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Evidences that Progesterone Receptor B decreases Estrogen Receptor α gene expression through its interaction to a half-PRE site at Estrogen Receptor α gene promoter.

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SUMMARY

Regulated expression of Progesterone Receptor-A (PR-A) and Progesterone Receptor-B (PR-B) is critical for appropriate mammary gland responsiveness to Progesterone (Pg) which has a broad impact on target cells. There is considerable biological evidence for cross talk between the progesterone and estrogen hormone receptor signalling pathways even though the molecular mechanisms remains to be further elucidated.

In the present study we demonstrate that PR mediates the down-regulation of the E2 induced cell proliferation due to Pg cotreatment in MCF-7 breast cancer cells. In order to assess weather increased PR-B/PR-A ratio could affect response to E2 through alteration of ERα expression levels in MCF-7 cells, we transient over-expressed PR-B isoform. We show that PR-B isoform over-expression, robustly inhibits ERα expression in terms of mRNA and protein content, causing concomitantly the repression of the estrogen regulated genes cyclin D1 and pS2, addressing a specific effect of PR/PR-B on ERα gene transcription. For instance ERα gene promoter activity was drastically inhibited by PR-B over-expression. ERα gene promoter analysis reveals a responsive region containing a half-PRE site located at –1757 bp to –1752 bp as putative effector of PR action. In presence of mutation of the half-PRE, the down-regulatory effect induced by PR/PR-B over-expression was abrogated. Moreover chromatin immunoprecipitation

analyses, reveal an increase of PR bound to the ERα regulatory region and recruitment of a corepressor complex containing NCoR but not SMRT and DAX1, concomitantly with hypoacetilation of the chromatin and displacement of RNA polymerase II. Furthermore PR-B ablation studies using small interfering RNA demonstrate the specific recruitment of PR-B isoform on ERα promoter.

Our study provide evidences for a mechanism by which over-expressed PR-B is able to actively repress ERα gene expression in vivo. The antiestrogenic effect of over-expressed PR-B also provides potential therapeutic strategy for breast cancer treatment.

INTRODUCTION

The sex steroid hormones, Estrogen (E2) and Progesterone (Pg), play an important role in normal mammary gland development, and it is believed that breast cancer progression is influenced by them and their receptors (1, 2). The level of these steroid hormones receptors is a strong prognostic factor for patients with breast cancer and has been used in clinical management as an indicator of endocrine responsiveness (3, 4).

Approximately 75% of primary breast cancers express ER, and more than half of these cancers also express PR.(5) Both ER and PR are prognostic factors, although both are weak and lose their prognostic value after long-term followup.(6) PR is an estrogen-regulated gene, and its synthesis in normal and cancer cells requires estrogen and ER. Therefore, it is not surprising that ER positive/PR-positive tumors are more common than ER-positive/PR-negative tumors. The etiology of ER-positive/PR-negative tumors is currently unclear. Some studies have shown that ER and PR status can change over the natural history of the disease or during treatment.(7) For instance, sequential breast cancer biopsies have shown that ER levels are reduced slightly with intervening endocrine therapy, although complete loss is uncommon. In contrast, PR levels decrease more dramatically during tamoxifen therapy, with up to half of tumors completely losing PR expression when resistance develops.(8) These ER-

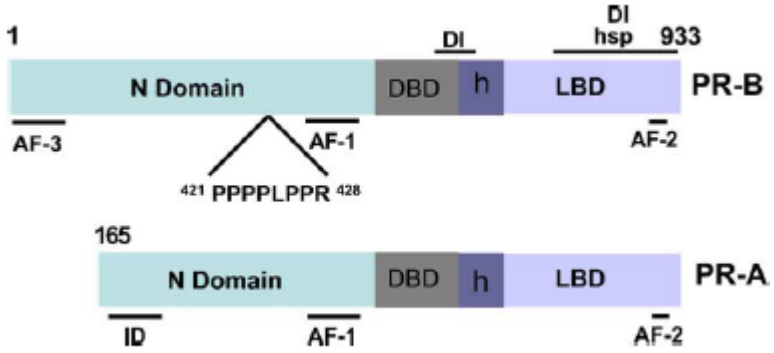
positive/PR-negative metastatic tumors then display a much more aggressive course after loss of PR compared with tumors retaining PR, and patients then have a worse overall survival, indicating a change in tumor cell–regulatory mechanisms.(9,10) Whether and how the loss of PR affects the poor clinical course of these tumors is at present unclear.

Although Estrogen Receptor (ER) is an accepted predictor of response to endocrine therapy the role of Progesterone Receptor (PR) has been more controversial. Recent studies published on the largest retrospective analysis of early breast cancer treated with tamoxifen found that patients with ER+/PR+ tumors benefited much more from adjuvant tamoxifen therapy than those with ER+/PR- tumors (11,12). Importantly, multivariate analyses, including lymph node involvement, tumor size, and age, show that PR status was independently associated with disease-free and overall survival.

Progesterone Receptors belong to the subfamily of classical nuclear steroid receptors; human PR proteins exist as two isoforms, termed PR-A and PR-B.

Both PR-A and PR-B contain three functional domains including the N-terminus, a centrally located DNA binding domain (DBD), and C-terminal ligand binding domain (LBD). Three-dimensional structures of isolated DBD and LBDs have revealed common motifs for these regions. By comparison, little is known about the structure of the N-terminal domain. This is the least conserved region among family members with re-spect to both length and amino acid sequence. The N-domainis functionally important, as it is required for full transcriptional activity of

steroid hormone receptors and for many cell- and target gene-specific responses. Other functional and structural determinants have been identified within these broader three domains. In addition to binding steroid hormone, the LBD contains determinants for dimerization (DI) in the absence of DNA, binding of heat shock proteins(hsps) and for nuclear localization sequence (NLS). The DBD contains a second NLS and dimerization domain that is dependent on DNA binding. Steroid receptors contain at least two transcription activation domains (AFs), AF-1 in the N-terminal domain and highly conserved AF-2 in the C-terminal LBD. These are autonomous transferable domains required for the DNA bound receptor to transmit a transcriptional activation response and they function as specific binding sites for coactivators. AF-2 located in the LBD is hormone-dependent and becomes activated as a result of the steroid hormone inducing a conformational change that creates a hydrophobic binding pocket for members of the p160 family of steroid receptor coactivators (SRCs). The coactivators that bind to and mediate the activity of AF-1 are yet not well defined.



The human PR is expressed as two isoforms from a single gene by alternate promoter usage. PR-A (94 kDa) differs from PR-B (120 kDa,) by lacking 164 amino acids (aa) at the N-terminus . Although the two forms of PR have similar steroid hormone and DNA binding activities, they have distinct transcriptional activities. PR-B in general, is a much stronger activator than PR-A. However, PR-A can be a strong activator under specific cell and target gene contexts. The stronger activation potential of PR-B is due in part to the existence of a third activation domain (AF-3) within the first N-terminal 164 aa that is unique to PR-B. Under certain cell and target promoter contexts PR-A is inactive as a transcription factor and can function as a ligand-dependent transdominant repressor of other steroid receptors including PR-B and the estrogen receptor (ER). PR-A can act in this repressor mode in response to the binding of either progestin agonists or antagonists. An inhibitory domain (ID) responsible for this transrepressor function has been mapped to the first 140 N-terminal (aa 165–305) amino acids of PR-A. Since the sequence within ID is present in both PR isoforms but is only active in the context of PR-A suggests the PR-B specific N-terminal segment plays a role in suppressing the ID domain.

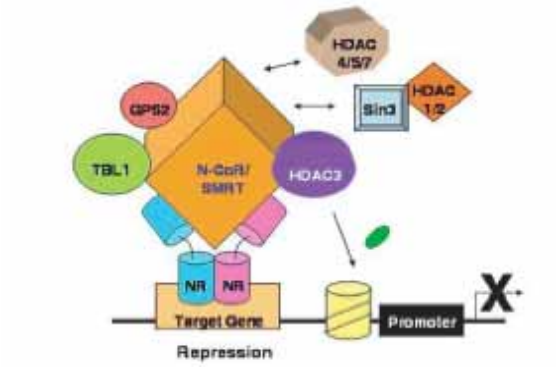
Despite structural similarities, PR-A and PR-B regulate different subsets of genes and although PR-B is transcriptionally more active, there are genes, known to be involved in breast cancer progression, that are uniquely regulated by PR-A (13,14,15).

In vivo the two PR isoforms are usually co-expressed at similar levels in normal cells, yet their ratio varies dramatically in different tissues, physiological states and disease (16,17). With regard to the mammary gland, in transgenic mice, 3:1 over-expression of PR-A over PR-B results in extensive epithelial cell hyperplasia, excessive ductual branching, and disorganized basement membrane, all features associated with neoplasia. In contrast, over-expression of PR-B leads to premature ductual growth arrest and inadequate lobulo-alveolar differentiation (18,19). Moreover the loss of coordinated PR-A and PR-B expression is an early event in carcinogenesis and is evident in pre-malignant lesions (20). A significant proportion of carcinomas expresses a predominance of PR-A isoform and this is associated with poor clinical outcomes: in endometrial cancer, PR-A isoform predominance is significantly more common in tumors of higher grade, indicating an association between PR-A isoform predominance and poor prognosis (21,22).

Although ER and PR are members of different steroid hormone receptor sub-families and recognize distinct hormone response elements, there is considerable biological evidence for cross-talk between the estrogen and progestin hormone receptor signaling pathways. In many cases, progestins suppress the stimulatory effects of estrogens in target cells, for example estrogen increases the expression of both c-*fos* and PR mRNA in uterine cells, and progestins block this effect (23,24) which appear to be mediated via PR, but it is unclear if ER or some other component of the estrogen-ER signaling pathway is the target for repression. Also liganded PR can suppress estradiol-stimulated ER activity, with the

magnitude of repression dependent on the PR isoform, progestin ligand, promoter, and cell type (25). The exact molecular mechanisms regulating Estrogen Receptor α (ERα) expression in breast tumors are unclear, but studies suggest that they are partly at the level of transcription (26).

In the present study we examined whether increased PR-B/PR-A ratio could affect response to estradiol in ERα positive breast cancer cells, MCF-7. We demonstrate that PR-B over-expression down regulates ERα mRNA, protein content and gene promoter activity. The investigation of the ERα gene promoter, site directed mutagenesis of half-PRE region in Estrogen Receptor α promoter, and chromatin immunoprecipitation provide evidence for an active mechanism by which the PR-B is able to negatively regulate expression of Estrogen Receptor α gene by recruitment to the Estrogen Receptor α promoter of a corepressor complex containing NCoR but not SMRT and DAX1, causing hypoacetilation of the chromatin and displacement of RNA polymerase II. Furthermore PR-B ablation studies using small interfering RNA demonstrated the critical involvement of PR-B isoform in the recruitment on Estrogen Receptor α promoter in MCF-7 PR-B overexpressing cells.



MATERIAL AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM/F-12), L-glutamine, Eagle's non-essential amino acids, penicillin, streptomycin, calf serum, bovine serum albumin, and phosphate-buffered saline were purchased from Eurobio (Les Ullis Cedex, France). Triazol reagent was obtained from Invitrogen (Carlsbad, CA), and FuGENE 6 was from Roche Applied Science (Indianapolis, Indiana). Taq DNA polymerase, 100-bp DNA ladder, dual luciferase kit, thymidine kinase and *Renilla* luciferase plasmid were provided by Promega (Madison, Wisconsin). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMFS), and sodium orthovanadate were purchased from Sigma (Milan, Italy)

Antobodies used in this study anti-ERα, anti-cyclin D1, anti-GAPDH, anti-Pol II, anti-NCoR, anti SMRT, anti DAX-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-acetyl histone4-K16, from Upstate (Lake Placid, NY); anti PR, from NeoMarkers (Labvision, Freemont, CA). Salmon sperm DNA was from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated horse anti-goat IgG and ABC complex/horseradish peroxidase were provided by Vector Laboratories (Burlingame, CA). Chromogen, 3-diaminobenzidine tetrachloride dihydrate, was purchased from Bio-Optica, MI. ECL System was

purchased from Amersham Pharmacia (Buckinghamshire, UK). VCX500 ultrasonic processor was provided by Sonics (Newtown, CT).

Plasmid Firefly luciferase reporter plasmid XETL is a construct containing an estrogen-responsive element from the *Xenopus* vitellogenin promoter (27) The wild type human ERα expression vector (HEGO) consists of the full-length ERα cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (28). The full-length PR-B expression plasmid consists of the full-length PR-B cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (gift from Dr. D. Picard University of Genève, Switzerland). The PR DNA-binding mutant (PR-B DBD mutant C587A) expression plasmids was previously described by Faivre et al (29) (gift from Dr. C. Lange University of Minnesota Cancer Center, Minneapolis-USA). The full-length PR-A expression plasmid was previously described by Kastner *et al.* (30). The deletion fragments of the ERα gene promoter are described by L.A. deGraffenried (31) (gifts from Dr. SAW Fuqua, Baylor College of Medicine, Houston, Texas-USA). The Renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Cell Culture Wild type human breast cancer MCF-7 cells were gifts from Dr. B. Van der Burg (Utrecht, The Netherlands). Human uterine cervix adenocarcinoma (HeLa) cells were obtained from the ATCC (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 medium containing 5% fetal calf serum (5% FCS), 1% L-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/ml

penicillin/streptomycin in a 5% CO_2 humidified atmosphere. Cells were cultured in phenol red-free DMEM, 0.5% bovine serum albumin (0,5% BSA), and 2 mM L-glutamine (serum-free medium, SFM), for 48 h before each experiment. Hormone stimulation was performed in DMEM containing 5% charcoal-treated fetal calf serum to reduce the endogenous steroid concentration. (32)

Proliferation assays For quantitative proliferation assays, 10,000 cells were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 5 % charcoal-treated FCS with the indicated treatments; medium was renewed every 2 days (with treatments) and cells were trypsinized and counted in a hemocytometer on day 6.

Reverse Transcription-PCR Assay Total cellular RNA was extracted from MCF-7 cells using Triazol reagent as suggested by the manufacturer. 2 µg of total RNA were reverse-transcribed using 200 IU of Moloney murine leukemia virus-reverse transcriptase in a reaction volume of 20 µl (0.4 µg of oligo(dT), 0.5 mM deoxy-NTP, and 24 IU of RNasin) for 30 min at 37 °C, followed by heat denaturation for 5 min at 95 °C. 2 µl of cDNA were then amplified with a pair of 5' and 3' 50-pmol primers for either ER α or pS2 or acidic ribosomal phosphoprotein P0 (36B4) by using 2 units of Taq DNA polymerase. The primers used are as follows: ERα forward, 5'-GGAGACATGAGAGCTGCCA-3', and reverse, 5'-CCAGCAGCATGTCGACGATC-3', to amplify a 438-bp fragment; pS2 forward, 5'-TTCTATCCTAATACCATCGACG-3', and reverse, 5'-

TTTGAGTAGTCAAAGTCAGAGC-3' to amplify a 210-bp fragment; cyclin D1 5'-TCTAAGATGAAGGAGACCATC-3', and reverse, 5'-GCGGTAGTAGGACAGGAAGTTGTT-3', to amplify a 400 bp fragment; PR-B forward, 5'-TAGTGAGGGGGCAGTGGAAC-3', and reverse, 5'-AGGAGGGGGTTTCGGGAATA-3' to amplify a 442 bp fragment; 36B4, forward, 5'-CTCAACATCTCCCCCTTCTC-3', and reverse, 5'-CAAATCCCATATCCTCGTCC-3', to amplify a 408-bp fragment. PCR amplification was performed in 10 mM Tris-HCl, pH 9.0, containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. Equal amounts of PCR product were electrophoresed on a 1% agarose gels and visualized by ethidium bromide staining. To check out for the presence of DNA contamination, a reverse transcription-PCR was performed without Moloney murine leukemia virus-reverse transcriptase (negative control).

Western blotting Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 μL of 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, 1% SDS, a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. Protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy), probed overnight

at 4°C with the antibody as indicated in the figure legends, followed by anti-goat or anti-mouse horseradish peroxidase-conjugated antibody and then revealed using the enhanced chemiluminescence system (Amersham Biosciences).

Transfections and luciferase assays Cells (1 x 10^5) were plated into 24-well dishes with 500 μL of regular growth medium per well the day before transfection. The medium was replaced with that lacking serum on the day of transfection, which was done using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 μg of reporter plasmid, alone or in combination with ERα and/or PR-B or mDBD PR or PRA expression plasmids as indicated in the figure legends, and 5 ng of pRL-TK. Upon transfection, the serum-free medium containing the indicated treatment was renewed and then cells were treated for 24 h. Luciferase activity was measured with the Dual Luciferase kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by the *Renilla* luciferase activity.

For whole cell extracts and chromatin immunoprecipitation (ChIP) assay, cells shifted to serum-free medium for 24 h were plated on a 10-cm dish and transfected after 24 h using the FuGENE 6 reagent, with an appropriate amount of the various plasmids as indicated in the figure legends. Cells were treated as indicated in the figure legends or left untreated in DMEM containing 5% charcoal-treated fetal calf serum for 48 h.

Empty vector was used to ensure that DNA concentrations were constant in each transfection.

Lipid-Mediated Transfection of siRNA Duplexes RNA oligonucleotides directed against PR-B or NCoR, were purchased from Invitrogen (Carlbad, CA), and transfection was performed as described previously (33,34). Briefly, MCF-7 cells were plated in medium lacking antibiotics 2 days before transfection so that they would be 30–50% confluent at the time of transfection. On the day of transfection (d 0), siRNA duplexes (200 nM) and Lipofectamine (8 μl per well; Invitrogen) were diluted in Optimem (Invitrogen) according to the manufacturer's instructions. The medium was changed 24 h later (d 1**).** On day 5, cells were placed in serum-free medium and treated as indicated in the figure legends.

Site-directed mutagenesis Was performed on the D fragment of the ERα promoter using the QuikChange mutagenesis kit (Stratagene) following the manufacturer's instructions. The mutagenesis sense and antisense primers were designed using the Stratagene web site.

The sequences for the primers were: 5'-AGCAGGGAGATGAGGATTGCTGaagTCCATGGGGGTATGT-3' and 5'-ACATACCCCCATGGACttcAGCAATCCTCATCTCCCTGCT-3'. The plasmids were then sequenced to confirm the mutation of the desired site.

Chromatin immunoprecipitation (ChIP) Assay We followed the ChIP methodology described by Shang *et al.* (35) with minor modifications. MCF-7 cells were grown in 100-mm plates. 90% confluent cultures were shifted to serum-free medium for 24 h and then treated left untreated as indicated in the figure legends. Following treatment, the cells were washed twice with phosphate-buffered saline and cross-linked with 1% formaldehyde at 37 °C for 10 min. Next the cells were washed twice with phosphate-buffered saline at 4 °C, collected, and resuspended in 200 µl of Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1) and left on ice for 10 min. Then the cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4 °C for 10 min at 14,000 rpm. Supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 µl of sonicated salmon sperm DNA/protein A-agarose for 1 h at 4 °C. The precleared chromatin was immunoprecipitated for 12 h either with PR, RNA Pol II, acetyl histone4-K16, NCoR, SMRT and DAX-1 antibodies or with normal goat IgG as the negative control. After that, 60 µl of salmon sperm DNA/protein A-agarose was added, and precipitation was continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1

mM EDTA, 10 mM Tris-Cl, pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). The eluates were reverse cross-linked by heating at 65 °C for 12 h and digested with 0.5 mg/ml proteinase K at 45 °C for 1 h. DNA was obtained by phenol and phenol/chloroform extractions. 2 µl of 10 mg/ml yeast tRNA were added to each sample, and DNA was precipitated with ethanol for 12 h at –20 °C and resuspended in 20 µl of TE buffer. 5 µl of each sample were used for PCR with the following ER α promoter primers: forward 5'-ACGTTCTTGATCCAGCAGGGTA-3' and reverse 5'-ACCTGCCAAATTATATGCAAATGGCAG-3' containing the half-PRE site and forward 5'-GTGGCCATTGTTGACCTACAG-3' and reverse 5'-CTGTAGGTCAACAATGGCCAC-3' upstream the half-PRE site. The amplification products, obtained in 30 cycles, were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without DNA sample.

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

Progesterone decreases estradiol induced cell proliferation in MCF-7 breast cancer cells.

Reports about the effects of progestins on cell proliferation are contradictory and fuel the debate around the actions on breast tissue.

In order to understand the eventual ability of progestins to interfere with cell proliferation stimulated by estradiol (E2), we evaluated the effects of different concentrations of progesterone (Pg 1 nmol/L, Pg 10 nmol/L , Pg 100 nmol/L) on the proliferation of MCF-7 cells which express low levels of Progesterone Receptor (PR) inducible by estradiol treatment.

The results obtained revealed that after 6 days, as expected 10 nmol/L E2 treatment significant increased the cell number, Pg alone at all tested concentrations had no significant effect compared with the control experimental conditions, however 10 nmol/L Pg caused the maximal inhibitory effect (48 %) on the E2 induced cell proliferation (Fig. 1A). These data evidenced that the action of Pg in inhibiting cell proliferation is likely to be due on prior exposure to E2 inducing PR-A and PR-B expression as evidenced by western blotting analysis (Fig. 1B) RU 486 is an antagonist of progestins action in human and binds with high affinity to the corresponding receptors (36). In our experimental conditions 1 μmol/L RU 486 did not affect the growth of the MCF-7 cells under control treatment conditions (Fig. 1C).

However, in combination with 10 nmol/L E2 and 10 nmol/L Pg significantly reduced the inhibitory effect due to Pg treatment on cell proliferation, suggesting that these inhibitory action is mediated by PR.

It is also interesting to note that 1 μmol/L OH-Fl, an androgen receptor (AR) antagonist, did not modify the inhibitory action of Pg on E2 stimulated proliferation, indicating that AR is not involved.

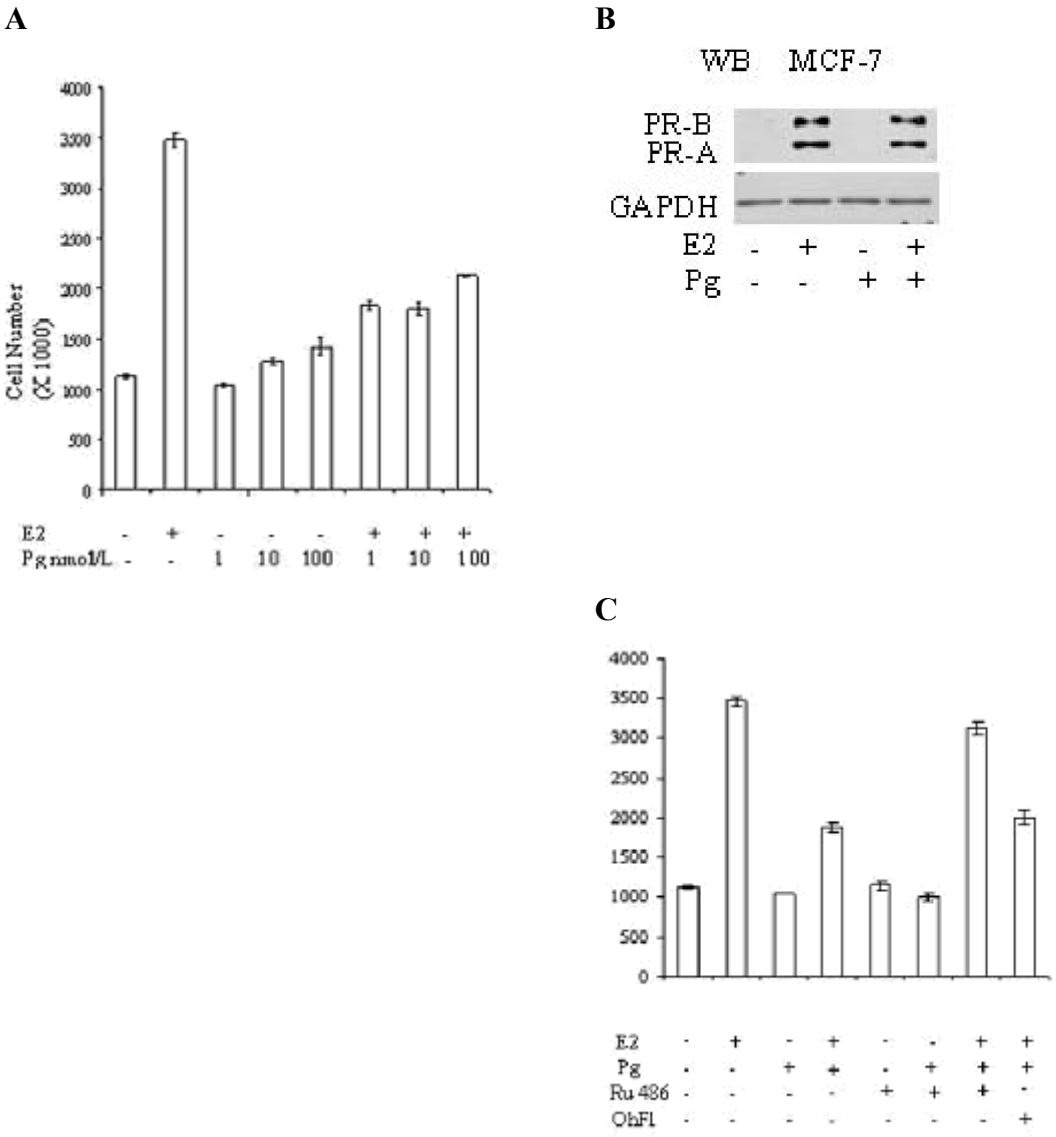


Figure 1. Progesterone decreases the estradiol induced cell proliferation in MCF-7 breast cancer cells. *A,* MCF-7 cells were treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or increasing amount of Progesterone (1 nmol/L, 10 nmol/L , 100 nmol/L) [Pg] in medium containing 5% charcoal-stripped FBS (medium was refreshed and treatments were renewed every 2 d) and then counted on day 6. *B,* Immunoblot analysis of PR. MCF-7 cells were treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg) for 48 hours. GAPDH levels were used as a loading control. *C,* Cells cultured in the experimental conditions described in *A,* were also treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg) in combination with RU 486 1 µmol/L or OHFl 1 µmol/L and counted on day 6. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD.

Progesterone Receptor B over-expression represses the E2/ ERα transcriptional activity.

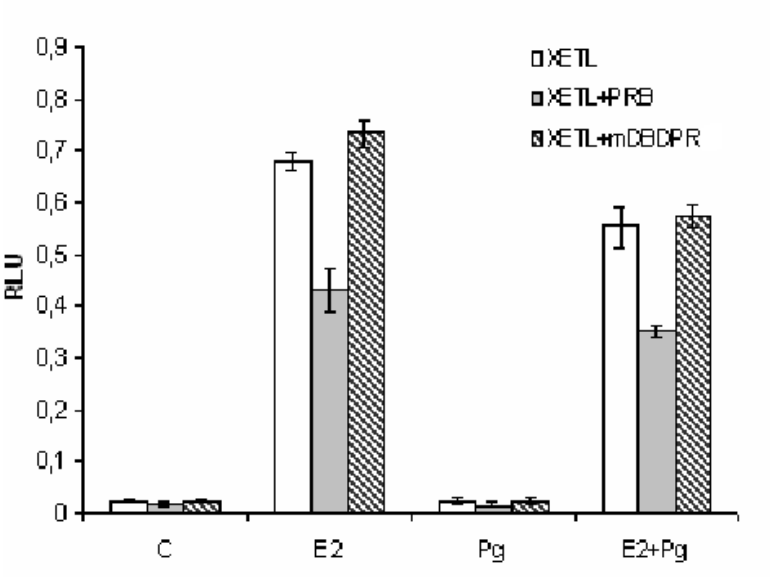
The PR system is characterized by several complexities; one is that PR is an estrogen receptor dependent gene product (3) and in MCF-7 cells the action of Pg requires priming treatment of E2 inducing PR-A and PR-B isoforms expression.

To study whether changes in relative expression levels of PR-B and A isoforms we analyze the specific effects of PR-B over-expression on genomic activity induced by estradiol treatment. To this aim a luciferase reporter plasmids containing a consensus estrogen-responsive element (ERE) sequence was transiently co-transfected into MCF-7, in presence or absence of expression plasmids encoding the full length PR-B. Cells were treated with 10 nmol/L E2 and/or 10 nmol/L Pg, as indicated in Fig. 2A for 18 h. Treatment with E2 alone resulted, as expected, a substantial increase in luciferase activity and interestingly PR-B transient over-expression repressed this effect as much as 36%; moreover when the cells were co-treated with E2 and Pg the inhibitory effect was of 49%. Conversely, over-expression of a PR mutant with a disrupted DBD (Cys587 to Ala) cannot mediate transcriptional responses and did not affect luciferase activity in the same experimental conditions.

To confirm these findings, the same experiments were carried out in HeLa cells expressing ectopic ERα. As shown in Fig. 2B, PR-B co-expression repressed the 10 nmol/L E2 induced luciferase activity by 75% and 73 % when cells were co-treated

with E2 and 10 nmol/L Pg; over-expression of a PR mutant with a disrupted DBD had no inhibitory effects.

A



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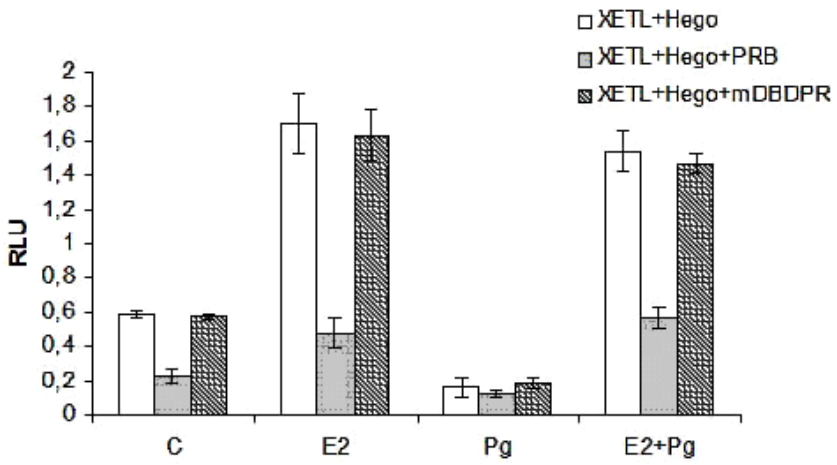


Figure 2. Progesterone Receptor B over-expression represses the E2/ ERα transcriptional activity. ERE luciferase reporter assay. MCF-7 cells *(A)* and HeLa cells expressing ectopic ERα *(B)*, were transiently co-transfected with XETL in the presence or absence of full-length PR B expression plasmid or mDBD PR expression plasmid. After transfection cells were treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg), harvested after 18 h, then luciferase activities were determined. *Columns*, mean of luciferase activities observed in three independent experiments. *bars,* SD.

Progesterone Receptor B over-expression down-regulates ER α protein levels and mRNA

After demonstrating the inhibitory effects of PR-B over-expression on the estrogen receptor induced signal we examined whether this action might be due to a decrease in the expression levels of ERα protein.

Immunoblot analysis of lysates from MCF-7 cells treated with 10 nmol/L E2 and/or 10 nmol/L Pg for 48 h, shown that as expected E2 treatment repressed the ER α expression levels while Pg alone had no effect (Fig 3A).

Moreover, in MCF-7 cells over-expressing PR-B, a marked repression of ERα content was observed in control experimental conditions in absence of treatment, as well as after ligands exposure compared with the examined conditions in absence of exogenous PR-B. These effects were not observed in cells overxpressing PR-A.

Previous studies provide strong evidences that ERα expression in breast cancer is regulated at both the transcriptional as well as the translational level. (38)

To further investigate the molecular basis of the PR-B mediated regulation of ERα we examined the effect of PR-B over-expression on ERα mRNA levels in MCF-7 cells treated for 48 h with 10 nmol/L E2 and/or 10 nmol/L Pg. As shown in Fig. 3B, ERα mRNA was reduced by E2 treatment compared to control, moreover the over-expression of PR-B further decreased ERα mRNA compared with the examined conditions in absence of exogenous PR-B.

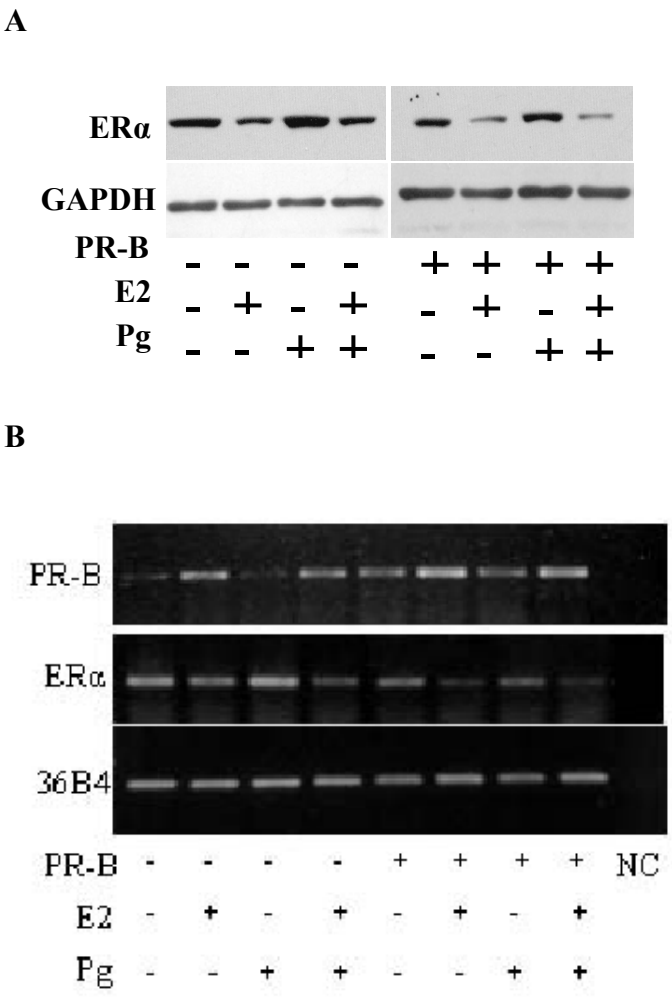


Figure 3. Progesterone Receptor over-expression decreases the ER α protein and mRNA levels. *(A)* Immunoblot analysis of ERα. MCF-7 cells and MCF-7 transient over-expressing PR-B were treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg) for 48 hours. GAPDH levels were used as a loading control. *(B)* Semiquantitative RT-PCR. mRNA expression of ERα in MCF-7 cells and MCF-7 cells transient over-expressing PR-B. The expression of ERα was evaluated in cells treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg) for 48 h; the housekeeping gene 36B4 was determined as a control.

PR-B overexpression decreases the levels of ERα-regulated genes in MCF-7 cells.

The effects of E2 hormone are mediated by ER through its gene regulation activities. To determine weather Pg could inhibit the effect of E2 by modifying the transcriptional activity of ERα, the mRNA of well known estrogen target genes, cyclin D1 and pS2, were studied. (Fig. 3C) As expected after 48 h treatment, 10 nmol/L E2 marked increased the mRNA levels of the estrogen-regulated cyclin D1 and pS2 in MCF-7 cells. However, the induction of cyclin D1 levels was impaired by 20% after concomitant 10 nmol/L E2 and 10 nmol/L Pg treatment; similarly, E2 induced pS2 expression was impaired by 15%. These data evidenced that the inhibitory action of Pg in MCF-7 cells is concomitant to high PR expression levels due to prior exposure to E2.

To analyze whether changes in relative expression levels of PR B and A isoforms causing the above described down-regulatory effect on ERα protein and mRNA levels, could affect the expression of estrogen regulated genes, plasmids encoding full length PR-B were transient transfected into MCF-7 cells. After 48 h of ligands treatment, cells were harvested and subjected to semiquantitative RT-PCR experiments comparing mRNA levels encoding cyclin D1, and pS2 after standardization with a housekeeping gene encoding the ribosomal protein 36B4. As depicted in Fig. 3C, PR-B over-expression, represses both cyclin D1 and pS2 mRNA levels .

C

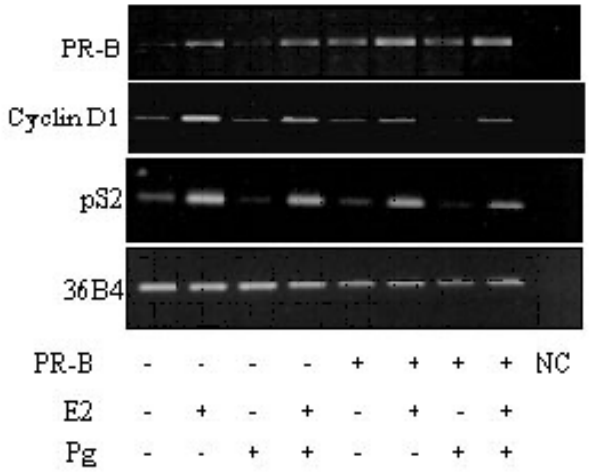


Figure 3. PR-B overexpression decreases the levels of ERα-regulated genes in MCF-7 cells. *(C)* mRNA expression of IRS-1 Cyclin D1 and pS2 and PR-B in MCF-7 cells and MCF-7 transient overexpressing PR-B. Cells were treated with vehicle (–) or Estradiol 10 nmol/L (E2) and/or Progesterone 10 nmol/L (Pg) for 48 hours. The housekeeping gene 36B4 was determined as a control.

The PR-B overexpression mediates the down-regulation of ER α promoter activity by the region between −2769 bp to −1000 bp and mutation of the half-PRE site in ERα promoter abrogates the PR-B induced inhibitory effect.

To analyze whether PR-B could interfere with ERα gene transcription the promoter of ERα was investigated first with a bioinformatics approach using the NCBI Genome data base (www.ncbi.nlm.nih.gov). The region examined in this study covered from −4100 bp to +212 bp and in this portion one half-PRE (−1757 bp to −1752 bp) was identified as putative effector of PR action.

To evaluate weather this region was involved in Progesterone Receptor mediated down-regulation of ERα expression, five overlapping ERα promoter deletion constructs, −245 bp to +212 bp (fragment A), −735 bp to +212 bp (fragment B), −1000 bp to +212 bp (fragment C), −2769 bp to +212 bp (fragment D), and −4100 bp to +212 bp (fragment E), all relative to the first transcriptional start site, sub-cloned into the luciferase reporter vector pGL3-basic, were analysed as depicted in Fig. 4 A. The five fragments were transiently transfected into the ER positive MCF-7 cells. Data are shown as relative promoter activity in luciferase units. Consistent with previous ERα promoter studies of L.A. de Graffenried et al. 2002, fragment A exhibited high levels of activity compared to vector alone (Fig. 4B), the activity of fragments B and C was slightly increased relative to A and the activity of fragment D significantly decreased relative to the activity seen with A, the activity of fragment E increased. In the same experimental conditions PR-B over-expression had no effect on

the promoter activity of A, B and C lacking the half-PRE site; in contrast was able to significantly and reproducibly reduce by 64 % the activity of D and by 50 % the activity of E tested, indicating that the region between −2769 bp to −1000 bp appeared to be responsible for the PR-B mediated down-regulation. These inhibitory effects were not observed co-transfecting an expression plasmid encoding PR mutant with a disrupted DBD (Cys587 to Ala) into MCF-7 cells.

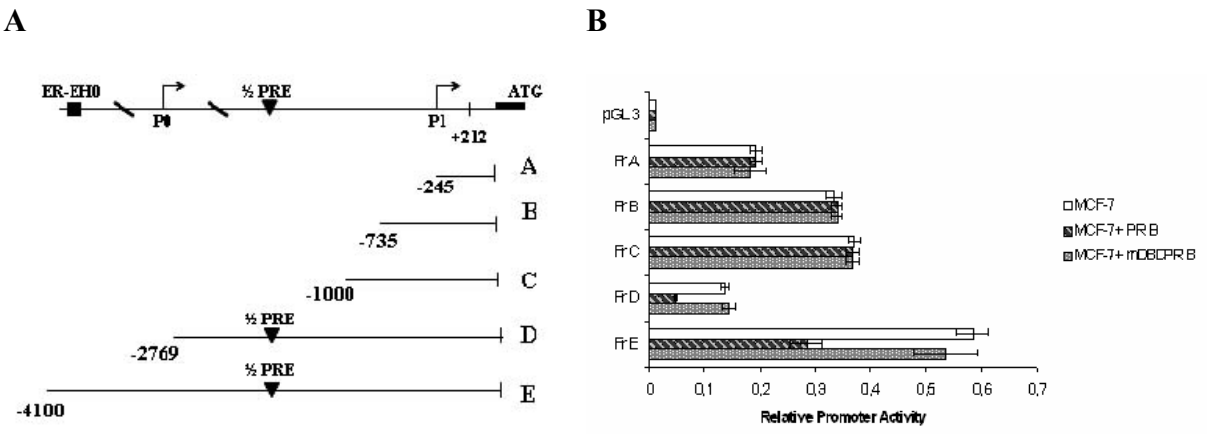
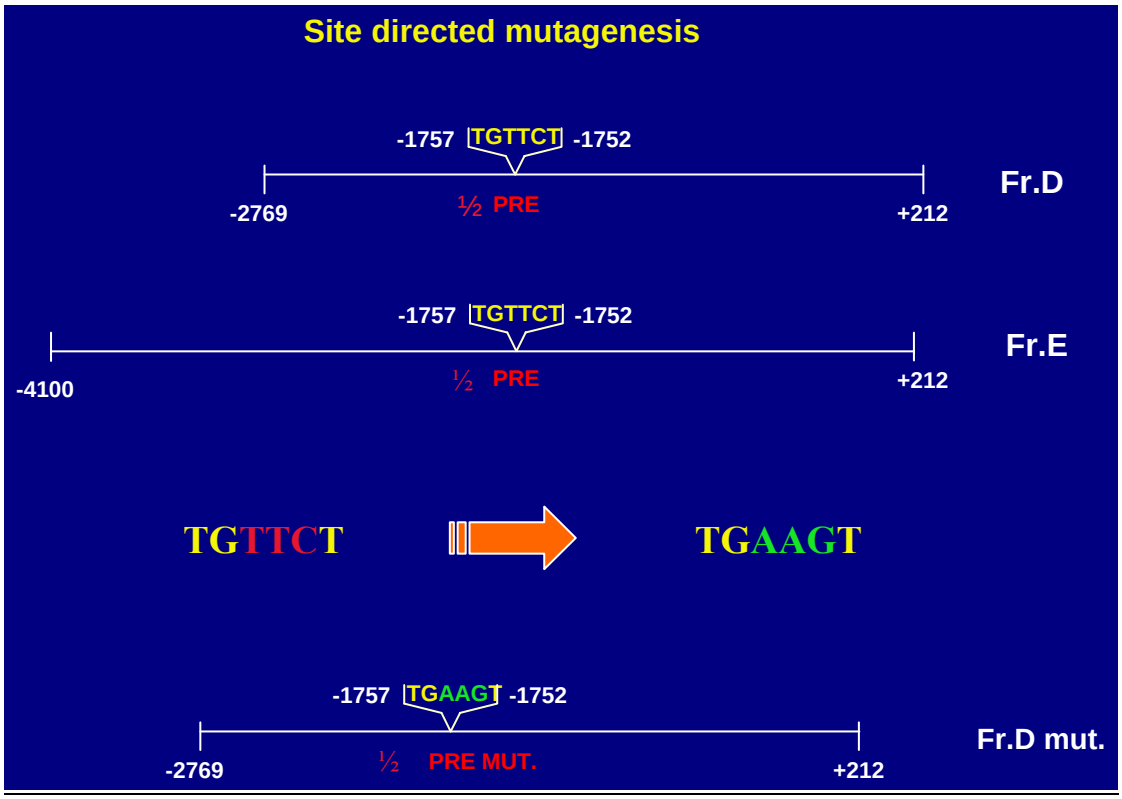


Figure 4. E**R α promoter activity is down-regulated by PR-B over-expression and mutation of the half-PRE abrogates this effect** *A,* Schematic representation of deletion fragments of the ERα gene promoter. Fragment coordinates are expressed relative to the primary transcription start site *B,* Promoter activity of the ER α 5'-flanking region. Constructs described in *A* were transiently co-transfected in MCF-7 cells in presence or absence of full-length PR-B expression plasmid or mDBD PR expression plasmid. After 24 h, cells were harvested, then luciferase activities were determined. Columns, mean of luciferase activities observed in three independent experiments. *bars,* SD.

Site directed mutagenesis reveals a role for the half-PRE in the fragment D of the ER α promoter

To evaluate the role of the half-PRE present in the fragment D of ER α gene promoter (−1757 bp to −1752 bp), we used site-directed mutagenesis to alter this site. We changed 3 of the half-PRE to ensure that the altered binding site would not be recognizable by the PR. Transient transfections were performed in MCF-7 cells with multiple independent clones containing the desidered mutation. We found that all of the half-PRE mutated clones (D mut) were unaffected by PR-B over-expression (Fig. 4 C). These results indicate that the half-PRE element in the estrogen receptor α promoter is necessary for repression due to PR-B over-expression.



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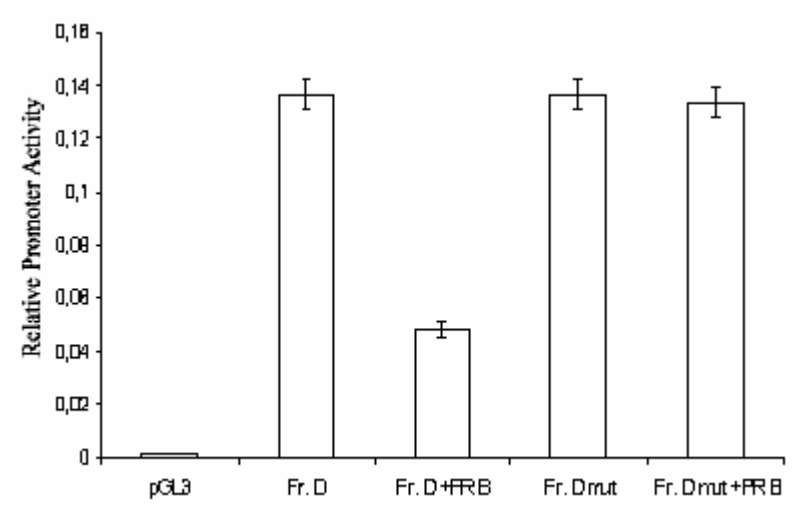

Figure 4. Site directed mutagenesis reveals a role for the half-PRE in the fragment D of the ER α promoter *C* The half-PRE site in the Fr D promoter construct was mutated using site-directed mutagenesis. The Fr D and Fr D mut promoter constructs were co-transfected into MCF-7 cells, and promoter activity was assessed in the absence or presence of full-length PR-B expression plasmid. Luciferase assay was performed after 24 h. *Columns*, mean of luciferase activities observed in three independent experiments. *bars,* SD.

A corepressor complex containing N-CoR bound PR-B is recruited at the ERα promoter "in Vivo"

To examine PR recruitment to the ER α gene promoter *in vivo*, we used ChIP assays. MCF-7 cells overexpressing or not PR-B were treated for 1 h with either control vehicle or 10 nmol/L Pg, after which chromatin was cross-linked with formaldehyde, and protein-DNA complexes were immunoprecipitated with antibodies against PR, RNA polymerase II, acetyl histone H4-K16 , NCoR, DAX-1 or SMRT. The PCR primers used encompass the half-PRE site of ERα promoter. Results obtained in MCF-7 PR-B overexpressing cells and depicted in Fig. 5A, show an increased recruitment of PR to the ERα promoter encompassing the half-PRE site in a ligand independent manner, but not to the unrelated region located upstream the half-PRE site. Moreover Histone H4 became deacetilated and RNA polymerase II was released from the ER α promoter upon PR-B overexpression, indicating that consequently to the PR interaction the chromatin in this region becomes a less permissive environment for transcription. To examine whether corepressor could be recruited by PR bound ERα promoter we tested NCoR, SMRT and DAX-1, previously described as PR corepressors (39) by ChIP assay. Of note NCoR showed hormone independent recruitment to the ERα promoter in MCF7 cells overexpressing PR-B, highlighting a possible new role of this corepressor in the down regulation of ERα expression. SMRT and DAX 1 were not detected in the examined experimental conditions.

To determine which PR isoform mediates the interaction with ER α promoter, MCF-7 cells were transfected with PR-B siRNAs to achieve knochdown of PR-B proteins (Fig. 5 B). Remarkably the PR recruitment on half-PRE site of ER α promoter was strongly reduced by PR-B specific siRNA (Fig.5 A) indicating the specific recruitment of PR-B isoform on ERα promoter .

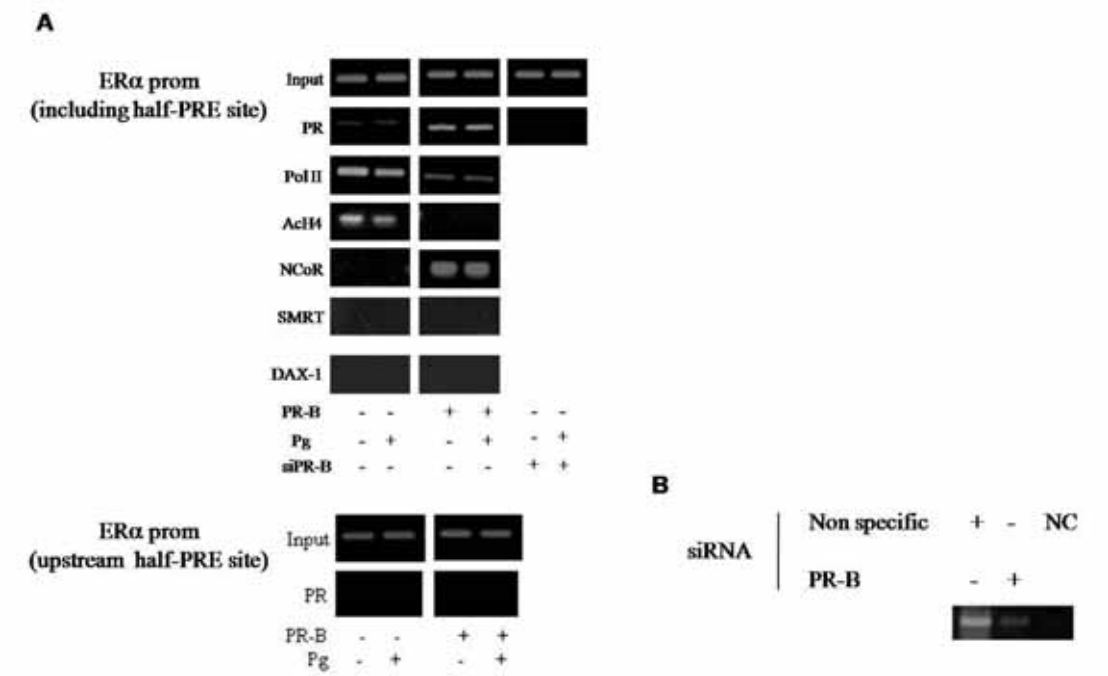

Figure 5. PR is recruited to the ERα promoter, RNA polymerase II is dismissed. *A* ChIP was performed on the PR-responsive region of the ERα promoter using various antibodies as indicated. *A* nonspecific region upstream of the half-ERE site in the ERα promoter was used as negative control. The cells were treated for 45 min with vehicle (–) or Progesterone 10 nmol/L (Pg). Similar results were obtained in multiple independent experiments. *B* Knockdown of PR-B. MCF-7 cells were transfected with siRNA targeted against PR-B. The medium was changed 6 h later and cells were treated with vehicle (–) or Progesterone 10 nmol/L (Pg) for 48 hrs. RNA was isolated and the expression of PR-B was analyzed by semiquantitative RT-PCR.

DISCUSSION

Approximately 75% of primary breast cancers express ER and more than half of these cancers coexpress PR. ER and PR are considered prognostic factors for early breast cancer therapeutic management although both are weak and lose their prognostic value after long-term follow-up. PR exists as two isoforms, called PR-A and PR-B, which have distinct roles in regulating the effects of progesterone and there is increasing evidence that enhanced PR-A/PR-B ratio is coincident with a major breast cancer growth and progression.(40)

It is well documented that in hormone dependent breast cancer cells, progesterone may inhibit the induction of classically estrogen regulated genes even though the molecular mechanisms underlying these effects remain to be fully elucidated.(41, 42)

In the present study we demonstrated that PR-B isoform overexpression represses Estrogen Receptor α expression in MCF-7 breast cancer cells.

We first demonstrated that Progesterone Receptor mediates the down-regulatory effect induced by progesterone treatment on estradiol stimulated cell proliferation, since RU486 prevents this inhibitory effect. In addition we show that the overexpression of PR-B produces an inhibition of estrogen induced ERE reporter gene activity due to the down-regulatory effect of PR-B on ERα expression. The same findings were reproduced in Hela cells expressing ectopic ERα and PR-B.

The latter event may explain the reduced expression of classically ERα regulated genes as pS2 and Cyclin D1 upon PR-B overexpression.

In order to clarify the molecular mechanisms through which PR-B may interfere with ERα gene transcription we explored the ERα promoter sequence. We identify the existence of a PRE emisite as a putative effector of the inhibitory action of PR-B on ERα expression. Utilizing five deleted constructs we observed that the down-regulatory effect induced by PR-B overexpression on ERα promoter activity was still present but was no longer noticeable in the deleted construct lacking the half PRE site that we considered as putative effector site of PR-B inhibitory action. For instance when we performed site directed mutagenesis in the above mentioned region of ERα promoter, the effects due to PR-B overexpression on ERα promoter activity were completely abrogated. The inhibitory action of PR-B isoform over-expression on ERα promoter trascriptional machinery address most likely the capability of PR-B to recruit in these circumstances some corepressors interfering with ERα gene trascription.

To determine which PR isoform could mediate the interaction of specific coregulators with ERα promoter, siRNA directed against PR-B alone was used. Our ChIP assay results demonstrate the key role of PR-B isoform in the interaction with half-PRE site on ER α gene promoter.

Although there are far fewer nuclear receptor corepressors, these molecules serve important roles in negatively regulating receptor-dependent gene expression. It was reported that unliganded nuclear receptors as TR and RXR can repress basal

transcription in the absence of their cognate ligands, and this function is mediated, at least in part, by two nuclear proteins, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR). (43) The latter has been recently documented to be also recruited by both estrogen and progesterone receptors in the presence of ligand antagonist. Here we demonstrated that PR-B bound to ERα promoter, in presence or absence of its own natural ligand, is able to recruit a repressor of cell transcriptional machinery as NCoR. Recent data regarding PR transcriptional regulation of different gene promoter mostly address its capability to recruit in those circumstances NCoR. We tested if the latter event may also occur in our experimental conditions during the binding of PR-B isoform to the ERα promoter. Our ChIP experiments proved that among different corepressor molecules able to interact with PR, NCoR is the only present on PR-B/ DNA complex. These results could support a model in which elevated expression levels of PR-B increase the interaction of the receptor with NCoR on the half-PRE site of ERα promoter, an event incompatible with PR-coactivator interactions.

In conclusion we highlight the importance of regulated expression of PR-A and PR-B for cellular properties in breast cancer cells and we have provided a new insight into the molecular mechanism through which PR-B mediates the antiestrogenic effect in breast cancer cells. We think that the inhibition of Estrogen Receptor α by PR-B is a critical regulatory pathway in ER-positive cells, and we can speculate that deregulation of this repression in breast cancer may

have dramatic effects such as the promotion of transformation and metastasis. Our combined results suggest that this antiestrogenic mechanism is of therapeutic relevance for breast cancer treatment.

REFERENCES

1. Clarke CL. Sutherland RL Progestin regulation of cellular proliferation. Endocr Rev 1990; 11: 266-30
2. Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res. 2007;9(1):R6
3. Fuqua SAW and Cui Y. Estrogen and progesterone receptor isoforms: clinical significance in breast cancer. Breast Cancer Research and Treatment 2004;87: S3–S10.
4. Knight WA 3rd, Osborne CK, Yochmowitz MG, McGuire WL. Sterod hormone receptors in management of human breast cancer. Ann Clin Res 1980;12 :202-7
5. McGuire WL: Hormone receptors: Their role in predicting prognosis and response to endocrine therapy. Semin Oncol 5:428-433,1978
6. Bardou VJ, Arpino G, Elledge RM, et al: Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. J Clin Oncol 21:1973-1979, 2003
7. Hull DF, Clark GM, Osborne CK, et al: Multiple estrogen receptor assays in human breast cancer. Cancer Res 43:413-416, 1983

8. Gross GE, Clark GM, Chamness GC, et al: Multiple progesterone receptor assays in human breast cancer. Cancer Res 44:836-840,1984
9. Balleine RL, Earl MJ, Greenberg ML, et al: Absence of progesterone receptor associated with secondary breast cancer in postmenopausal women. Br J Cancer 79:1564-1571, 1999
10. Rossouw JE, Anderson GL, Prentice RL, et al: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. JAMA 288:321-333, 2002
11. Bardou VJ, Arpino G, Elledge RM, Osborne CK, Clark GM. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. J Clin Oncol 2003;21:1973-9
12. Kim HJ, Cui X, Hilsenbeck SG, Lee AV. Progesterone receptor loss correlates with human epidermal growth factor receptor 2 overexpression in estrogen receptor-positive breast cancer. Clinical Cancer Research 2006; 1013s-1018s
13. Kastner P, Krust A, Turcotte B, et al. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 1990; 9:1603–14
14. Kraus WL, Katzenellenbogen BS. Regulation of progesterone receptor gene expression and growth in the rat uterus: modulation of estrogen

actions by progesterone and sex steroid hormone antagonists. Endocrinology 1993;132:2371–9.

15. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 2002; 277:5209-5218
16. Boyd- Leinen PA Fournier D, Spelsberg TC. Nonfunctioning progesterone receptors in the developed oviducts from estrogen-withdrawn immature chicks and in aged nonlaying hens. Endocrinology 1982; 111:30-36
17. Kato J, Hirata S, Nozawa A, Mouri N. The ontogeny of gene expression of progestin receptors in the female rat brain. J Steroid Biochem Mol Biol. 1993 ; 47:173-82.
18. Shyamala G, Yang X, Silberstein G, Barcellos-Hoff MH, Dale E. Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. Proc Natl Acad Sci U S A. 1998; 20;95(2):696-701
19. Shyamala G, Yang X, Cardiff RD, Dale E. Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci U S A. 2000; 97(7):3044-9.
20. Mote PA, Bartow S, Tran N, Clarke CL. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat 2002; 72:163–172,

21. Kraus WL, and Katzenellenbogen BS. Regulation of progesterone receptor gene expression and growth in the rat uterus: modulation of estrogen action by progesterone and sex steroid hormone antagonists. Endocrinology 1993; 132:2371–2379.
22. Arnett-Mansfield RL, de Fazio A, Wain GV, Jaworski RC, Byth K, Mote PA, Clarke CL. Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium. Cancer Res 2001; 61: 4576–4582,
23. Kirkland JL, Murthy L, Stancel G. M. Progesterone inhibits the estrogen-induced expression of c-fos messenger ribonucleic acid in the uterus. Endocrinology 1992; 130(6):3223-30
24. Loose-Mitchell DS, Chiappetta C, Stancel G. M.. Estrogen regulation of c-*fos* messenger ribonucleic acid. Mol. Endocrinol. 1988; 2:946–951.
25. Katzenellenbogen BS. Mechanisms of action and cross-talk between estrogen receptor and progesterone receptor pathways. J Soc Gynecol Investig. 2000;7:S33-7.
26. Weigel RJ, deConinck EC. Transcriptional control of estrogen receptor in estrogen receptor negative breast carcinoma. Cancer Res 1993; 53: 3472–3474,
27. Bunone G, Briand PA, Miksicek R J, and Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. 1996; 15, 2174–2183

28. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, and Chambon P. The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J. 1989; 8**,** 1981–1986
29. Faivre EJ and Lange CA 2007 Progesterone Receptors Upregulate Wnt-1 To Induce Epidermal Growth Factor Receptor Transactivation and c-Src-Dependent Sustained Activation of Erk1/2 Mitogen-Activated ProteinKinase in Breast Cancer Cells. Mol and Cell Biol 27: 466–480
30. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. 1990; 9(5):1603-14
31. deGraffenried L.A., Hilsenbeck SG., Fuqua S.A.W. Sp1 is essential for estrogen receptor α gene transcription. Journal of Steroid Biochemistry & Molecular Biology 2002; 82, 7–18
32. van der Burg, B., Rutteman, G. R., Blankenstein, M. A., de Laat, S. W., and van Zoelen, E. J. Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. J. Cell. Physiol. 1988; 34**,** 101–108
33. Hardy DB, Janowski BA, Corey DR, Mendelson CR Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. Mol Endocrinol. 2006 Nov;20(11):2724-33.

34. Dennis AP, Lonard DM, Nawaz Z, O'Malley BW. Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol. 2005 Mar;94(4):337-46.

35. Shang, Y., Hu, X., Di Renzo, J., Lazar, M. A., and Brown, M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000; 103**,** 843–852

36. Baulieu EE. Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. Science 1989; 245(4924):1351-7

37. Graham JD, Roman SD, McGowan E, Sutherland RL, Clarke CL. Preferential stimulation of human progesterone receptor B expression by estrogen in T-47D human breast cancer cells. J Biol Chem. 1995; 270(51):30693-700

38. Martin MB, Saceda M, Garcia-Morales P, Gottardis MM. Regulation of estrogen receptor expression. Breast Cancer Res Treat 1994; 31: 183–189

39. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev. 1999 Jun;20(3):321-44.

40. Hopp TA, Weiss HL, Hilsenbeck SG, Cui Y, Allred DC, Horwitz KB and Fuqua SAW Breast Cancer Patients with Progesterone Receptor PR-A-Rich Tumors Have Poorer Disease-Free Survival Rates .Clinical Cancer Research 200410, 2751-2760

41. Alexander IE, Shine J, Sutherland RL. Progestin regulation of estrogen receptor messenger RNA in human breast cancer cells. Mol Endocrinol 1990; 4:821-828

42. Read LD, Greene GL, Katzenellenbogen BS . Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol Endocrinol. 1989 Feb;3(2):295-304

43. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev. 2004 Feb;25(1):45-71.

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

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Chemicals

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

Semen samples and spermatozoa preparations

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

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

LNCaP cells culture

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

RNA isolation and RT–PCR

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

Table 1: Oligonucleotide sequences used for RT–PCR

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

Gel extraction and DNA sequence analysis

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

Western blot analysis of sperm proteins

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

Immunofluorescence assay

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

Immunoprecipitation of sperm proteins and LNCaP cells proteins

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

Evaluation of sperm viability

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

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

PI3K activity

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

DNA laddering

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

Statistical analysis

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

Results

AR mRNA and protein were detected in human sperm

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

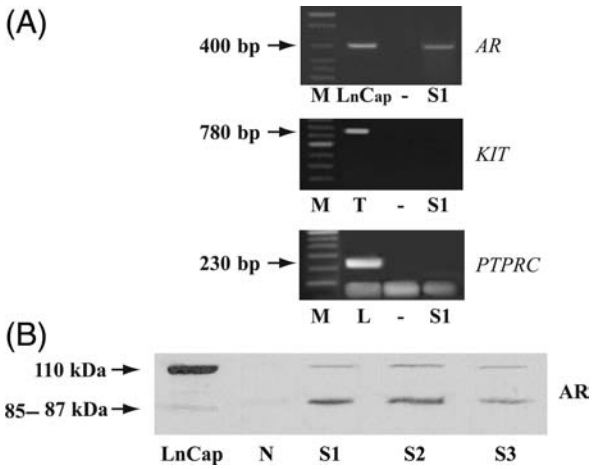

Figure 1: AR expression in human ejaculated spermatozoa (**A**) reverse transcription-PCR analysis of human *AR* gene, *KIT* and *PTPRC* in percolled human ejaculated spermatozoa (S1), negative control (no M-MLV reverse transcriptase added) (-), positive control (LnCap, prostate cancer cell; T, human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

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

Immunolocalization of AR in human sperm

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

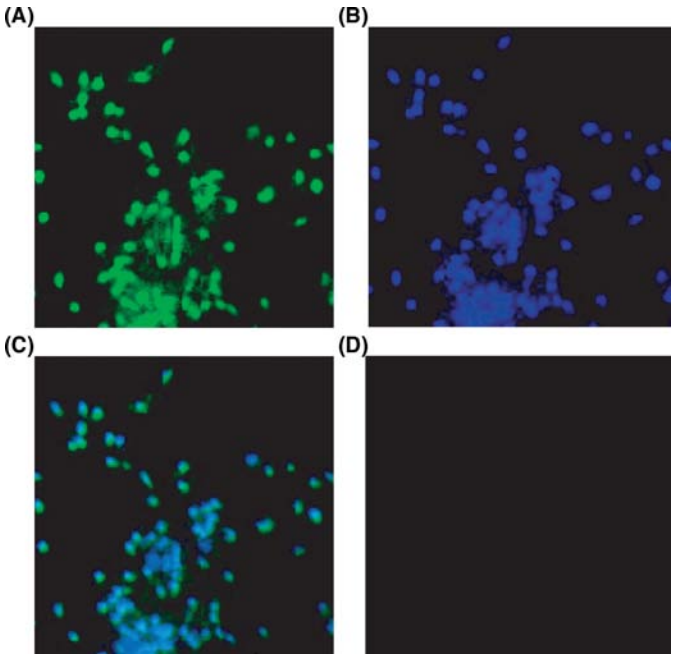

Figure 2: Immunolocalization of AR in human ejaculated spermatozoa

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

AR is phosphorylated in human sperm

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

Androgens effect on sperm viability

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

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

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

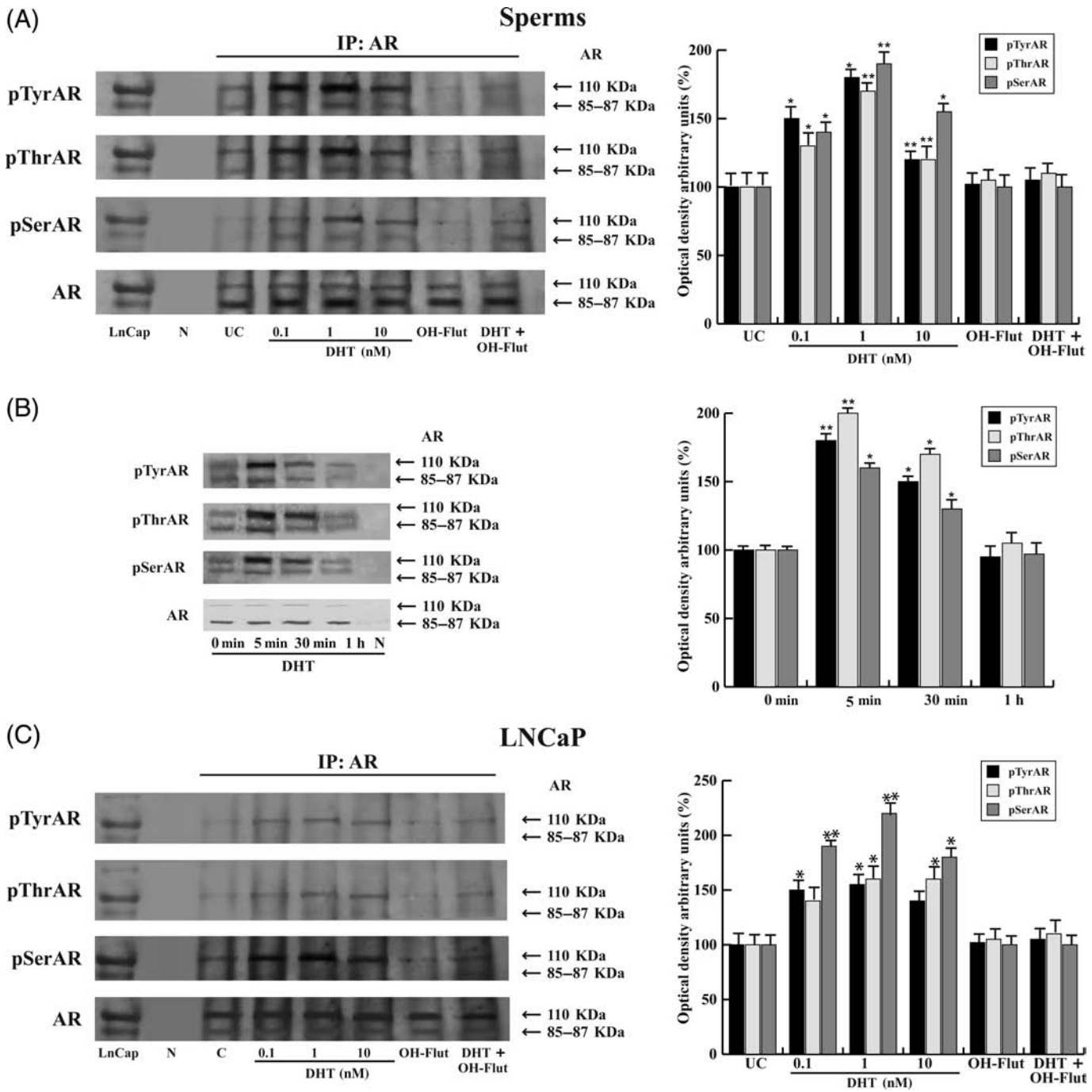

Figure 3: AR is phosphorylated in human sperm

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

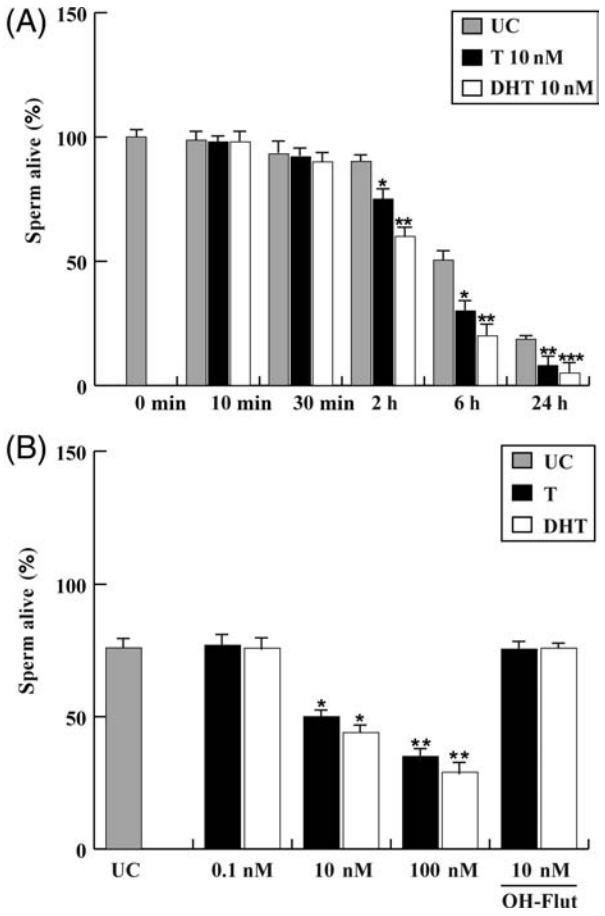

Figure 4: Effect of androgen on sperm viability
Viability was assessed by using Eosin Y as described in *Materials and Methods*. (**A**) time course of sperm incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 without (UC) or in the presence of 10 nM T or 10 nM DHT. (**B**) sperm were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

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

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

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

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

Androgens effects on caspases are mediated by AR

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

Discussion

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

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

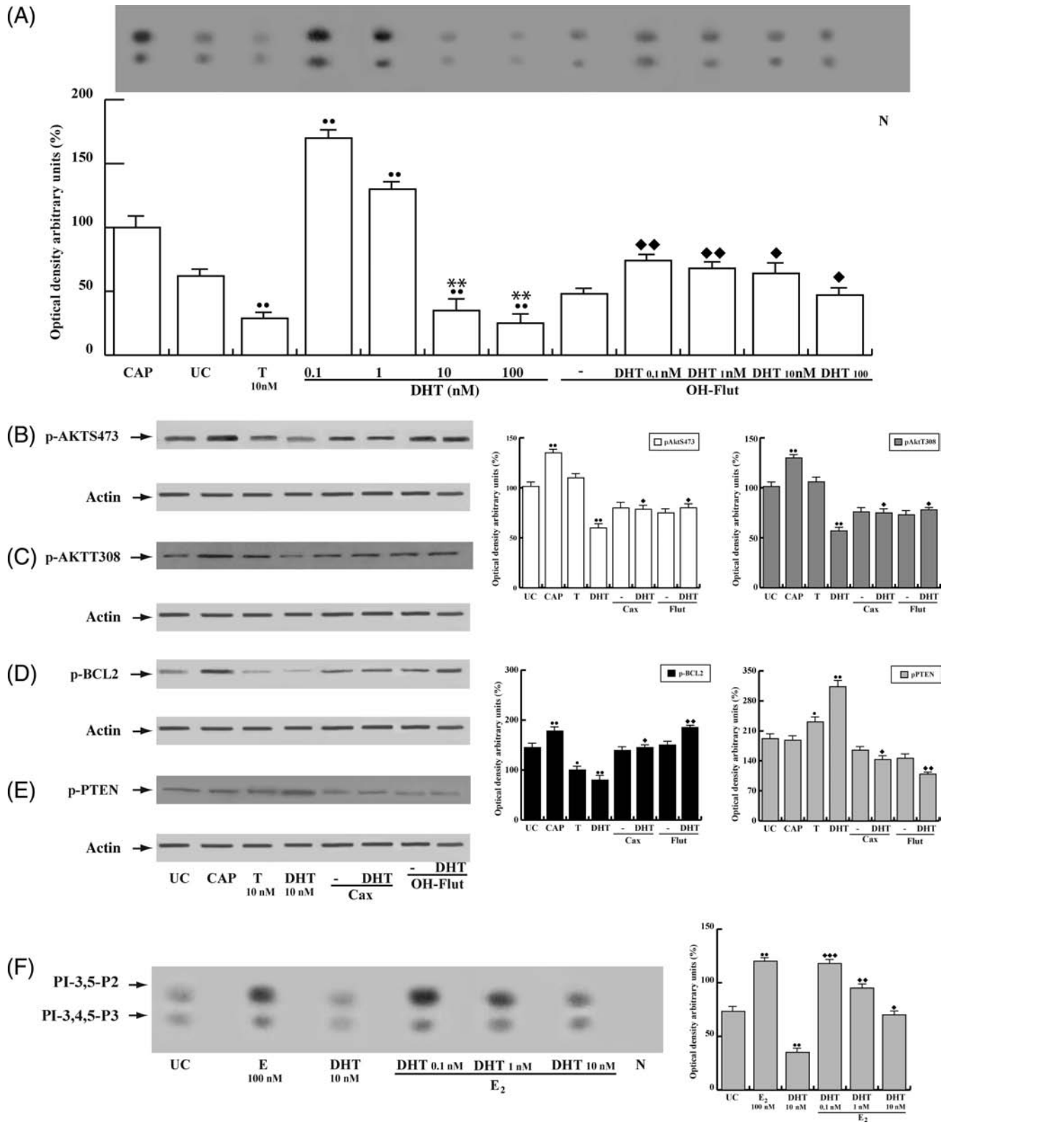

Figure 5: Androgens action on PI3K activity, p-AKT, p-BCL2 and p-PTEN is mediated by AR

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

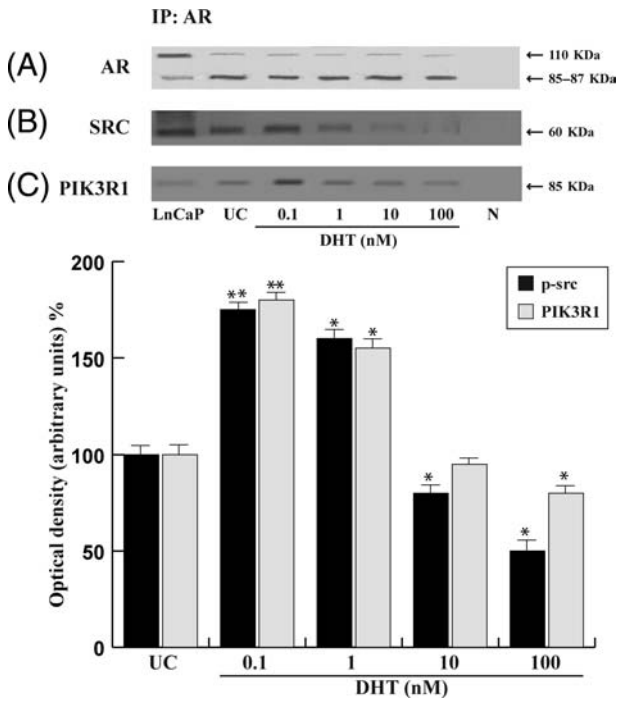

Figure 6: AR, PIK3R1 and phospho-SRC tyrosine kinase coimmunoprecipitate in human sperm
Washed spermatozoa from normal samples were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, without (UC) or in the presence of DHT at increasing concentrations (0.1, 1, 10 and 100 nM).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

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

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

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

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

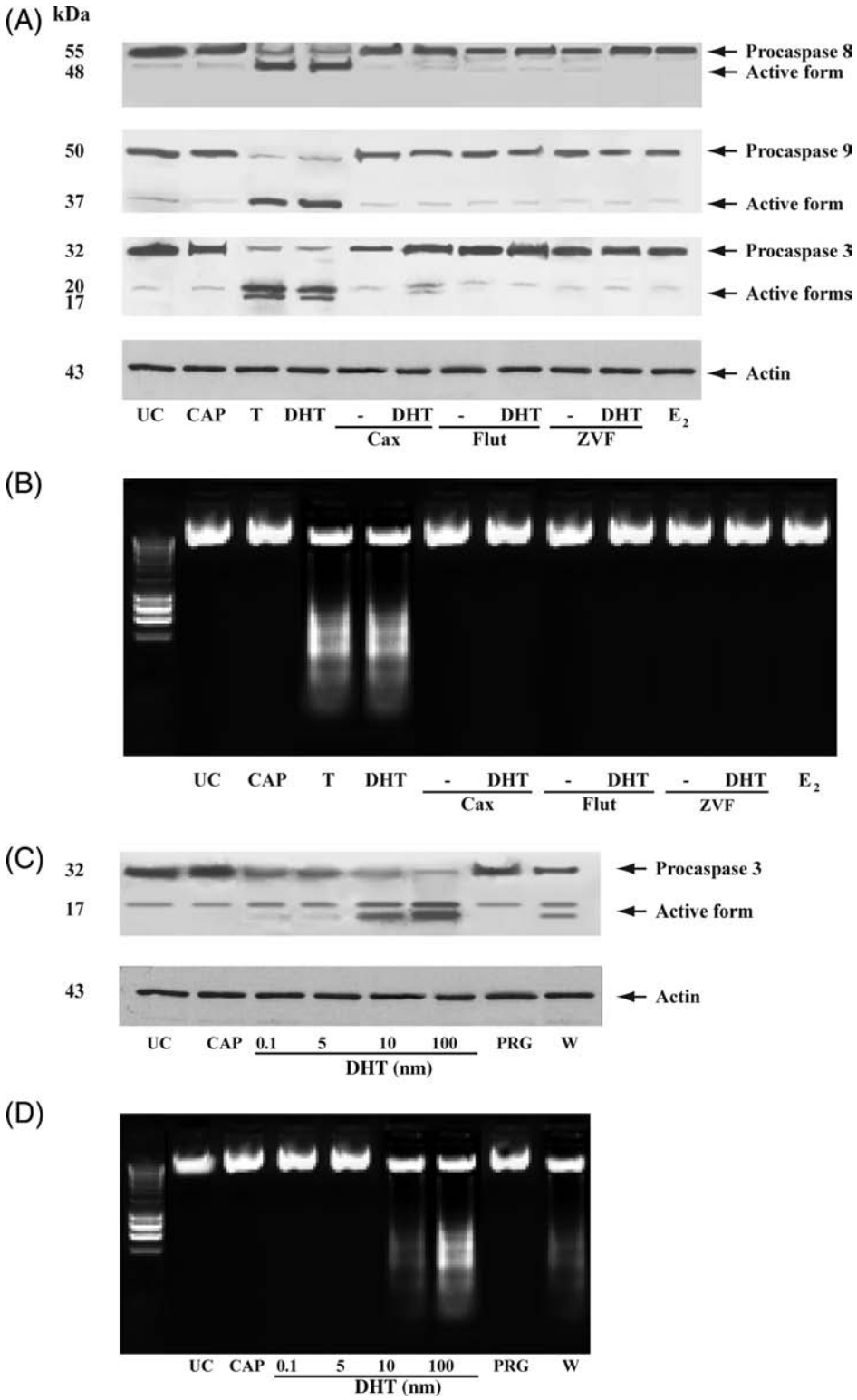

Figure 7: Androgens effects on caspases are mediated by AR
(**A**) washed pooled sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 (UC) in the presence of 10 nM T or 10 nM DHT or 100 nM E for 30 min. Some samples were washed with the unsupplemented Earle's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

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

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

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

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

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References

Andò S, Aquila S. Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Mol Cell Endocrinol* 2005;**245**:1–6.

Andò S, Giacchetto C, Beraldi E, Panno ML, Carpino A, Sposato G, Lombardi A. Testosterone and dihydrotestosterone seminal plasma levels in varicocele patients. *Andrologia* 1983;**15**:374–379.

Aquila S, Sisci D, Gentile ME, Middea E, Catalano S, Carpino A, Rago V, Andò S. Estrogen receptor (ER) α and ER β are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J Clin Endoc Metab* 2004;**89**:1443–1451.

Aquila S, Sisci D, Gentile ME, Middea E, Siciliano L, Andò S. Human ejaculated spermatozoa contain active P450 aromatase. *J Clin Endocrinol Metab* 2002;**87**:3385–3390.

Aquila S, Gentile M, Middea E, Catalano S, Andò S. Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* 2005a;**146**:552–557.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V, Andò S. Leptin secretion by human ejaculated spermatozoa. *J Clin Endocrinol Metab* 2005b;**90**:4753–4761.

Calogero AE, Burrello N, Barone N, Palermo I, Grasso U, D'Agata R. Effects of progesterone on sperm function: mechanisms of action. *Hum Reprod* 2000;**15**:28–45.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998;**282**:1318–1321.

Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, Bottero D, Vitale F, Migliaccio A, Auricchio F. Androgen-stimulated DNA synthesis and cytoskeletal changes in fibroblasts by a nontranscriptional receptor action. *J Cell Biol* 2003;**161**:547–556.

Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, De Falco A, Varricchio L, Bottero D, Nanayakkara M, Migliaccio A *et al.* Rapid signalling pathway activation by androgens in epithelial and stromal cells. *Steroids* 2004;**69**:517–522.

Cato ACB, Nestl A, Mink S. Rapid actions of steroid receptors in cellular signaling pathways. *Sci STKE* 2002;**138**:RE9.

Cheng CY, Boettcher B, Rose RJ, Kay DJ, Tinneberg HR. The binding of sex steroids to human spermatozoa. An autoradiographic study. *Int J Androl* 1981;**4**:1–17.

Cohen GM. Caspases—the executioners of apoptosis. *Biochem J* 1997;**326**: 1–16.

Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, Chang C. The androgen receptor in spermatogenesis. *Cytogenet Genome Res* 2003;**103**:299–301.

Coviello AD, Bremner WJ, Matsumoto AM, Herbst KL, Amory JK, Anawalt BD, Yan X, Brown TR, Wright WW, Zirkin BR *et al.* Intratesticular testosterone concentrations comparable with serum levels are not sufficient to maintain normal sperm production in men receiving a hormonal contraceptive regimen. *J Androl* 2004;**25**:931–938.

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C *et al.* A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc Natl Acad Sci* 2004;**101**:1327–1332.

Gao T, McPhaul MJ. Functional activities of the A and B forms of the human androgen receptor in response to androgen receptor agonists and antagonists. *Mol Endocrinol* 1998;**12**:654–663.

Goyal HO, Bartol FF, Wiley AA, Neff CW. Immunolocalization of receptors for androgen and estrogen in male caprine reproductive tissues: unique distribution of estrogen receptors in efferent ductule epithelium. *Biol Reprod* 1996;**56**:90–101.

Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol Cell Endocrinol* 1997;**126**:59–73.

Hyne RV, Boettcher B. The selective binding of steroids by human spermatozoa. *Contraception* 1977;**15**:163–174.

Ito T, Deng X, Carr BK, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 1997;**272**:11671–11673.

Iwamura M, Abrahamsson P-A, Benning CM, Cockett AT, Di Sant'Agnese PA. Androgen receptor immunostaining and its tissue distribution in formalin fixed, paraffin-embedded sections after microwave treatment. *J Histochem Cytochem* 1994;**42**:783–788.

Kimura N, Mizokami A, Oonuma T, Sasano H, Nagura H. Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* 1993;**41**:671–678.

King KJ, Nicholson HD, Assinder SJ. Effect of increasing ratio of estrogen: androgen on proliferation of normal human prostate stromal and epithelial cells, and the malignant cell line LNCaP. *Prostate* 2006;**66**:105–114.

Kuwahara M. The effects of ligation of vas deferens, corpus epididymidis and vasa efferentia on the testicular function in rats. *Tohoku J Exp Med* 1976;**120**:251–257.

Luboshitzky R, Kaplan-Zverling M, Shen-Orr Z, Nave R, Herer P. Seminal plasma androgen/oestrogen balance in infertile men. *Int J Androl* 2002;**25**:345–351.

Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998;**273**:13375–13388.

McPhaul MJ, Young M. Complexities of androgen action. *J Am Acad Dermatol* 2001;**45**:S87–S94.

Miller D, Ostermeier GC, Krawetz SA. The controversy, potential and roles of spermatozoal RNA. *Trends Mol Med* 2005;**11**:156–163.

Olsen NJ, Viselli SM, Fan J, Kovacs WJ. Androgens accelerate thymocyte apoptosis. *Endocrinology* 1998;**139**:748–752.

Olson M, Kornbluth S. Mitochondria in apoptosis and human disease. *Curr Mol Med* 2001;**1**:91–122.

Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004;**81**:802–809.

Parsons R. Human cancer, PTEN and the PI-3 kinase pathway. *Semin Cell Dev Biol* 2004;**15**:171–176.

Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. Rapid signalling by androgen receptor in prostate cancer cells. *Oncogene* 1999;**18**:6322–6329.

Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND, van der Kwast TH. Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* 1991;**39**:927–936.

Said TM, Paasch U, Glander HJ, Agarwal A. Role of caspases in male infertility. *Hum Reprod Update* 2004;**10**:39–51.

Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* 2003;**7**:428–432.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. *Eur J Biochem* 1998;**254**:439–459.

Shetty G, Wilson G, Hardy MP, Niu E, Huhtaniemi I, Meistrich ML. Inhibition of recovery of spermatogenesis in irradiated rats by different androgens. *Endocrinology* 2002;**143**:3385–3396.

Shim D, Kang HY, Jeon BW, Kang SS, Chang SI, Kim HY. Protein kinase B inhibits apoptosis induced by actinomycin D in ECV304 cells through phosphorylation of caspase 8. *Arch Biochem Biophys* 2004; **425**:214–220.

Solakidi S, Psarra AM, Nikolaropoulos S, Sekeris CE. Estrogen receptors α and β (ERα and ERβ) and androgen receptor (AR) in human sperm: localization of ERβ and AR in mitochondria of the midpiece. *Hum Reprod* 2005;**20**:3481–3487.

Steers WD. 5alpha-reductase activity in the prostate. *Urology* 2001;**58**:17–24.

Suarez-Quian CA, Martinez-Garcia F, Nistal M, Regadera J. Androgen receptor distribution in adult human testis. *J Clin Endocrinol Metab* 1999;**84**:350–358.

Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ. Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of PIK3R1, androgen receptor, and Src. *J Biol Chem* 2003;**278**:42992–43000.

Vornberger W, Prins G, Musto NA, Suarez-Quian CA. Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* 1994;**134**:2307–2316.

Wang LG, Liu XM, Kreis W, Budman DR. Phosphorylation/ dephosphorylation of androgen receptor as a determinant of androgen agonistic or antagonistic activity. *Biochem Biophys Res Commun* 1999;**259**:21–28.

Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc Natl Acad Sci USA* 1994;**91**:1234–1238.

Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are expressed in a variety of human tissues. *Mol Cell Endocrinol* 1996;**120**:51–57.

World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions, 4th edn. Cambridge: Cambridge University Press, 1999.

Wu X, Senechal K, Neshat MS, Whang YE, Sawyers CL. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1998;**95**:15587–15591.

Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X *et al.* Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci USA* 2002; **99**:13498–13503. Erratum in: *Proc Natl Acad Sci USA* 2002;**12**:15245.

Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl* 2002;**23**:870–881.

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Title page

Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival.

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Abstract

Much effort has recently focused on understanding the role of leptin, the obese gene product secreted by adipocytes, in regulating growth and reproduction in rodents, humans and domestic animals. The present study was designed to investigate the expression of leptin and its receptor in pig spermatozoa. Successful western blot evidenced a 16 kDa band for leptin and six isoforms ranging from 120 to 40 kDa for the leptin receptor, suggesting a possible biological effects in these cells. Intriguingly, leptin and leptin receptor were both located at the acrosomal level, addressing an action in the events that render the sperm able to fertilize the oocyte. In fact, both capacitation indexes and acrosin activity were enhanced upon 10 nM leptin and these effects were reduced by the anti-leptin receptor antibody. Afterwards, we explored the main transduction pathways regulated by the hormone. Our results showed that in pig sperm leptin can trigger the signal transducer and activator of transcription 3, a classical component of cytokine signal transduction pathways, which expression was never reported in male gamete and that we found constitutively activated. Besides, leptin was able to induce the activation of Phosphatidylinositol Phosphate Kinase 3 and Mitogen-Activated Protein Kinase pathways as well as of Bcl-2, a known antiapoptotic protein. Altogether, these data also address a role for leptin and its receptor on pig sperm survival. The presence of leptin and its receptor in pig sperm suggests that they, through an autocrine short loop, may induce signal transduction and molecular changes associated to sperm capacitation and survival.

INTRODUCTION

Leptin (Lep) is known to be the hormonal link between energy stores and several vital functions, including food intake and energy homeostasis. It is a 16-kDa adipocyte-derived hormone that suppresses food intake, stimulates energy expenditure, increases metabolic rate, and ultimately causes loss of body fat. (Boulounie *et al.* 1998; Kamohara *et al.* 1997; Rossetti *et al.* 1997). Much attention has focused on the effects of Lep as a central satiety agent and its action was reported to be involved in the regulation of fatness via feed intake. Although originally postulated to act largely via the central nervous system, several recent studies have demonstrated that Lep exerts a wide repertoire of peripheral effects through direct actions on target tissues. These direct effects include stimulation of fatty acid oxidation in adipocytes (Muller *et al.* 1997; Boulounie *et al.* 1998; Lord *et al.* 1998; Sierra-Honigmann *et al.* 1998; Zhao *et al.* 1998) and increased glucose uptake in skeletal muscle and brown adipose tissue in vivo (Kamohara *et al.* 1997; Yaspelkis *et al.* 1999) and in a myotube cell line in vitro (Berti *et al.*1997).

The pig is emerging rapidly as an important biomedical research model, and whereas genetic influences may impact the degree of similarity between pig and human systems, the regulation of some endocrine and metabolic processes in the pig may be more similar to humans than are rodents (Tsiaoussis *et al.* 2001; Vilei *et al.* 2001). Lep plays a key role in the regulation of energy homeostasis and is also linked to mechanisms controlling reproductive processes. With respect to reproductive function, the importance of Lep has grown rapidly in the past few years in fact, numerous lines of evidence point to the participation of this peptide in the regulation of neuroendocrine axis and thereby, among others , in reproductive processes of different species (Campfield *et al.* 1996; Foster *et al*. 1999; Ahima *et al.* 2000; Amstalden *et al*. 2000),

including pigs (Barb *et al.* 1998; 1999). However, data concerning the involvement of Lep in controlling reproductive functions at the level of hypothalamus and pituitary in the pig are very limited. The relationship between nutrition and reproduction in swine has been extensively reviewed (Zurek *et al.* 1995; Quesnel & Prunier 1995). Nutrition may influence reproductive performance by a number of mechanisms, including central effects on gonadotropin secretion (Booth *et al.* 1994) and local effects on ovarian function (Cosgrove *et al.* 1992; Booth *et al.* 1996). Consistent with the data reported by Zak (Zak *et al*. 1997), the different feeding regimens caused differential sow body weight changes. In the pig nutritional signals such as Lep are detected by the central nervous system (CNS) and translated by the neuroendocrine system into signals, which regulate appetite, hypothalamic gonadotropin-releasing hormone (GnRH) release and subsequent luteinizing hormone (LH) secretion. Changes in body weight or nutritional status are characterized by altered adipocyte function, a reduction in adipose tissue leptin expression, serum leptin concentrations and a concurrent decrease in LH secretion.

In both male and female mice it has been shown that leptin have direct effects on fertility since the hormone is able to reverse the infertility of *ob/ob* mice, lacking of the leptin gene (Cunningham *et al.* 1999). The biological actions of Lep on body weight homeostasis, neuroendocrine function and fertility are carried out through interactions with its specific obese receptor (Ob-R), in target tissues. The pattern of leptin receptor expression in the testis is specie-specific. Several isoforms of Ob-R exist that differ mainly in the length of the cytoplasmic domain (Takaya *et al.* 1996; Dieterichet *et al.* 1996; Lee *et al.* 1996; Lollmann *et al.* 1997; Murakami *et al.* 1997; Bjorbaek *et al.* 1998; Yamanita *et al.* 1998). The Ob-R, a single membrane-spanning glycoprotein, belongs to the class I cytokine receptor superfamily, sharing sequence homologies for interaction with Janus kinase as well as signal transducers and activators of transcription

(STATs) (Tartaglia *et al.* 1997). To date, the human Ob-R is identified as a full-length Ob-Rb form (Tartaglia *et al.* 1995) and as several short form receptors, generated by alternative splicing, OB-ra, OB-rc, OB-rd , OB-rf and OB-re (Bennet *et al.* 1996; Cioffi *et al.* 1996, Tartaglia *et al.* 1997). The hypothalamus is supposed to be the only tissue with predominantly expressed full-length Ob-R. However, an increasing number of peripheral tissues are described to express the long form as well as several isoforms (Cioffi *et al.* 1996; Tartaglia *et al.* 1997; Glasow *et al.* 1998; Breidert *et al.* 1999), including liver, heart, kidneys, lungs, small intestine, pituitary cells, testes, ovaries, spleen, pancreas, adrenal gland and adipose tissue (Margetic *et al.* 2002). Furthermore studies on different OB-r splice variants indicated that many tissues may contain a heterologous mix of OB-r subtypes. In a variety of porcine tissues Ob-R mRNA (long form) has been detected, including adipose tissue (Lin *et al.* 2000). Using *in situ* hybridisation techniques, other groups have shown that leptin receptor mRNA is expressed in Sertoli cells of adult rats (Tena-Sempere *et al.* 2001).

Rodent testis revealed the Ob-R expression but with a differential cellular site, in fact, the immunoreactivity was confined in Leydig cells of rat but in germ cells of mouse (El-Hefnawy *et al.* 2000, Caprio *et al.* 2003). This discrepancy may be due to interspecie difference. Lep and Ob-R were found to be present in the human seminiferous tubules (Soyupek *et al.* 2005), in human seminal plasma (Jope *et al.* 2003), in human sperm (Aquila *et al.* 2005) and recently Ob-R was also identified in boar sperm (De Ambrogi *et al.* 2007). In the present study we investigated Lep and Ob-R expression in pig sperm, evaluating their potential role on sperm capacitation and survival by the assessment of the main molecular markers involved in these processes.

Materials and Methods

Chemicals

BSA protein standard, Laemmli sample buffer, prestained molecular weight marker, Percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, dimethyl sulfoxide, Earle's balanced salt solution, triethanolamine buffer, $MgCl_2$, propidium iodide, LY294002 and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Recombinant porcine leptin was purchased from Protein Laboratories Rehovot (Rehovot, Israel), acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, ECL Plus Western blotting detection system, Hybond™ ECL™, were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antibodies (Abs) polyclonal rabbit anti-leptin (A-20), rabbit anti-Ob-R(H-300), rabbit anti-p-Akt1/Akt2/Akt3 (Ser473), rabbit anti-p-Akt1/Akt2/Akt3 (Thr308), rabbit anti-phosphotyrosine (PY99), mouse ant-p-signal transducer and activator of transcription-3 p-STAT3 (B-7), peroxidase-coupled anti-rabbit, and FITC/ Texas Red conjugated anti-rabbit IgG were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-p-Bcl-2 and p-ERK1/2 Abs were from Cell Signalling (Milan, Italy). Cholesterol-oxidase (CHOD)-peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Italia Srl, Catania, Italy).

Animals and semen samples

The investigation has been conducted on semen from 7 fertile male pigs (*Sus scrofa domestica*, Large White) kept at " Swine Artificial Insemination Centre " (Rende, Cosenza, Italy). The animals were 24 to 30 month-old and their weights were from 280 to 320 kg. Individual fresh ejaculates were collected by the gloved hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Semen was transported within half an hour to the laboratory, it was diluted 1:10 with TBS

buffer and centrifuged on a discontinuous Percoll density gradient (72 % / 90 %) to remove bacteria and debris (Kuster *et al*. 2004).

Evaluation of sperm viability

Viability of pig sperm was assessed using the DNA-specific fluorochrome propidium iodide (PI). Sperm suspension ($1x10^6$ mL) was exposed to PI (12 µmol/ L) for 5 min at room temperature Then spermatozoa were fixed by adding 1 mL of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris /l (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41) observing a minimum of 200 spermatozoa × slide (100× objective).

Sample treatments

Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 minutes (min) at 39 °C and 5 % CO_2, without (control) or with increasing concentration of Lep (10 and 60 nM). These doses were chosen on the basis of physiological concentrations to reproduce the environment of the sperm journey. In fact, Lep concentration in porcine semen ranged from 9.2 - 13.38 ng/ml (considering a mean of 10 ng/ml we obtain 62 nM) (Lackey *et al.* 2002) while the value for Lep in follicular fluid was about 1.9 ng/ml (12 nM) (Gregoraszczuk *et al.* 2004). Some cells were also pre-treated (15 min) with the inhibitor, LY 294002, or with the anti-Ob-R Ab (autocrine blockage). No adverse effects among the different treatments have been observed on pig sperm viability.

Then, samples were centrifuged(3000 g x 5min) using the upper phase for further determinations and sperm pellet for western blot analysis.

Western blot (WB) analysis of sperm proteins

Western blot analysis was used to identify leptin and Ob-R in individual pig sperm. Sperm samples were washed twice with Earle's balanced salt solution (uncapacitating medium) and then centrifuged for 5 min at 5000 x g. The pellet was resuspended in lysis

buffer as previously described (Aquila *et al.* 2002). Equal amounts of proteins (80 µg) were boiled for 5 min, separated by 10 % polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the (indicated) specific antibody (Ab). The bound of the secondary antibody was revealed with the ECL Plus WB detection system according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of leptin or Ob-R (i.e. lysates pre-incubated with anti-leptin Ab or anti-Ob-R Ab for 1 hour at room temperature and immunoprecipitated with Protein A/G-agarose).

To further validate the results for Ob-R, as negative control, non-immune rabbit serum, instead of the first antibody, was used at the same dilution ratio (1 : 1000).

Western blot was also performed to identify STAT3, Bcl-2, ERK 1/ 2, Akt1/ Akt2 in pig sperm extracts and to evaluate the cell signaling induced by Lep (p-Akt1/Akt2/Akt3, p-STAT-3, p-Bcl-2 and p-ERK1/2). MCF7 cell lines were used as positive controls while negative controls were performed as above indicated These last blots were stripped (glycine 0.2M ,pH2.6 for 30 min at room temperature) and riprobed with anti-βactin Ab as loading control.

The experiments were repeated at least four times for each sample.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5mM Tris-HCl buffer, pH 7.5; then 10µl of concentrated cell suspension was added to 250µl drop of warm (37°C) TBS and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 minutes at –20 °C. After methanol removal, sperm cells were washed in TBS, containing 0.1% Triton X-100 and were treated for immunofluorescence

Leptin and Ob-R stainings were carried out, after blocking with normal goat serum (10 %), using anti leptin(1:100) and anti-Ob-R (1:100) as primary antibodies, followed by

anti-rabbit IgG Texas Red conjugated/ FITC conjugated antibodies (1:200) respectively. Sperm cells incubated without the primary antibodies were utilized as negative controls. The slides were immediately examined under an epifluorescence microscope (Olympus BX41) observing a minimum of 200 spermatozoa × slide (100× objective).

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured (in duplicate) in the incubation medium from pig spermatozoa by a CHOD - POD enzymatic colorimetric method according to manufacturer's instructions Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control, NC) for 30 minutes (min) at 39 °C and 5 % CO_2. Other samples were incubated in the presence of 10 and 60 nM Lep concentrations . Some samples were incubated with anti-Ob-R combined with 10nM Lep. At the end of the sperm incubation, the culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 ml of buffer reaction. The samples were incubated for 10 minutes at room temperature, then the cholesterol content was measured with the spectrophotometer at 505 nm. Cholesterol standard used was 200 mg/dl. The limit of sensitivity for the assay was 0.05 mg/dl. Inter- and intra-assay variations were 0,71 % and 0,57 % respectively.

Acrosin activity assay

Acrosin activity was assessed by the method of Glogowski (Glogowski *et al.* 1998). Briefly, sperm were washed in Earle's medium and centrifuged at 800 *g* for 20 min, then they were resuspended (final concentration of 100 x 10^3 sperm/ml) in different tubes containing no treatment (control) or the indicated treatments (experimental). 1 ml of substrate–detergent mixture (23 mmol/l BAPNA in DMSO and 0.01% Triton X-100 in 0.055 mol/l NaCl, 0.055 mol/l HEPES at pH 8.0 respectively) was added for 1 hour at room temperature. After incubation, 0.5 mol/l benzamidine was added (0.1 ml) to each of the tubes and then they were

centrifuged at 1000 *g* for 30 min. The supernatants were collected and the acrosin activity measured spectrophotometrically at 410 nm. In this assay, the total acrosin activity is defined as the amount of the active (non-zymogen) acrosin associated with sperm plus the amount of active acrosin that is obtained by pro-acrosin activable. The acrosin activity was expressed as mIU/10^6 sperms.

RESULTS

Western Blot showed both Lep and Ob-R expression in pig sperm

The presence of Lep protein in pig spermatozoa was investigated by WB using an antibody raised against the carboxyl-terminus of the human protein. One immunoreactive band was observed at 16 kDa in the lysates from pig sperm samples at the same mobility of the adipocyte extract used as positive control (Fig 1A).

Ob-R by WB, using the same antibody as used for immunohistochemistry (Fig. 1B) showed six different immunoreactive isoforms (120, 90, 80, 65, 60 and 40 kDa) (Fig 1B,b1). The bands were not detected by non immune rabbit serum indicating that these proteins are specific for Ob-R (Fig. 1B,b2).

Immunolocalization of Lep and OB-R in pig sperm

An intense red fluorescence localized Leptin in the acrosoma of pig sperm while the other cellular regions were unlabelled (Fig 2 A). In addition, a brilliant green light revealed that Ob-R was confined in the apical portion of sperm acrosoma (2 B). No fluorescent signal was obtained when primary antibodies (anti-Lep Ab or anti-Ob-R Ab) were omitted (inserts) thus confirming the specificity of the antibody binding.

Lep influences pig sperm capacitation enhancing both cholesterol efflux and protein tyrosine phosphorylation

One of the early events associated with capacitation of mammalian spermatozoa is the cholesterol efflux, after which protein phosphorylation begins. Our results showed a significant increase in cholesterol efflux (Fig 3 A). Protein tyrosine phosphorylation pattern of pig sperm, similar to that previously reported in porcine (Tardif *et al.* 2003), was also induced by leptin treatment (Fig 3 B). However, leptin effect on both processes

was not dose-dependent, because only 10 nM lep was able to sustain the increases. Further, anti-Ob-R Ab was able to abolish Lep effect.

Lep stimulates pig sperm acrosin activity

Capacitation prepares the sperm for the acrosome reaction and acrosin is a sperm representative acrosomal enzyme. Acrosin activity showed that 10 nM Lep were able to stimulate the enzymatic activity, while no effect was observed with 60 nM Lep. Anti-Ob-R Ab combined with 10 nM Lep were able to abolish Lep effect (Fig 4).

STAT3 is activated by Lep/Ob-R in pig sperm

The JAK/STAT pathway is one of the main signalling cascades activated by Lep (Thomas 2004). Our results showed in pig sperm the expression of the STAT3 (Fig 5 A) which is a critical mediator of leptin action. Therefore, we aimed to investigate whether this important factor of Lep signal is activated in pig male gamete. As shown in Fig 5 B,C constitutively activated (phosphorylated) STAT3 is present in pig sperm and 10 nM Lep treatment induced a slight but significant activation that was reversed by the anti-Ob-R Ab.

ERK 1/2 is activated by Lep/Ob-R in pig sperm

Activation of MAPK has also been implicated as a signalling intermediate for Lep in various cell types (Takahashi *et al.* 1997; Tanabe *et al.* 1997). Besides, several lines of evidence indicate that components of the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPK) are present in spermatozoa and are involved in motility and capacitation (Naz *et al*. 1992; Luconi *et al.* 1998; de Lamirande & Gagnon 2002). Our results showed the expression of ERK 1/2 (Fig 6 A) and evidenced that Lep, through its receptor, is able to activate ERK 1/2 (Fig 6 B,C).

Lep/Ob-R signalling modulates pig sperm survival

Fig 7 A shows in pig sperm the expression of Bcl-2, a key protein in survival signaling. Furthermore, our data (Fig 7 B, C) revealed that leptin induced the Serine 70 phosphorylation of Bcl-2, that is the physiologically relevant phosphorylation site, necessary for its full and potent antiapoptotic function. This effect was inhibited by the anti-Ob-R Ab.

PI3-K plays an important role in cell survival and metabolism in somatic cells as well as in sperm (Aquila *et al.* 2004; Aparicio *et al*. 2006). The PI3-K main downstream effector is the Akt, identified in human and boar spermatozoa. Fig 7 D shows the expression of Akt in pig sperm. Then we investigated the possible Akt phosphorylation by Lep evaluating the dual serine 473 and threonine 308 aminoacidic residues that give the full Akt activation. Our results showed that Akt is constitutively activated and that Lep induced an increase in the kinase phosporylation, which was reduced by using anti-Ob-R Ab (Fig 7 E, F). To ensure that the increase in Akt activation is driven by PI3-K activation, the effect of a specific PI3-K inhibitor, 10μM LY294002 was assessed. The inhibitor abolished Lep-induced Akt S473 and Akt T308 phosphorylations (Fig 7 E, F).

Statistical analysis

Data, presented as mean ± SEM, were evaluated by the one –way analysis of variance (ANOVA) The differences in mean values were calculated at a significance level of $P \leq 0.05$.

DISCUSSION

In recent years, it has been suggested that Lep is a metabolic signal to the reproductive system (Barash *et al.* 1996). To date, the mechanisms by which Lep regulate reproductive function remain to be determined. Lep and Ob-R were recently found in human sperm (Jope *et al.* 2003; Aquila *et al.* 2005) and the Ob-R in boar sperm (De Ambrogi *et al.* 2007). However, Lep and its signaling as well as its relationship with male gamete function across non-human species were not investigated. In the present study, we investigated, for the first time, the presence of Lep and Ob-R in pig sperm and evaluated their potential action on capacitation and sperm survival. The chief aim of our study was also to identify the potential signalling molecules set in motion by Lep in pig sperm.

First we evidenced the presence of Lep and OB-R by WB and immunofluorescence assay. Lep as protein was evidenced at the same size as it was observed in human sperm (Aquila *et al.* 2005), whereas different isoforms were obtained for the Ob-R. However, the pattern of leptin receptor expression is specie-specific and different Ob-R isoform sizes were reported in several tissues (Margetic *et al.* 2002). Interestingly, both Lep and Ob-R in pig sperm were found to be present exclusively at the acrosomial level. Since spermatozoa are highly polarized cells, they compartmentalize specific metabolic and signaling pathways to the regions where they are needed. On the basis of our data it is reasonably to hypothesize a role for Lep/Ob-R in the events that render the sperm able to fertilize the oocyte. As it concerns Ob-R, our data are similar to that of De Ambrogi (De Ambrogi *et al.* 2007), where the receptor was prevalently located on the acrosome of boar sperm, although they found weak immunofluorescence also in the subequatorial area and either on the whole tail or just on the midpiece. Whereas, in human sperm the receptor was visualized at the tail (Jope *et al.* 2003). These differences may be due to the sample management or they are specie-specific. Indeed, particularly intriguing is the

difference in the leptin expression site between human and pig, although the use of the same antibody. In fact, the hormone was specifically compartmentalized in the equatorial segment and the midpiece in human sperm (Aquila *et al*. 2005), whereas in pig on the acrosome. We can only hypothesize that leptin is differently involved in the regulation of sperm events in the two species.

In order to fertilize, the mammalian spermatozoa must undergo capacitation in the female tract. The mechanisms of capacitation remain poorly understood; although most studies have been conducted on mammals. During capacitation various biochemical and biophysical changes occur in the gamete and the molecular details of capacitation appear to vary somewhat among species. Up-stream of these events are subtle but all-determining changes to the sperm plasma membrane, among which may be mentioned the efflux of cholesterol (Visconti *et al.* 1999 a, b; Shadan *et al.* 2004). Cholesterol efflux from spermatozoa destabilizes lipid raft structures in the plasma membrane thereby initiating protein phosphorylation and acquisition of the capacitated state. Tyrosine phosphorylation of sperm proteins occurs during capacitation in mouse, human, bull, hamster (Visconti *et al.* 1995; Leclerc *et al.* 1996; Galatino-Homer *et al.* 1997) and also in the pig (Kalab *et al.* 1998; Flesch *et al.* 1999). It appears from our data that Lep through its receptor is able to affect both capacitation and acrosome reaction addressing their role in the pig sperm acquisition of fertilizing ability.

During the last few years, the study of the signaling events derived from Lep binding to its receptor has promoted a better understanding of the biochemical and molecular mechanisms of Lep function. The Ob-R is a member of the class I cytokine receptor superfamily that prevalently signals via association with non-receptor tyrosine kinases. Since Ob-R does not have intrinsic enzymatic activity, it activates associated JAK2, which auto-phosphorylates numerous tyrosine residues at the same time as it phosphorylates tyrosine residues on the functional Ob-R, upon ligand binding. The

phosphorylated intracellular domain then provides a binding site for STAT proteins. Although signals mediated directly by motifs on the Ob-R-associated JAK2 are poorly characterized, it is well known that Tyr_{985} recruits the binding of the protein tyrosine phosphatase SHP-2 and the inhibitory suppressor of cytokine signaling-3, whereas Tyr_{1138} specifically binds and activates the latent STAT3 (Munzberg & Myers 2005). The activation of STAT proteins in response to Lep was assayed in a variety of mouse tissues or cells known to express Ob-R. In our study, for the first time, we have demonstrated that STAT3 is expressed in the pig sperm, it is constitutively activated and its phosphorylation increased following Lep binding to its receptor. Here, we provide new information to indicate that Lep can stimulate the JAK-STAT pathway in pig sperm, resulting in a phosphorylation of STAT-3 depending on Lep concentration.

Since capacitation is a crucial step in the acquisition of sperm fertilizing ability, it is likely that it is controlled by redundant mechanisms and that cross talks between different pathways occur during this process (de Lamirande *et al*. 1997; Leclerc *et al.* 1998). The Ras-Raf-MAPK (mitogen-activated protein kinase) pathway is also a downstream target of Ob-Rs (Takahashi *et al.* 1997; Tanabe *et al.* 1997) and several lines of evidence indicate that components of the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPK), as well as the key players in this cascade (Shc, Grb2, Ras, Raf-1, mitogen-activated protein kinase [MEK], ERK1, and ERK2), are present in spermatozoa and are involved in capacitation (Naz *et al.* 1992; Luconi *et al*. 1998; de Lamirande & Gagnon 2002). Therefore, the activation of this pathway upon Lep was examined in pig sperm. Interestingly, we have shown that Lep positively modulates ERK1/2 through its receptor indicating that MAPK-dependent processes are involved in the hormone action.

Several pathways are also activated by JAKs including PI3K. Indeed, Lep signals via PI 3-kinase in insulinoma cells (Harvey *et al.* 2000), muscle cells (Berti *et al.* 1997) and

hepatocytes (Zhao *et al.* 2000). Sperm capacitation is regulated by multiple pathways and among these signalling cascades the PI3K and its role in modulating sperm postejaculatory changes remain elusive. In somatic cells PI3K is implicated in many biological processes, including cell survival and chemotaxis, membrane ruffling and DNA synthesis, receptor internalization, and vesicular trafficking (Wymann & Pirola 1998; Cantrell 2001). A role for PI3K has been also suggested in sperm functions such as survival and metabolism (Fisher *et al.* 1998; Luconi *et al*. 2001, Aquila *et al*. 2004, Aquila *et al*. 2005). In somatic cells, PI3K phosphorylates a large spectrum of protein substrates (Wymann & Pirola 1998). One of the main effectors of the PI3K, is the Akt (also named protein kinase B [PKB]), identified as a serine/threonine (Ser/Thr) protein kinase and it was also found in human ejaculated spermatozoa (Aquila *et al.* 2004). From our result it emerges that PI3K and Akt are implicated in mediating Lep signals in pig sperm, suggesting that this hormone may be involved in sperm survival. Concomitantly, Bcl-2 (Ito *et al.* 1997), a key protein in survival signaling, is enhanced upon Lep exposure and this effect was reduced by the anti-Ob-R Ab. Previous work have shown that Lep attenuates apoptosis of different cell types, such as osteoblasts, granulosa cells, and pancreatic islet cells (Almong *et al.* 2001; Okuya *et al.* 2001; Gordeladze *et al.* 2002). Moreover, very recently, Lep has been found to inhibit stress-induced apoptosis of T lymphocytes in vivo (Fujita *et al.* 2002). Data presented here clearly demonstrate that Lep, inducing the phosphorylation of classical key survival proteins, such as p42/44 MAPK, PI3K, Akt and Bcl-2, allows pig sperm survival process.

It is important to point out that in all our experiments we obtained different responses by using low or high Lep doses, in a pattern in which only 10nM are stimulatory. The outcome of signalling activation can depend on differences in ligand concentration as it was demonstrated in human sperm (Marshall 1995; Aquila *et al.* 2005). Besides,

recently it was hypothesized that the net effect of Lep upon male reproductive function may depend on the circulating level of the molecule. Thus, predominant stimulatory effects are observed at Lep levels above a minimal threshold, in contrast, direct inhibitory actions may take place in the presence of a significantly elevated Lep concentration (Tena-Sempere & Barreiro 2002; Caprio *et al.* 2001).

It has been demonstrated that pig seminal plasma contains a significant amount of Lep, which decreases a lot in the follicular fluid (Lackey *et al.* 2002). As sperm leave seminal plasma during their transit in the female reproductive tract, they are exposed to decreased leptin concentrations. It may be speculated that the high Lep observed in seminal plasma may contribute to maintain sperm in a quiescent metabolic condition. Instead, the low Lep concentrations of the pig female reproductive tract secretions could reduce its inhibitory effect on sperm, thus rendering them suitable for capacitation and fertilizing ability acquisition.

Lep regulates food intake and energy expenditure via central and peripheral mechanisms. Fatness in pigs is of prime economic importance due to market incentives for production of lean pork and elevated fatness increasing the feed costs. Lep was identified as a metabolic signal affecting central regulation of reproduction in the pig and our data have shown that this hormone affects pig sperm acquisition of fertilizing ability. Therefore, on the basis of our results the manipulation of Lep concentration, suggesting a strategy for altering body composition, may affect pig reproduction.

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REFERENCES

Ahima RS, Flier JS 2000 Leptin. *Annual Review of Physiology* **62** 413-437.

Almog B, Gold R, Tajima K, Dantes A, Salim K, Rubinstein M, Barkan D, Homburg R, Lessing JB, Nevo N, Gertler A, Amsterdam A. 2001 Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. *Molecular and Cellular Endocrinology* **183** 179-91

Amstalden M, Garcia MR, Williams SW, Stanko RL, Nizielski SE, Morrison CD, Keisler DH,Williams GL 2000 Leptin gene expression, circulating leptin, and luteinizing hormone pulsatility are acutely responsive to short-term fasting in prepubertal heifers: relationship to circulating insulin and insulin-like growth factor I. *Biology of Reproduction* **63** 127-133.

Aparicio IM, Bragado MJ, Gil MC, Garcia-Herreros M, Gonzalez-Fernandez L, Tapia JA, Garcia-Marin LJ 2006 Phosphatidylinositol 3-kinase pathway regulates sperm viability but not capacitation on boar spermatozoa. *Molecular Reproduction and Development* **74** 1035-1042.

Aquila S, Gentile M, Middea E, Catalano S & Andò S. 2004 Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **146** 552–557.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V & Andò S 2005 Leptin secretion by human spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **90** 4753–4761.

Aquila S, Sisci D, Gentile ME, Middea E, Siciliano L, Andò S 2002 Human ejaculated spermatozoa contain active P450 aromatase. *Journal of Clinical Endocrinology & Metabolism* **87** 3385–3390.

Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK, and Steiner RA 1996 Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137** 3144-3147.

Barb CR, Kraeling RR, Barrett JB, Rampacek GB 1999 Role of leptin in modulating neuroendocrine function: a metabolic link between the brain-pituitary and adipose tissue. *Reproduction of Domestic Animal* **34** 111-125.

Barb CR, Yan X, Azain MJ, Kraeling RR, Rampacek GB, Ramsay TG 1998 Recombinant porcine leptin reduces feed intake and stimulates growth hormone secretion in swine. *Domestic Animal Endocrinol*ogy **15** 77-86.

Bennett BD, Solar GP, Yuan JQ, Mathias J, Thomas GR, Matthews W 1996 A role for leptin and its cognate receptor in hematopoiesis. *Current Biology* **6** 1170–1180.

Berti L, Kellerer M, Capp E, Haring HU 1997 Leptin stimulates glucose transport and glycogen synthesis in C2C12 myotubes: evidence for a PI3kinase mediated effect .*Diabetologia* **40** 606-609.

Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, McCall AL, Flier JS 1998 Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology* **139** 3485–3491.

Booth PJ, Cosgrove JR, Foxcroft GR 1996 Endocrine and metabolic responses to re-alimentation in feed-restricted pre-pubertal gilts: associations among gonadotropins, metabolic hormones, glucose, and utero ovarian development. *Journal of Animal Science* **74** 840-848.

Booth PJ, Craigon J, Foxcroft GR 1994 Nutritional manipulation of growth and metabolic and reproductive status in pre-pubertal gilts. *Journal of Animal Science* **72** 2415-2424.

Bouloumie A, Drexler HC, Lafontan M, and Busse R 1998 Leptin, the product of Ob gene, promotes angiogenesis. *Circulation Research* **83**: 1059-1066.

Breidert M, Miehlke S, Glasow A, Orban Z, Stolte M, Ehninger G, Bayerdorffer E, Nettesheim O, Halm U, Haidan A, Bornstein SR 1999 Leptin and its receptor in normal human gastric mucosa and in *Helicobacter pylori*-associated gastritis. *Scandinavian Journal of Gastroenterology* **34** 954–961.

Campfield LA, Smith FJ, Burn P 1996 The OB protein (leptin) pathway: a link between adipose tissue mass and central neural networks. *Hormone of Metabolic Research* **28** 619-32.

Cantrell DA 2001 Phosphoinositide 3-kinase signalling pathways. *Journal of Cell Science* **114** 1439-1445.

Caprio M, Fabbrini E, Isidori AM, Aversa A, Fabbri A 2001 Leptin in reproduction. *Trends in Endocrinology & Metabolism* **12** 65–72

Caprio M, Fabbrini E, Ricci G, Basciani S, Gnessi L, Arizzi M, Carta AR, De Martino MU, Isidori AM, Frajese GV, Fabbri A 2003 Ontogenesis of leptin receptor in rat Leydig cells. *Biology of Reproduction* **68** 1199–1207.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D, Snodgrass HR 1996 Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nature Medicine* **2** 585–589.

Cosgrove JR, Tilton JE, Hunter MG, Foxcroft GR 1992 Gonadotropin-independent mechanisms participate in ovarian responses to re-alimentation in feed-restricted prepubertal gilts. *Biology of Reproduction* **47** 736-745.

Cunningham MJ, Clifton DK, Steiner RA 1999 Leptin's Actions on the Reproductive Axis: Perspectives and Mechanisms. *Biology of reproduction* **60** 216–222.

De Ambrogi M, Spinaci M, Galeati G, Tamanini C 2007 Leptin receptor in boar spermatozoa. *International Journal of Andrology* **9** [Epub ahead of print].

de Lamirande E, Gagnon C. 2002 The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. *Molecular of Human Reproduction* **8**124-35.

de Lamirande E, Leclerc P, Gagnon C. 1997 Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular of Human Reproduction* **3** 175-94.

Dieterich KD, Lehnert H 1998 Expression of leptin receptor mRNA and the long form splice variant in human anterior pituitary and pituitary adenoma. *Experimental and Clinical Endocrinology & Diabetes* **106** 522–525.

El-Hefnawy T, Ioffe S, Dym M 2000 Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology* **141** 2624–2630.

Fisher HM, Brewis IA, Barratt CL, Cooke ID, Moore HD 1998 Phosphoinositide 3-kinase is involved in the induction of the human sperm acrosome reaction downstream of tyrosine phosphorylation. *Molecular Human of Reproduction* **4** 849 -855.

Flesch FM, Colenbrander B, van Golde LM, Gadella BM 1999 Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochemical and Biophysical Research Communications* **262** 787–792.

Foster DL, Nagatani S 1999 Physiological perspectives on leptin as a regulator of reproduction: role in timing puberty. *Biology of Reproduction* **60** 205-215.

Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, Nakao K, Mimori T 2002 Leptin inhibits stress-induced apoptosis of T lymphocytes *Clinical and Experimental Immunology* **128** 21-26.

Galantino-Homer HL, Visconti PE, Kopf GS 1997 Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3959-monophosphate-dependent pathway. *Biology of Reproduction* **56** 707–719.

Glasow A, Haidan A, Hilbers U, Breidert M, Gillespie J, Scherbaum WA, Chrousos GP, Bornstein SR. 1998 Expression of Ob receptor in normal human adrenals: differential regulation of adrenocortical and adrenomedullary function by leptin. *Journal of Clinical Endocrinology and Metabolism* **83** 4459–4466.

Glogowski J, Demianowicz W, Piros B, Ciereszko A. 1998 Determination of acrosin activity of boar spermatozoa by the clinical method: optimization of the assay and changes during short-term storage of semen. *Theriogenology* **50:** 861-72.

Gordeladze JO, Drevon CA, Syversen U, Reseland JE 2002 Leptin stimulates human osteoblastic cell proliferation, *de novo* collagen synthesis, and mineralization: impact on differentiation markers, apoptosis, and osteoclastic signalling. *Journal of Cellular Biochemistry* **85** 825-836.

Gregoraszczuk EL, Ptak A, Wojtowicz AK, Gorska T, Nowak KW. 2004 Estrus cycle-dependent action of leptin on basal and GH or IGF-I stimulated steroid secretion by whole porcine follicles. *Endocrine Regulations* **38** 15-21.

Harvey J, McKay NG, Walker KS, Van der Kaay J, Downes CP, Ashford ML 2000 Essential role of phosphoinositide 3-kinase in leptin-induced K(ATP) channel activation in the rat CRI-G1 insulinoma cell line. *Journal of Biological Chemistry* **275** 4660-4669.

Ito T, Deng X, Carr BK, May WS 1997 Bcl-2 phosphorylation required for anti-apoptosis function. *Journal of Biological Chemistry* **272**:11671–11673.

Jope T, Lammert A, Kratzsch J, Paasch U, Glander HJ. 2003 Leptin and leptin receptor in human seminal plasma and in human spermatozoa. *International Journal of Andrology* **26** 335-41.

Kalab P, Peknicova J, Geussova G, Moos J 1998 Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Molecular Reproduction and Development* **51** 304–314.

Kamohara S, Burcelin R, Halaas JL, Friedman JM, and Charron MJ 1997 Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* **389** 374-377.

Kuster CE, Hess RA, Althouse GC 2004 Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplet on ejaculated porcine spermatozoa. *Journal of Andrology* **25** 340-347.

Lackey BR, Gray SL, Henricks DM. 2002 Measurement of leptin and insulin-like growth factor-I in seminal plasma from different species. *Physiological Research* **51** 309-11.

Leclerc P, de Lamirande E, Gagnon C. 1996 Cyclic adenosine 39,59 monophosphate dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biology of Reproduction* **55** 684–692.

Leclerc P, de Lamirande E, Gagnon C. 1998 Interaction between Ca2+, cyclic 3',5' adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. *Journal of Andrology* **19** 434-43.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM 1996 Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379** 632–635.

Lin J, Barb CR, Matteri RL, Kraeling RR, Chen X, Meinersman RJ, Rampacek GB 2000 Long for leptin receptor mRNA expression in the brain, pituitary and other tissues in the pig. *Domestic Animal Endocrinology* **19** 53-61.

Lollmann B, Gruninger S, Stricker-Krongrad A, Chiesi M 1997 Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochemical and Biophysical Research Communications* **238** 648–652.

Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, and Lechler RI 1998 Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* **394** 897-901.

Luconi M, Barni T, Vannelli GB, Krausz C, Marra F, Benedetti PA, Evangelista V, Francavilla S, Properzi G, Forti G, Baldi E. 1998 Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biology of Reproduction* **58** 1476-89.

Luconi M, Marra F, Gandini L, Filimberti E, Lenzi A, Forti G, Baldi E 2001 Phosphatidylinositol 3-kinase inhibition enhances human sperm motility. *Human of Reproduction* **16** 1931 -1937.

Margetic S, Gazzola C, Pegg GG, Hill RA. 2002 Leptin: a review of its peripheral actions and interactions. *International Journal of Obesity and Related Metabolic Disorders* **26** 1407-33.

Marshall CJ 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80** 179–185.

Muller G, Ertl J, Gerl M, and Preibisch G. 1997 Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *Journal of Biological Chemistry* **272** 10585-10593.

Munzberg H, Myers MG . 2005 Molecular and anatomical determinants of central leptin resistance. *Nature Neuroscience* **8** 566-570.

Murakami T, Yamashita T, Iida M, Kuwajima M, Shima K 1997 A short form of leptin receptor performs signal transduction. *Biochemical and Biophysical Research Communications* **231** 26–29.

Naz RK, Ahmad K, Kaplan P. 1992 Expression and function of ras proto-oncogene proteins in human sperm cells. *Journal of Cell Science* **102** (Pt 3) 487-494.

Okuya S, Tanabe K, Tanizawa Y, Oka Y. 2001 Leptin increases the viability of isolated rat pancreatic islets by suppressing apoptosis. *Endocrinology* **142** 4827-4830.

Quesnel H, Prunier A. 1995 Endocrine bases of lactational anoestrus in the sow. *Reproduction Nutrition Development* **35** 395-414.

Rossetti L, Massillon D, Barzilai N, Vuguin P, Chen W, Hawkins M, Wu J, and Wang J 1997 Short term effects of leptin on hepatic gluconeogenesis and in vivo insulin action. *Journal of Biological Chemistry* **272** 27758-27763.

Shadan S, James PS, Howes EA, Jones R. 2004 Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biology of Reproduction* **71** 253-265.

Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, and Flores-Riveros JR 1998 Biological action of leptin as an angiogenic factor. *Science* **281** 1683-1686.

Soyupek S, Armagan A, Serel TA, Hoscan MB, Perk H, Karaoz E, Candir O. 2005 Leptin expression in the testicular tissue of fertile and infertile men. *Archives of Andrology* **51** 239-246.

Takahashi Y, Okimura Y, Mizuno I, Iida K, Takahashi T, Kaji H, Abe H & Chihara K 1997 Leptin induces mitogen-activated protein kinase dependent proliferation of C1H10T1/2 cells. *Journal of Biological Chemistry* **272** 12897-12900

Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Mori K, Tamura N, Hosoda K, Nakao K 1996 Molecular cloning of rat leptin receptor isoform complementary DNAs–identification of a missense mutation in Zucker fatty (*fa/fa*) rats. *Biochemical and Biophysical Research Communications* **225** 75–83.

Tanabe K, Okuya S, Tanizawa Y, Matsutani A, Oka Y. 1997 Leptin induces proliferation of pancreatic beta cell line MIN6 through activation of mitogen-activated protein kinase. *Biochemical and Biophysical Research Communications* **241** 765-768.

Tardif S, Cubè C, Bailey JL 2003 Porcine Sperm Capacitation and Tyrosine Kinase Activity Are Dependent on Bicarbonate and Calcium but Protein Tyrosine

Phosphorylation Is Only Associated with Calcium . *Biology of Reproduction* **68**: 207-213.

Tartaglia LA 1997 The leptin receptor. *Journal of Biological Chemistry* **272** 6093–6096.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI. 1995 Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83 1263–1271.

Tena-Sempere M, Barreiro ML 2002 Leptin in male reproduction: the testis paradigm. *Molecular and Cellular Endocrinology* **188** 9–13.

Tena-Sempere M, Manna PR, Zhang FP, Gonzalez LC, Dieguez C, Huhtaniemi I, Aguilar E 2001 Molecular mechanism of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *Journal of Endocrinology* **170** 413–423.

Thomas T 2004 The complex effects of leptin on bone metabolism through multiple pathways. *Current Opinion in Pharmacology* **4** 295–300.

Tsiaoussis J, Newsome PN, Nelson LJ, Hayes PC, and Plevris JN 2001 Which hepatocyte will it be? Hepatocyte choice for bioartificial liver support systems. *Liver Transplantation* **7** 2-10.

Vilei MT, Granato A, Ferraresso C, Neri D, Carraro P, Gerunda G, and Muraca M 2001 Comparison of pig, human and rat hepatocytes as a source of liver specific metabolic

functions in culture systems implications for use in bioartificial liver devices. *The International Journal of Artificial Organs* **24** 392-396.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS 1995 Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121** 1129–1137.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS 1999 Cholesterol efflux-mediated signal transduction in mammalian sperm. b-cyclodextrins initiate trans-membrane signalling leading to an increase in protein tyrosine phosphorylation and capacitation. *Journal of Biological Chemistry* **274** 3235–3242.(a)

Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, Kopf GS 1999 Colesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation *Developmental Biology* **214** 429–443. (b)

Wymann MP, Pirola L. 1998 Structure and function of phosphoinositide 3-kinases. *Biochimica and Biophysica Acta* **1436** 127-150.

Yamashita T, Murakami T, Otani S, Kuwajima M, Shima K 1998 Leptin receptor signal transduction: OBRa and OBRb of fa type. *Biochemical and Biophysical Research Communications* **246** 752–759.

Yaspelkis BB III, Ansari L, Ramey EL, Holland, GJ, and Loy SF. 1999 Chronic leptin administration increases insulin-stimulated skeletal muscle glucose uptake and transport. *Metabolism* **48** 671-676.

Zak LJ, Cosgrove JR, Aherne FX, Foxcroft GR. 1997 Pattern of feed intake and associated metabolic and endocrine changes differentially affect post weaning fertility in primiparous lactating sows. *Journal of Animal Science* **75** 208-211.

Zhao AZ, Bornfeldt KE, and Beavo JA. 1998 Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. *Journal of Clinical Investigation* **102** 869-873.

Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG, Beavo JA, Bornfeldt KE. 2000 Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. *Journal of Biological Chemistry* **275** 11348-11354.

Zurek E, Foxcroft GR, Kennelly JJ. 1995 Metabolic status and interval to first ovulation in postpartum dairy cows. *Journal of Dairy Science* **78** 1909-1920.

FIGURE LEGENDS

Figure 1 : Western blot analysis of leptin and leptin receptor from pig sperm extracts **A:** Immunoblot of leptin. *lane 1*: adipocyte cells used as positive controls(+), *lane 2*: negative control (-) *lanes* P1, P2: representative pig sperm samples **B:** Immunoblot of Ob-R from two representative pig sperm samples (P1, P2). **b1:** membrane was incubated with primary anti Ob-R Ab, **b2:** membrane was tested using the normal rabbit serum at the same dilution of the anti Ob-R Ab.

Figure 2: Representative immunofluorescence labelling of leptin and leptin receptor (Ob-R) in pig spermatozoa: **A** A red intense fluorescence localized leptin in the sperm acrosomal region. **B** A green brilliant light showed Ob-R in the apical acrosome. *Inserts*: immunonegative controls. *Scale bars*: 5µm

Figure 3: Leptin affects cholesterol efflux and protein tyrosine phosphorylation of pig sperm. Spermatozoa were incubated in the absence (NC) or in the presence of leptin (10 and 60 nM) as well as in presence of anti Ob-R antibody + 10nM leptin. **A** Cholesterol in culture medium from pig sperm. Results are presented as mean ± SEM and are given per 10 x 10^6 spermatozoa. *, $P< 0.05$ *vs.*control **B** Western blot analysis of protein tyrosine phosphorylation from sperm lysates.

Figure 4: Leptin affects acrosin activity of pig sperm. Spermatozoa were incubated in the absence (NC) or in the presence of leptin (10 and 60 nM) as well as in presence of anti Ob-R antibody + 10nM leptin. Values are mean ± SEM *, $P< 0.05$ *vs.*control.

Figure 5: Western blot analysis of STAT 3 from one representative pig sperm lysate. **A**: Immunoblot of STAT3 in the pig sample (P). **B** p-STAT 3 band in sperm incubated in the absence (NC) or in the presence of leptin (10 and 60 nM) as well as in presence of anti Ob-R antibody + 10nM leptin (Ab Ob-R+lep). MCF7 cell lines were used as positive controls (+). Negative control (-) was performed as previously described. β-actin served as loading control. **C** Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± SEM *, $P< 0.05$ *vs.*control

Figure 6: Western blot analysis of ERK1/2 from one representative pig sperm lysate. **A** Immunoblot of ERK1/2 in the pig sample (P). **B** p-ERK1/2 band in spermatozoa incubated in the absence (NC) or in the presence of leptin (10 and 60 nM) as well as in presence of anti Ob-R antibody + 10nM leptin (Ab Ob-R+lep). MCF7 cell lines were used as positive controls (+).Negative control(-) was performed as previously described. β-actin served as loading control. **C** Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± SEM *, $P< 0.05$ *vs.*control

Figure 7: Western blot analysis of Bcl-2 and Akt from one representative pig sperm lysate. **A, D** Immunoblots of Bcl-2 and Akt, in the pig sample *(P)*. **B** p-Bcl-2 band when sperm cells were incubated in the absence *(NC)* or in the presence of leptin (*10 and 60 nM*) as well as in presence of anti Ob-R antibody + 10nM leptin (*Ab Ob-R+lep).* **E** p-AKTS (Akt ser) and p-AKTT (Akt thr) bands in sperm incubated in the absence *(NC)* or in the presence of leptin (*10 and 60 nM*) as well as in presence of anti Ob-R antibody + 10nM leptin (*Ab Ob-R+lep*) or LY 294002 *(LY*). **C, F**: Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± SEM *, $P< 0.05$ *vs.*control . *(+)* positive control. *(-)* negative control. β-actin served as loading control.

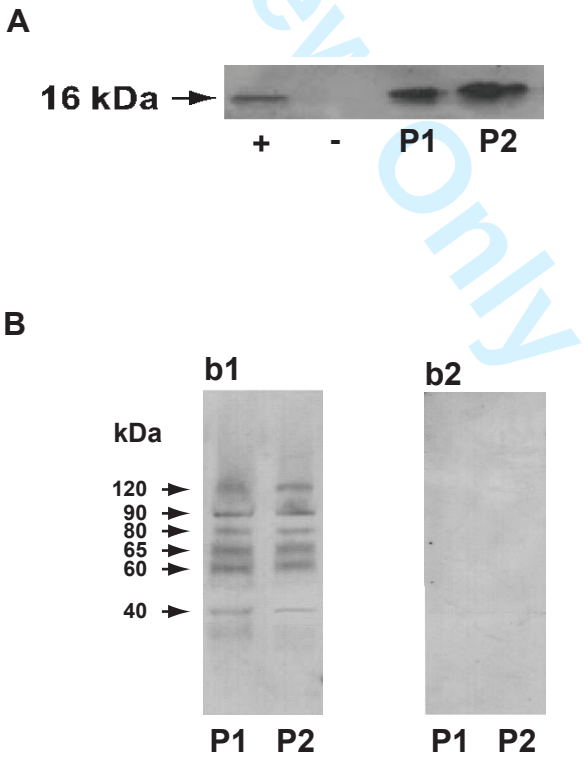


Fig. 1

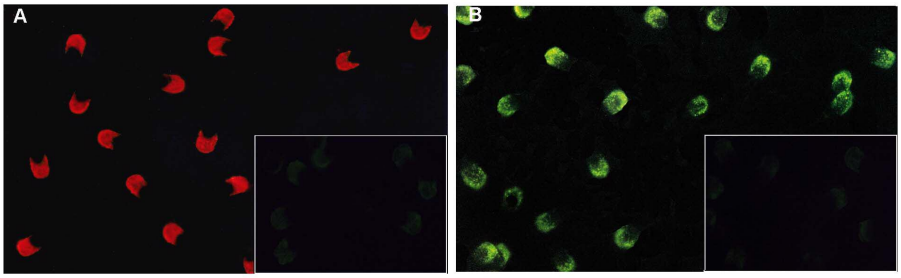
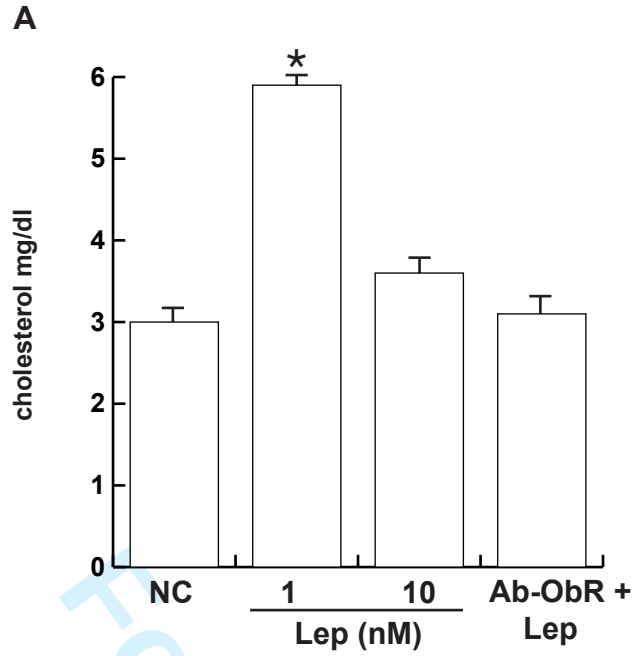


Figure 2: Representative immunofluorescence labelling of leptin and leptin receptor (Ob-R) in pig spermatozoa: A A red intense fluorescence localized leptin in the sperm acrosomal region. B A green brilliant light showed Ob-R in the apical acrosome. Inserts: immunonegative controls. Scale bars: 5 μm
180x180mm (300 x 300 DPI)



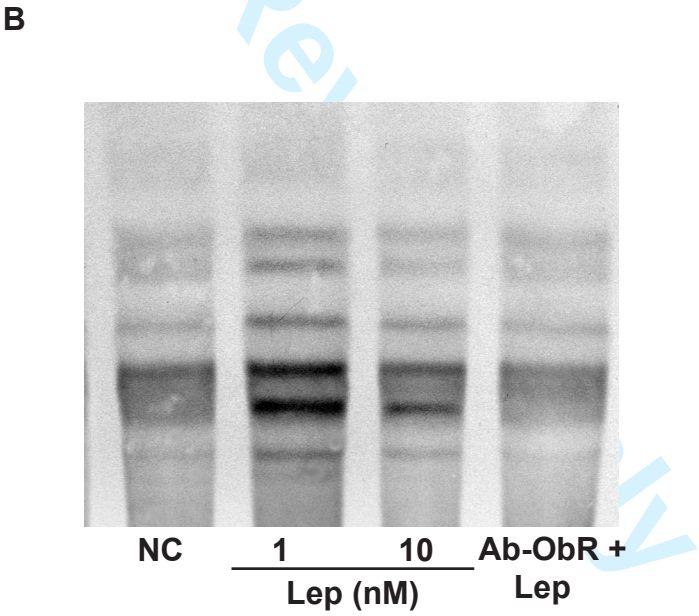


Fig. 3

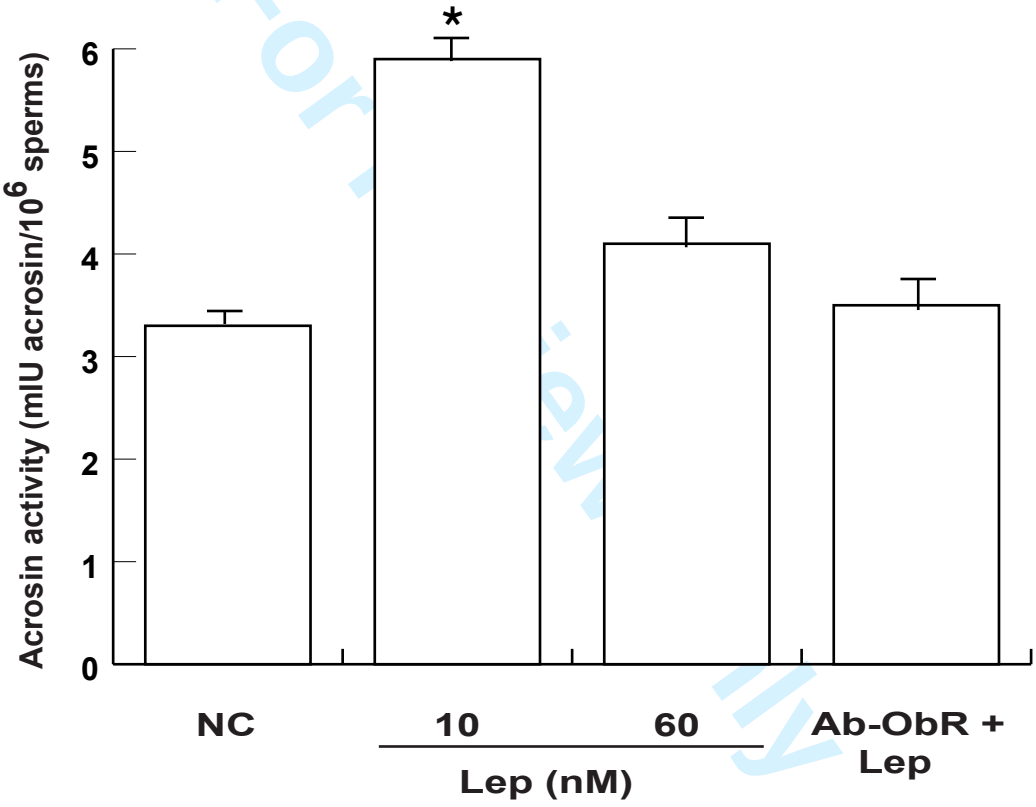
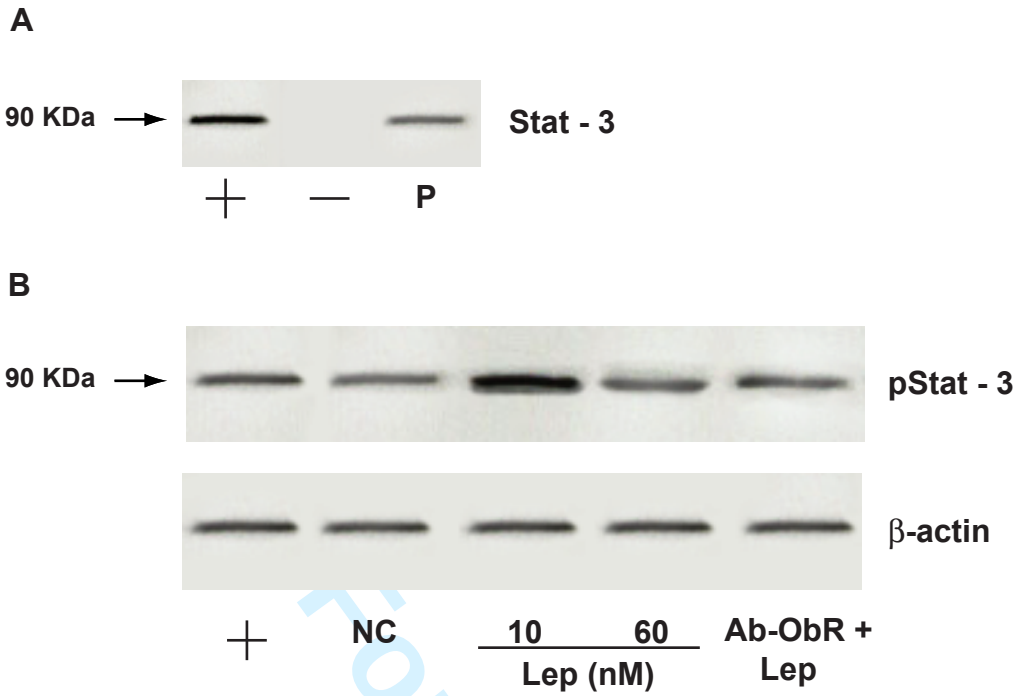

Acrosin activity (mIU acrosin/10^6 sperms)
0
1
2
3
4
5
6
*
NC
10
60
Ab-ObR + Lep
Lep (nM)

Fig. 4



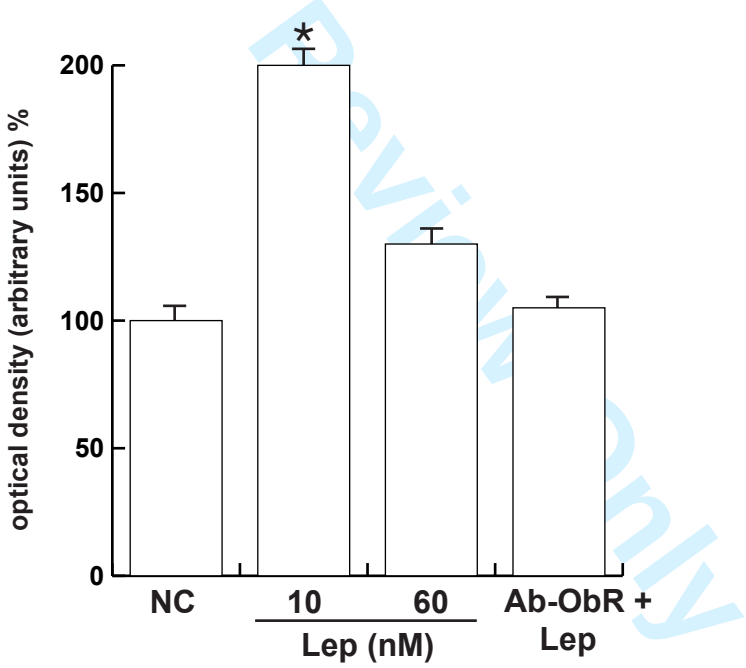


Fig. 5

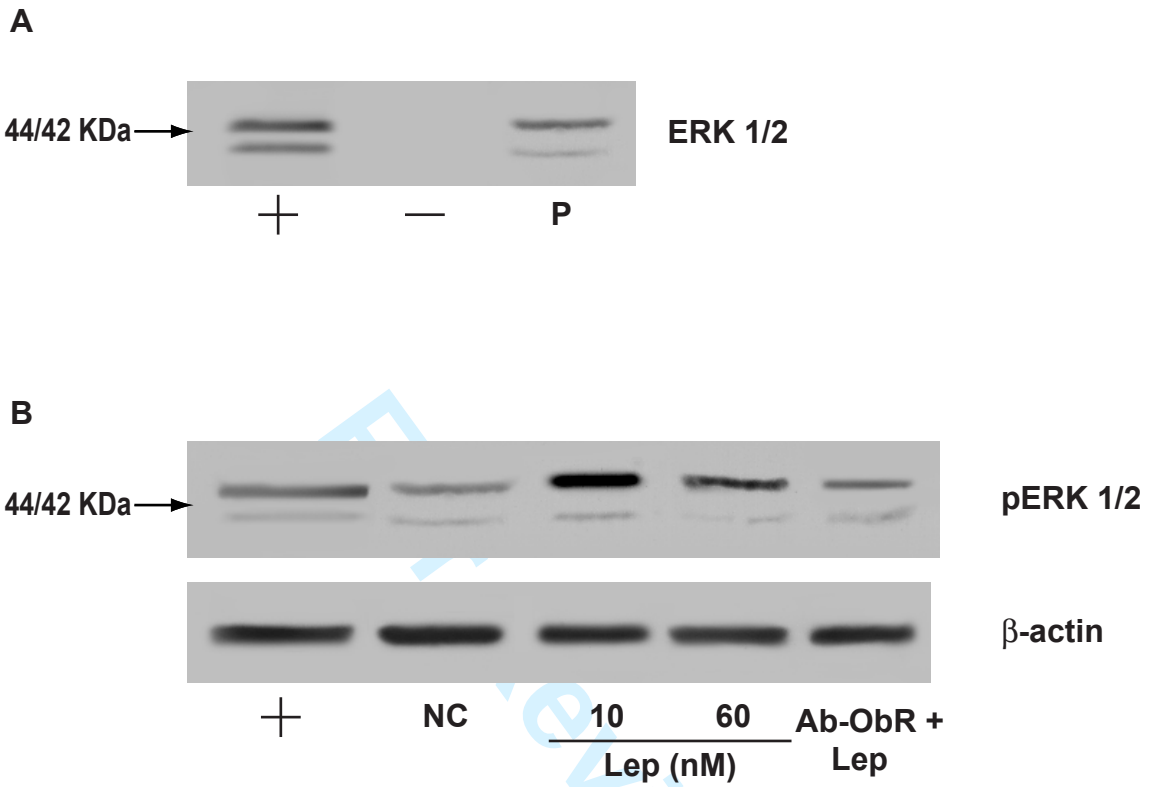


Fig. 6

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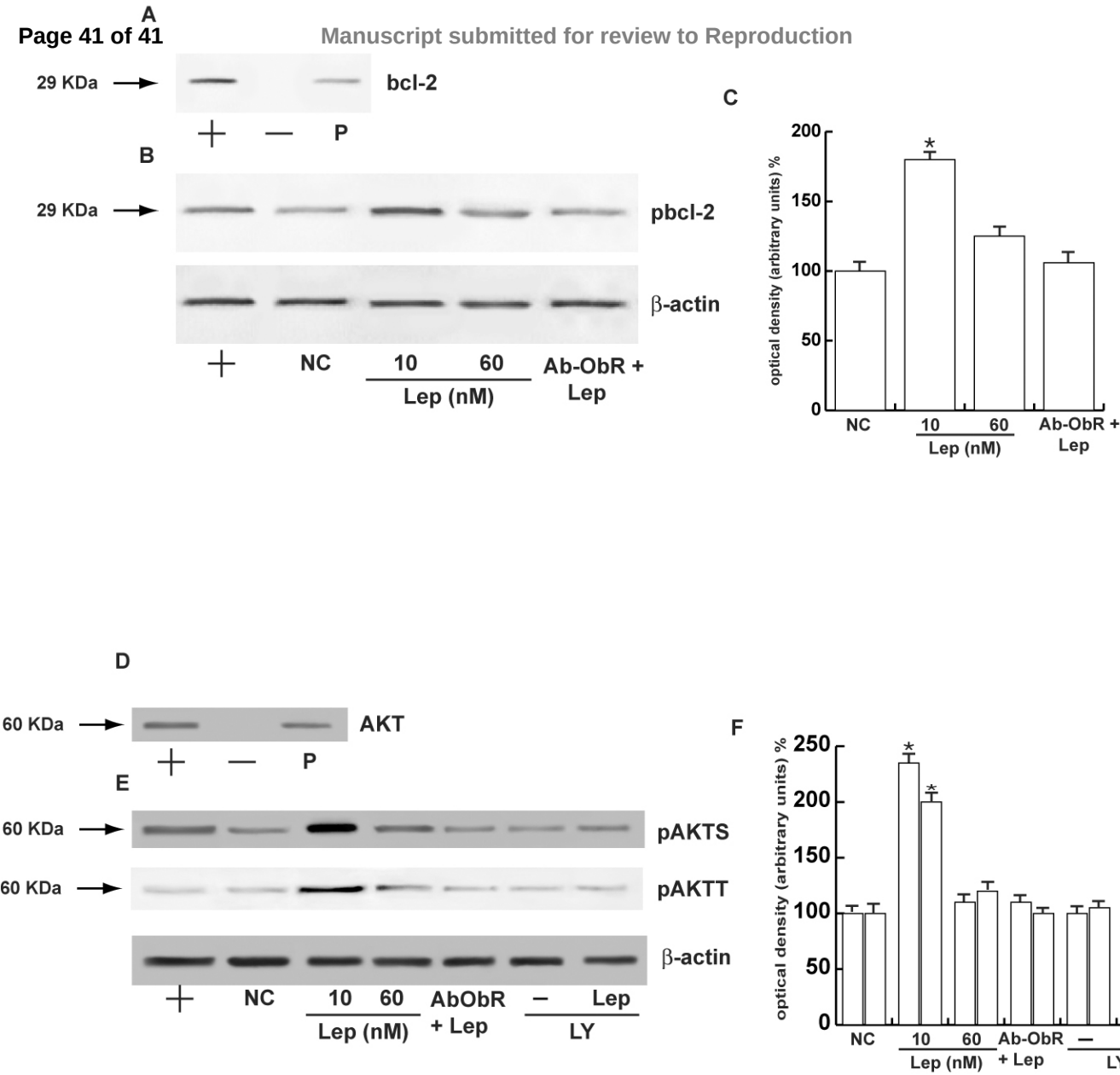

Fig. 7

ATTI DI CONVEGNO

1. Francesca De Amicis, Silvia Zupo, Rocco Malivindi and Sebastiano Andò. “Evidences that progesterone receptor b decreases estrogen receptor gene expression through its interaction to a half-PRE site at estrogen receptor gene promoter.” Atti del convegno “ 30th San Antonio Breast Cancer Symposium”, San Antonio 13-16 December 2007.

2. De Amicis F. , Zupo S. , Malivindi R. , Ando' S. , "Il Recettore Progestinico B decrementa l’espressione del Recettore Estrogenico interagendo con un emisito-PRE presente sul promotore del Recettore Estrogenico ". Atti del convegno "X Congresso Nazionale AIBG", Torino (Italy), 2007.

3. Lanzino M. , De Amicis F. , Marsico S. , Zupo S. , Ando' S. , " ARA70 interagisce con il recettore estrogenico e modula il cross-talk funzionale tra recettore estrogenico e recettore androgenico in cellule MCF-7". Contributo a VIII Congresso AIBG, Sirolo (AN), 15-17 settembre 2005.

4. Lanzino M. , De Amicis F. , Zupo S. , Tirotta E. , Ando' S. , " Endogenously expressed coactivator ARA70 interacts with ERalpha and modulates the functional interaction between ERalpha and AR in MCF-7 breast cancer cells". Atti del convegno "12th ICE", Lisbon, august 31-september, 2004.