 5α-reduction of testosterone to 5α-DHT in prostate tissue, resulting in decreased prostate volume. The aim of this study was to determine if DU affects P 5α-R and HSO activities and gene expression and if the resultant changes affect mammary cell proliferation and detachment. The effect of DU on progesterone metabolizing enzyme (PME) activities and mRNA expression were examined in tumorigenic (MCF-7) and non-tumorigenic (MCF-10A) human breast cell lines using radioisotopic metabolism and real-time PCR methods. DU (10^{-6} M) inhibited P conversion to 5α-pregnanes by >95% ($p<0.001$) and at the same time increased ($p<0.001$) 4-pregnene production. The DU actions were due to competitive inhibition of 5α-R and decreases ($p<0.01$-0.001) in 5α-R mRNA and concomitant increases in HSO activity ($p<0.001$) and mRNA expressio[n] ($p<$0.001). The resulting decrease in ratio of pro-cancer:anti-cancer hormo[nes] significant ($p<0.001$) inhibition of cell proliferation and detachment. This s[tudy is the] first to consider the effects of DU on P metabolism in human breast cancer cells, [and provides] the first evidence that DU not only suppresses P 5α-R but also increases P 3α[-] HSOs, leading to a marked shift toward production of anti-cancer hormon[es] inhibition of breast cancer cell growth and detachment.

[1] Cancer Res. 60:936-943, 2000.
[2] BMC Cancer 2003, 3:9
[3] BMC Cancer 2004, 4:27
[4] Endocrine 16:7-14, 2001.
[5] J Steroid Biochem Mol Biol 93:201-208, 2005

Supported by grants from GlaxoSmithKline and The Susan G. Komen Brea[st] Foundation.

OR31-4

Androgen Effects on Osteoblastic Differentiation of Prim[ary] Mouse Osteoblasts and Marrow-Derived Mesenchymal S[tem] Cells: Modulation by Prostate Cancer Cells (LAPC-4).

R Jasuja*[1,2], E Fu[3], C Magyar[4], J Flanagan[2], J Refela[3], R Miki[3], J Tchieu[3], F Petrigliano[5], R Singh[1], N K LeBrasseur[2], C A Morris[2], L Chao[3], P Cho[3], N C[...] J R Lieberman[5], S Bhasin[1,2], S Tetradis[3], C AM Jamieson[3]. *[1]Endocrinol, C R D[rew] Univ of Med, Los Angeles, CA; [2]Sect of Endocrinol, Boston Univ, Boston, MA; [3]Urology and Hum Genet, Univ of California, Los Angeles, CA; [4]Oral Radio[l] of California, Los Angeles, CA; [5]Orthoped Surg, Univ of California, Los An[geles,] CA; [6]Radiat Oncol, Univ of California, Los Angeles, CA.*

Androgens play a central role in prostate cancer growth and regulate the [...] between osteoblasts/ osteoclasts during bone growth. Prostate cancer often met[astasizes] to bone; these metastatic lesions are osteoblastic while metastatses from seve[ral] cancers are osteolytic. We therefore considered the possibility that prostate can[cer] might modulate osteoblastic differentiation of bone progenitor cells through [...] mediated pathway.

Objectives: We determined the effect of 5-α dihydrotestosterone (D[HT], a] nonaromatizable androgen, on osteogenic differentiation of primary mouse ost[eoblasts] (mOB) and mouse marrow-derived mesenchymal stem cells (mMSC). Addition[ally, we] evaluated the effects of co-culture with androgen-responsive prostate cancer cells (L[APC-] 4) on mMSC differentiation and responsiveness to DHT.

Results: Incubation of *ex-vivo* differentiated primary osteoblasts (deriv[ed from] calvariae) with DHT shifted their cell cycle profile primarily to Go/G1 from [...] absence of DHT. Concomitant with the changes in cell cycle, DHT treatment in[creased] the intensity of alkaline phosphatase staining, an osteogenic differentiation mar[ker, and] mRNA expression of key osteogenic differentiation genes, including oste[...] osteopontin, and alkaline phosphatase, as assessed by real time qPCR. Subse[quently,] using microarray chips containing the MEEBO (mouse exonic evidence-based oligonucleotides) set of 38,000 oligos, the cluster analyses were performed to [...] the genes involved in osteogenic differentiation program and markers of ce[ll cycle] progression. In co-culture experiments involving LAPC-4 and mMSCs, the pre[sence of] prostate cancer cells increased osteogenic differentiation of mMSCs several f[old over] corresponding controls. Remarkably, the androgenic modulation of ost[eogenic] differentiation in these co-cultures was LAPC4/mMSC cell ratio dependent.

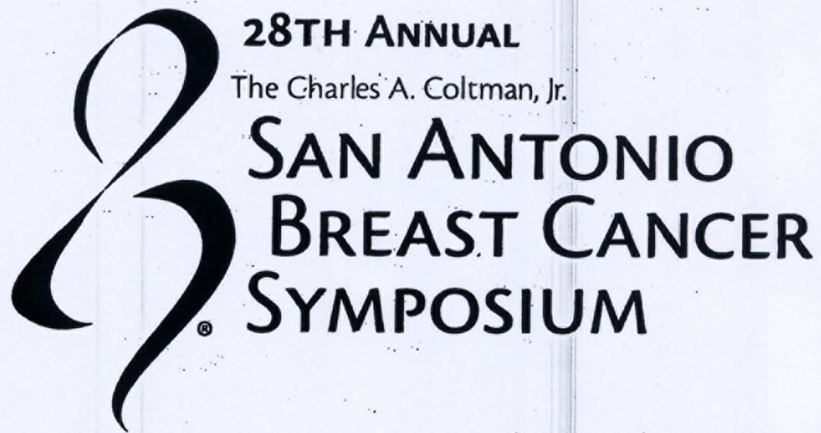
Conclusions: DHT treatment promotes osteogenic differentiation of [...] osteoblasts under osteogenic conditions. Prostate cancer cells stimulate primar[y] bone marrow derived MSCs towards osteogenic differentiation and modu[late the] osteoblastic response of mMSCs to DHT treatment. These data provide an in-vit[ro model] to examine the mechanism of androgen action on osteogenic differentia[tion and] androgenic modulation of osteoblastic lesions in prostate cancer bone metas[tases.]

OR31-5 ⊗

Function of Caveolin1 Phosphorylation in Breast Cance[r]

Ayesha N Shajahan*[1], Robert Clarke[1]. *[1]Oncol, Lombardi Comprehensive Ca[ncer Ctr,] Georgetown Univ Med Ctr, Washington, DC.*

Breast cancer is the most common type of cancer diagnosed in women in th[e] world. In breast tumorigenesis, disruption of the rate of cell prolifera[tion and] differentiation is partly mediated by the steroid hormone estrogen (E2). Blockin[g E2] effects in breast cancer by using antiestrogens, which act by binding to the e[strogen] receptor alpha (ERalpha), is a primary method of treatment for this disease. A[lthough] antiestrogen therapy is an effective treatment option for many, resistance to anti[estrogens] remains a major setback. Since E2 is also vital for the normal development of the m[ammary] gland, it is plausible that a parallel pathway exists that protects the gland from [...] effects of estrogen leading to carcinogenesis. Therefore, insight into the mecha[nism of] how breast epithelial cells become resistant to E2 could help uncover pathw[ays]



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

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

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

6106

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

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

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

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

6107

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

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

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

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

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

6108

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

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

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

...t study was supported by KOSEF grant.

...Nonclassic Progesterone Receptors Are Both ... Human Spermatozoa.

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

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

...y DFG and BMBF is gratefully acknowledged.

...Junction Dynamic Regulator during ... the Rat Testis.

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

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

...ICHD U01 HD045908

...re System for the Study of ...

...a Sim[1], Louisa Ludbrook[1], Michael J ... *...enry's Inst of Med Res, Clayton, Victoria, ... Biol, Monash Univ, Clayton, Victoria, ...*

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

P1-285

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

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

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

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

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

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

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

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

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

P1-286 ♦

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

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

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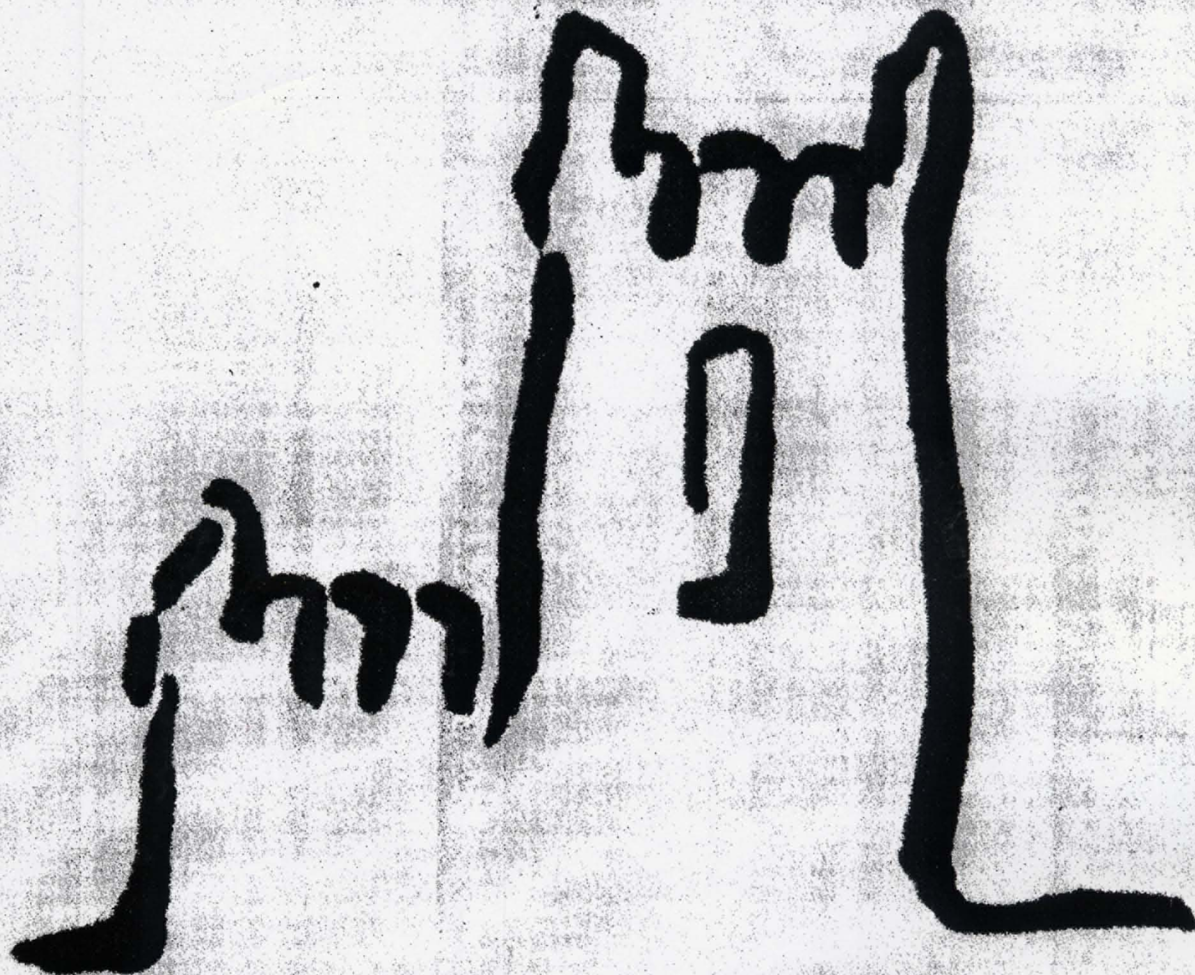

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P1015 Oncology - Series B

A new role of leptin as amplifier of estrogen signaling in breast cancer

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Background of Study: Leptin produced predominantly by adipose tissue, is able to stimulate normal and tumor cell growth. We demonstrated that leptin up-regulates aromatase gene expression in MCF-7 cells evidencing its important role in in situ estradiol production and estrogen-dependent breast cancer progression. Estrogen receptor (ERα) that plays an essential role in breast cancer development, can be transcriptionally activated in ligand-independent manner through MAPK signal.
Objectives: Sine leptin is able to activate the MAPK pathway, we investigated the ability of leptin to transactivate ERα.
Methods: In MCF-7 cells we examined through immunocytochemical staining, RT-PCR and Western Blotting the expression of ERα upon leptin treatment. Functional studies, in MCF-7 and HeLa cells, with a ERE reporter gene and ERα wild type or different deleted ERα constructs were performed under E2, TAM and/or leptin treatments.
Results: Leptin was able to reproduce the classical features of ERα transactivation: a nuclear localization and a down-regulation of its mRNA and protein levels. Transfection studies revealed that N-terminal transciptional activation functional 1 appears essential for ERα response to leptin. As result of the distinct action of E2 and leptin on the two functional domains respectively the combined exposure to the two hormones produced a sinergistic effect on the activation of ERα and reduced the action of some antiestrogens tested in the same circumstances.
Conclusions: We demonstrated a new role of leptin as amplifier of estradiol signal through a direct activation of ERα. Besides, high leptin levels, facilitating the functional activation of ERα, may work on a progressive loss of estrogen-dependence of ER+ cells.

P1016 Oncology - Series B

The androgen-regulated ZEB-1 gene is a biomarker for metastatic prostate cancer

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Background of Study: Steroid hormones regulate genes critical for both physiological and pathological processes. Our lab has shown that the ZEB (Zinc finger E-box Binding factor)-1 gene is a target of androgen signaling and may be involved in the etiology of male reproductive cancers.
Objectives: Having cloned the ZEB-1 promoter, our objectives are to map the androgen regulatory element(s) and to determine whether a correlation exists between ZEB-1 expression and prostate cancer progression.
Methods: A 974 base pair fragment upstream of the translation start site in the ZEB-1 gene was subcloned into a reporter vector. Two putative androgen response elements (AREs) in this region were then mutated alone and in combination. The wild-type and mutated vectors were transfected into the PC-3/AR prostate carcinoma cell line. Cells were cultured with or without 5 nM dihydrotestosterone (DHT) for 24 hours. To determine whether aberrant ZEB-1 expression is associated with prostate cancer progression, RNA was harvested from prostate biopsy tissues and subjected to real-time PCR using ZEB-1 specific probe and primers.
Results: Transfection experiments determined the wild-type ZEB-1 construct is induced 7- to 9-fold by DHT while none of the three mutated constructs were responsive. Real-time PCR assays revealed expression of ZEB-1 mRNA is unchanged in normal prostate, benign prostatic hyperplasia, and localized prostate cancer. A dramatic decrease in ZEB-1 expression (50- to 500-fold) correlates with progression of prostate cancer to the metastatic state.
Conclusions: The ZEB-1 gene is regulated by androgens acting through two AREs in its 5'-flanking region. ZEB-1 may serve as a biomarker for metastatic prostate cancer.

P1017 Oncology - Series B

Clinical studies of preclinical Cushing's syndrome?its clinical characteristics and postoperative course

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Background of Study: The diagnostic criteria of adrenal preclinical Cushing's syndrome(PCS) was almost established in Japan. However, the indication of adrenalectomy hasn't been quite established, and its natural history or postoperative prognosis remains to be elucidated.
Objectives: In order to clarify the above-mentioned, we studied clinical characteristics of patients with PCS and postoperative course of clinical abnormalities such as hypertension(HT), impaired glucose tolerance(IGT), hyperlipidemia(HL) and obesity(OB). Moreover, we compared them with overt Cushing's syndrome (CS).
Methods: Sixteen patients who satisfied Japanese diagnostic criteria for PCS, and 7 patients with CS were examined. Unilateral adrenalectomy was performed in 12 patients with PCS and all patients with CS, and all of them were histopathologically diagnosed as cortical adenoma. We retrospectively studied their clinical, endocrinological findings and postoperative clinical course. Postoperative observation periods were 39±23 months in PCS and 62±27 months in CS.
Results: Compared with CS, patients with PCS were older (55±11 vs. 51±10 y.o.), hi[illegible] percentage of male (38.5 vs. 0 %). Mean tumor size by CT was almost equal(26±8 vs [illegible] mm), but mean plain CT value of PCS was significantly lower than CS (5±16 vs. 31±1[illegible] endocrinological findings, the distinct difference between PCS and CS was found in C[illegible] test all of CS showed no response of plasma ACTH , but 40% of PCS showed normal response of plasma ACTH and the ratio of no response was only 13 % of PCS. The inc[illegible] dences of HT, IGT, HL and OB (BMI>25) in patients with PCS were 56%, 38%, 44% an[illegible] and those in CS were 100%, 57%, 57% and 43%, respectively. All of CS had some cli[illegible] abnormalities, but 19% of PCS had none of them. With regard to the postoperative cli[illegible] course, 50% of HT and 33% of IGT were improved in those with PCS. On the other ha[illegible] 86% of HT, 50% of IGT and 75% of HL were improved in those with CS. Patients reduc[illegible] more than 5% of body weight were 27% in PCS and 14% in CS. In PCS patients with [illegible] response of plasma ACTH after CRH load, their clinical abnormalities tended to be imp[illegible] by unilateral adrenalectomy.
Conclusions: Patients with PCS who showed low response of plasma ACTH after CRH or had clinical abnormalities should be considered to remove the adrenal tumor, becau[illegible] improvement of their clinical abnormalities may be expected by adrenalectomy.

P1019 Oncology - Series B

Activins, Hypoxia and PSA Expression in LNCaP Prostate Cancer Cells

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Background of Study: Androgen ablation therapy for prostate cancer (PCa) induces e[illegible] lial and tumour cell apoptosis, but also produces a hypoxic environment due to reduce[illegible] blood flow. The cellular response to hypoxia alters gene expression, resulting in a more aggressive phenotype. Activin is a multifunctional protein, and is elevated with hypoxia some tissues, including placenta. Despite prostate tumour cells being sensitive to activ[illegible] is not known whether the hypoxia response stimulates activin in PCa. If so, then unders[illegible] ing the functional role of activin in PCa and hypoxia is vital.
Objectives: To examine activin expression and its effect on tumour cell growth and PS[illegible] expression following androgen withdrawal and hypoxia.
Methods: LNCaP tumour cells were cultured with and without androgens and/or activin under normal or hypoxic (~5% O2) conditions. Activin and PSA expression were examin[illegible] real-time PCR. Activin and PSA protein was assessed by ELISA and/or Western blot.
Results: Following induced hypoxia in LNCaP cells, Activin A expression was unchange[illegible] with low mRNA and undetectable protein. PSA expression was decreased, but not signif[illegible] cantly. PSA expression was elevated in cells treated with either Activin A or testosterone further increased when treated in combination.
Conclusions: We have shown that Activin A stimulates PSA independently of androgens. However, the induced level of hypoxia and the treatment period did not alter Activin A or levels. Further work is underway to test whether increased hypoxia or treatment time ha[illegible] any effect on other forms of Activin that might regulate PSA expression.

P1020 Oncology - Series B

Establishment of an E_1, E_2 progesterone PTH and PTHrp secreting human ovarian small cell carcinoma hypercalcemic type cell line (JHOMS) expressing FSH recepto[illegible] – special reference to oocyte growth in vitro –

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The JHOMS cell line was established from ovarian small cell carcinoma hypercalcemic ty[illegible] in 26-year-old Japanese female. The JHOMS cell line was composed two kinds of cells, o[illegible] in angular adhesive cells and the other is spherical cells which covered on the angular adhesive cells. The spherical cells were collected with pipetting and cultured in fresh dish the adhesive and spherical cells were also observed. The diploidity of cell line was 80%, doubling time was 72 hours at 5th passage. They produced large amounts of E_1, E_2 and small amount of progesterone and 17β-hydroxyprogesterone. The JHOMS cells expressing FSH receptor (about 25,000/cell) respond to FSH which stimulated E_1, E_2 and progesteron[illegible] production. Furthermore, the cultures produced PTH and PTHrp. FSH also enhanced morph[illegible] logically the development of organelles of this cells. The graft in subcutis of nude mice showed small cell carcinoma hypercalcemic type resembled to the original tumor. The bio[illegible] tive factor(s) released by cell line can induced a significant stimulation of m[illegible] oocyte growth in vitro. ED_{50} for the steroid genic recponse to human FSH is about 150 pM, suggesting a tight coupling between receptor activation and the steroid genic response.

P1021 Oncology - Series B

Endothelial growth factors are elevated in human obesity

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Background of Study: Epidemiological data suggest that obesity is associated with increased risk of metastatic disease in patients with certain malignancies including brea[illegible] and prostate cancer.
Objectives: To investigate the relationship between body mass index and serum concentra[illegible] tions of vascular growth factors in human subjects.