Estrogen receptor beta displays cell cycle-dependent expression and regulates the G1 phase through a non-genomic mechanism in prostate carcinoma cells

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Abstract. Background: It is well known that estrogens regulate cell cycle progression, but the specific contributions and mechanisms of action of the estrogen receptor beta (ERβ) remain elusive.

Methods: We have analyzed the levels of ERβ1 and ERβ2 throughout the cell cycle, as well as the mechanisms of action and the consequences of the over-expression of ERβ1 in the human prostate cancer LNCaP cell line.

Results: Both ERβ1 mRNA and protein expression increased from the G1 to the S phase and decreased before entering the G2/M phase, whereas ERβ2 levels decreased during the S phase and increased in the G2/M phase. ERβ1 protein was detected in both the nuclear and non-nuclear fractions, and ERβ2 was found exclusively in the nucleus. Regarding the mechanisms of action, endogenous ERβ was able to activate transcription via ERE during the S phase in a ligand-dependent manner, whereas no changes in AP1 and NFκB transactivation were observed after exposure to estradiol or the specific inhibitor ICI 182,780.

Over-expression of either wild type ERβ1 or ERβ1 mutated in the DNA-binding domain caused an arrest in early G1. This arrest was accompanied by the interaction of over-expressed ERβ1 with c-Jun N-terminal protein kinase 1 (JNK1) and a decrease in c-Jun phosphorylation and cyclin D1 expression. The administration of ICI impeded the JNK1–ERβ1 interaction, increased c-Jun phosphorylation and cyclin D1 expression and allowed the cells to progress to late G1, where they became arrested.

Conclusions: Our results demonstrate that, in LNCaP prostate cancer cells, both ERβ isoforms are differentially expressed during the cell cycle and that ERβ regulates the G1 phase by a non-genomic mechanism.

Keywords: ERβ1, ERβ2, LNCaP, cell cycle, ERE, AP1, ICI 182,780

1. Introduction

The human prostate is an endocrine organ that depends on androgens to maintain its size and secretory function [50]. In the prostate, testosterone and 5α-dihydrotestosterone are metabolized to 17β-estradiol by P450-aromatase [16]. Although both ERα and ERβ have been identified in the prostate, only the latter is expressed in the epithelial cells [18,42], where it has been suggested to bind natural estrogenic ligands. Several isoforms of ERβ have been identified, resulting from exon skipping or the usage of alternative last exons encoding alternative carboxy-terminal peptides.
factors, such as AP1, NFκB, consisting in their interaction with other transcription get genes, (2) ERE-independent genomic mechanisms, the regulation of the transcriptional activity of tar-
cistical mechanism of action that require their binding oppositely associated with estrogen receptors, (1) the clas-
achieve these actions, any of the mechanisms previ-

opposite effects on cell proliferation [21,31,53]. To

streptomycin, as recommended. All of the compounds

containing 10% FCS, glutamine, and penicillin/
streptomycin, in the human prostate [20]. ERβ1 and ERβ2 proteins differ in the F domain, which includes helix 12 and is involved in binding transcriptional co-
regulators. ERβ2 was initially described as a protein unable to bind steroids; its function was thought to rely upon its ability to heterodimerize with the other estrogen receptors, preferentially ERα, causing their inactiv-
tion [40]. In the prostate, it has been shown that ERβ1 is clearly expressed in the nucleus of the epithelial cells in low-grade carcinomas while its expression is reduced in high-grade carcinomas [18,27]. A decreased expression of ERβ mRNA and protein has also been detected in breast, ovary and colon carcinomas [2,19,55], suggesting that ERβ might play a protective role in cancer development or progression. In con-
trast, it has been reported an increasing expression of ERβ2 with the transition from low-grade to high-grade prostate and breast carcinomas [17,20].

It is widely accepted that estrogens regulate cell cycle progression [14,49], but the specific contribution and mechanisms of action of the different estrogen receptors are still puzzling. With regard to their roles, there is evidence supporting that ERα and ERβ have opposite effects on cell proliferation [21,31,53]. To achieve these actions, any of the mechanisms previously associated with estrogen receptors, (1) the classical mechanism of action that require their binding to specific estrogen response elements (EREs) and the regulation of the transcriptional activity of target genes, (2) ERE-independent genomic mechanisms, consisting in their interaction with other transcription factors, such as AP1, NFrB, CRE and SP1, which in turn, bind to their respective DNA elements or (3) non-
genomic mechanisms, could be involved [6].

In the present study we tested the possibility that ERβ1 and ERβ2 were differentially expressed during the cell cycle and analyzed the mechanisms of action of ERβ1 in cell cycle regulation.

2. Material and methods

2.1. Cell cultures

The human prostate cancer cell lines, LNCaP and PC3, obtained from the American Type Culture Collection (Rockville, MD), were grown in RPMI 1640 containing 10% FCS, glutamine, and penicillin/ streptomycin, as recommended. All of the compounds were purchased from PAA Laboratories (Pasching, Austria). LNCaP cells were used between passages 10 and 16 and PC3 between passages 15 and 18. Cells were maintained in a humidified incubator with 5% CO2 at 37°C.

2.2. mRNA isolation and expression analysis by RT-PCR

Total RNA was extracted from the cell lines using the RNasea Kit (QIAGEN, Hilden, Germany), according to the supplier’s instructions. Four µg of RNA from each sample were reverse transcribed using Supercript II H- (Invitrogen, Carlsbad, CA), and 1 µl of the resulting cDNA was amplified in a 25 µl reaction in the presence of 1 U Taq polymerase (Eco-
gen, Barcelona, Spain), 0.05 mM MgCl2, 0.2 mM of dNTP mix and 0.1 µM of the specific primers for ERβ1, ERβ2 and S18 mRNA, as a control gene, in separate microtubes. For ERβ1, the upper and lower primers recognized, respectively, exon 7 (5′-AACGCCGTTACGCGTGGGG-3′) and the specific ERβ1 exon 8 (5′-ACGTGGGCAATCTATACCT-CC-3′). To amplify ERβ2, the same upper primer and a lower primer specific for ERβ2 exon 8 (5′-GGCAAGCCTGACCACACAATCC-3′) were used. For S18, specific primers were designed to amplify a 174 bp fragment (5′-GATGGGCGCGCGAAAT-3′, 5′-CTTGATCTGCGCGTAGTTGC-3′). Amplification was carried out in non-saturating conditions consisting of 39 cycles for ERβ1 and ERβ2 and 29 cycles for S18. Denaturation was performed at 94°C for 20 s, annealing for ERβ1/S18 at 59°C and for ERβ2/S18 at 58.7°C for 30 s, and extension at 72°C for 45 s. PCR products were separated on a 1.5% agarose gel and were quantified using an Image Analysis System (Bio-Rad Laboratories Inc., Her-
cules, CA). The amplified products were purified using a Qiaquick Gel Purification Kit (QIAGEN), cloned using the TOPO TA Cloning system (Invitrogen), and sequenced using an ABI Prism 310 genetic analyzer (PerkinElmer Corp., Wellesley, MA).

2.3. Protein extraction and western blot analysis

Trypsinized cells were washed twice in ice cold PBS, and the resulting pellets were resuspended in ice cold RIPA buffer (150 mM Tris HCl, 50 mM NaCl, 1% SDS, 1% NP-40 and 0.5% sodium de-
oxoycolate), supplemented with the protease inhibitor cocktail P2714 (Sigma, St. Louis, MI). Pellets were
dispersed by passing them through a 1-ml syringe, and they were centrifuged at 19,000 g at 4°C for 5 min. The protein concentration was assessed using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories Inc.). Equal amounts of proteins from each cell lysate were subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to Polyvinylidene Difluoride (PVDF) or nitrocellulose membranes. Individual membranes were blotted with rabbit polyclonal antibodies against the C-terminus of ERβ1 (PA1-313, at 1 µg/ml, Affinity Bioreagents, Golden, CO), the C-terminus of ERβ2 (diluted 1:200, see below for antibody description), cyclin D1 (1:1000, Upstate Biotechnology, Lake Placid, NY), cyclin A (1:1000, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), c-Jun (1:1000, Abcam, Cambridge, UK), phospho-c-Jun (Ser73) (1:1000, Abcam), and SAPK/JNK (1:200, Cell Signaling Technology, Inc., Beverly, MA), and with mouse monoclonal antibodies against cyclin B (1:1000, Santa Cruz Biotechnology, Inc.), retinoblastoma protein (1:400, Cell Signaling Technology, Inc.) and α-actin (1:2000, Santa Cruz Biotechnology, Inc.). Immunoreactive bands were visualized using anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Dako Cytomation, Glostrup, Denmark) and West Dura reagent (Pierce, Etten-Leur, The Netherlands). Specific bands were quantified using the Image Analysis System.

2.4. Antibody generation against ERβ2

A polyclonal antibody was generated by the Biotechnology and Biomedicine Institute (IBB) of the Autonomous University of Barcelona (Bellaterra, Spain) against a peptide (MKMETLLEATMEQ), corresponding to the 14 last residues of the human ERβ2 protein. The same peptide has previously been used by other authors [40]. A cysteine was added to the N-terminus, and the peptide, conjugated to keyhole limpet hemocyanin (KLH), was used to immunize two rabbits at 4 immunizations per rabbit. A recombinant GST-ERβ2 protein was synthesized to confirm its specificity. The ERβ2 cDNA was cloned into the pGEX-2T vector and sequenced to assure the absence of mutations. The GST-ERβ2 vector was used to transform E. coli BL21 cells, and protein expression was induced for 4 h at 37°C with 0.4 mM of IPTG (Sigma). The recombinant protein was purified with Glutathione-Sepharose beads (Sigma) and tested by the Enzyme-Linked Immunosorbent Assay (ELISA). Serum obtained prior to immunization was used as a negative control.

2.5. Plasmid construction and transfection

Four ERβ expression vectors were constructed: CMV-EGFP-ERβ1 530, which included the sequence encoding the long form of ERβ1 cloned into pEGFP-C3 (Clontech, BD Biosciences, Erembodegem, Belgium), CMV-FLAG-ERβ1 530, which included the same ERβ1 sequence cloned into pFLAG-CMV™-6a (Sigma), EGFP-ERβ1 EG167/168AA, a vector containing two mutations in the DBD that has been described previously [4] and CMV-HA-ERβ2 495, which contained the whole ERβ2 sequence cloned into pCMV-HA (Clontech). The two former ERβ1 and the ERβ2 cDNA fragments were generated by PCR from a human testis cDNA library (Marathon-Ready™, Clontech) and were inserted in frame downstream of the epitope tag. The EGFP-ERβ1 EG167/168AA cDNA was constructed by site-direct mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA). The correct DNA sequences were confirmed by sequencing. LNCaP cells were seeded into 100 mm plates to 70% confluence and were transfected with 4 µg of each construction vector using Fugene-6 (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer protocol. After overnight culture, fresh complete RPMI medium was added to each plate, and 24 hours later, cells were trypsinized, and the total protein was extracted to analyze the expression of ERβ1 and ERβ2 by western blot.

2.6. Flow cytometry acquisition and cell sorting

Trypsinized cells were washed twice in 10 mM of EDTA-PBS and 1% PBS-BSA, fixed by the drop-wise addition of 1 ml ice-cold 70% ethanol, washed again in 1% PBS-BSA, treated with 50 mg/ml ribonuclease A (Fermentas, St. Leon-Rot, Germany) at 37°C for 1 h, and stained with 5 µg/ml propidium iodide (PI). For cell cycle analysis, fluorescence was recorded using an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL), equipped with an argon ion laser tuned at 488 nm. Red fluorescence (620 nm) for PI and light scatter were measured simultaneously and plotted against each other. DNA analysis on single fluorescent histograms was carried out using Multicycle software (Phoenix Flow Systems, San Diego, CA). A minimum of 12,000 gated events per sample were analyzed. Cell sorting was performed using a FACSARia (BD Biosciences, San Jose, CA). In both cytometers, cell doublets and aggregates were discriminated. Cells were
analyzed based on their DNA content (PI fluorescence) and were sorted into three separate populations (G0/G1, S and G2/M). Sorted cells were centrifuged and washed twice with ice cold PBS. Protein extracts were blotted against cyclin D1, cyclin A, cyclin B, ER/β1, ER/β2 and α-actin. Each experiment was performed in duplicate.

2.7. Cell cycle synchronization

LNCaP cells were seeded and cultured to sub-confluence in 100 mm culture plates for the cell cycle synchronization experiments. The cells were synchronized at the G0/G1 phase by the isoleucine-deprivation method, as previously described [12].

2.8. Subcellular fractionation

A total of 10^6 cells were lysed by Dounce homogenization in 0.5 ml of cold hypotonic buffer (25 mM MES (morpholine ethane sulfonic acid), pH 6.5), containing protease inhibitors. The lysates were centrifuged at 3000g, at 4°C, yielding nuclear pellets and non-nuclear fractions. Pelleted nuclei were washed twice in hypotonic buffer to eliminate cytosolic contamination. Each fraction was adjusted to 1 × RIPA buffer. Quantification and protein extraction were performed as described above. Protein extracts were blotted against ER/β1, ER/β2, actin and the retinoblastoma protein.

2.9. Transient transfections and luciferase activity

LNCaP cells were seeded in RPMI medium supplemented with 10% fetal calf serum and cultured for 24 h prior transfection. Cells were then transiently transfected with 500 ng of each of the following reporter plasmids: ERE-LUC, NFκB-LUC and AP-1-LUC, kindly provided by Dr. M. Beato (Barcelona, Spain), Dr. M. Kracht (Hannover, Germany) and Dr. Anna Bigas (Barcelona, Spain), respectively, together with a control vector for β-galactosidase expression using FuGENE™ 6.0 (Roche Molecular Biochemicals). After transfection, cells were treated either with 10 nM E2 alone or 10 nM E2 plus 1 µM ICI 182,780 for 36 h, or they were left untreated in the same medium for two hours. The cells were stained with Hoechst 33342 dye (5 µg/ml medium) for 1 h in culture conditions, washed with PBS, trypsinized, eluted in culture medium as described previously [38], and prepared for cell sorting. Cells were analyzed based on their DNA content and sorted using the BD FACSAria flow cytometer into three separate populations (G0/G1, S and G2/M). Cells were then centrifuged, and both luciferase and β-galactosidase activities were analyzed.

2.10. Cell sorting of ERE-LUC transiently transfected cells

LNCaP cells were seeded in RPMI medium supplemented with 10% fetal calf serum and transiently transfected with the ERE-LUC plasmid overnight. Cells were then washed, cultured in 10% dextran-coated charcoal RPMI medium and treated either with 10 nM E2 alone or 10 nM E2 plus 1 µM ICI 182,780 for 6 h, or they were left untreated in the same medium for two hours. The cells were then centrifuged, and both luciferase and β-galactosidase activities were analyzed.

2.11. Detection of BrdU incorporation in ER/β1 transiently transfected LNCaP cells by confocal microscopy

LNCaP cells were seeded on microscope cover glasses (Marienfeld, Germany), transiently transfected with the EGFP-ER/β1 vector, as previously described, and treated with 10 µM BrdU for 2 h. Cells were then washed with PBS, fixed with PFA (4%, wt/vol in PBS) for 15 min, permeabilized with Triton X-100 (0.2%, vol/vol in PBS) for 15 min and treated with 5 mg/ml of DNase I (QIAGEN) diluted in distilled water for 1 h at 37°C. Cells were washed again with PBS, incubated with 1% BSA in PBS (wt/vol) for 1 h, and with a mouse anti-BrdU antibody (Sigma), diluted 1:100 in PBS containing 0.01% BSA, for 1 h at room temperature. After extensive washes in PBS, cover glasses were incubated for an additional hour with an anti-mouse antibody conjugated to Alexa Fluor 568 (Molecular Probes/Invitrogen) at 1:200 in PBS containing 0.01% BSA, washed with PBS and mounted in 50% glycerol/PBS solution. EGFP and Alexa Fluor 568 fluorescences were collected on a LEICA TCS-NT confocal laser-scanning microscope.
2.12. Detection of BrdU incorporation in diarylpropionitrile (DPN)-treated and in ERβ1 transiently transfected LNCaP cells by flow cytometry

LNCaP cells were seeded on 100 mm plates, transfected with the EGFP-ERβ1 vector and treated with BrdU for 2 h, as previously described [28]. 2 × 10^6 cells were trypsinized, fixed with ice-cold ethanol (70% vol/vol in PBS) at 4°C for 1 h and washed with PBS containing 0.01% BSA. DNA was denatured with 1 N HCl at room temperature for 20 min, washed in PBS containing 0.01% BSA and neutralized with 0.1 M sodium borate at room temperature for 2 min. Cells were then washed again in PBS containing 0.01% BSA and incubated with an anti-BrdU antibody conjugated to Alexa Fluor 660 (Molecular Probes–Invitrogen), diluted 1:5 in PBS containing 0.01% BSA, at 4°C overnight. After washing, cells were resuspended at 1 × 10^6 cells/ml in PBS containing 0.01% BSA, 200 mg RNAse A and 5 µg/ml PI. For cell cycle analysis, PI, EGFP, and BrdU were measured simultaneously using a MoFlo® flow cytometer (Dako Cytomation), equipped with an argon ion laser tuned at 488 nm. PI vs. EGFP dotplots (log scale) were used to collect a minimum of 10,000 events. The percentage of BrdU-positive cells was calculated as the ratio between the number of BrdU-positive cells and the total number of cells in transfected and non-transfected cellular populations separately.

The BrdU incorporation was also analyzed in LNCaP cells treated with DPN (Tocris Bioscience), using the APC BrdU Flow KIT (BD Biosciences PharMingen, San Diego, CA), according to the supplier instructions. Briefly, cells were seeded in RPMI medium supplemented with 10% FCS and 24 h later the medium was changed to RPMI supplemented with 5% FCS for the rest of the experiment. After 24 h, cells were either treated with 10^{-6} or 10^{-8} M DPN dissolved in ethanol, vehicle or non-treated. The maximum ethanol concentration per Petri dish was 0.05%. At 24, 48 and 72 h of DPN administration, cells were pulsed with 1 mM BrdU in DPBS buffer (2.7 mM KCl, 1.46 mM KH₂PO₄, 136.9 mM NaCl, 8.1 mM Na₂HPO₄) supplemented with 3% FBS for 2 h, and washed in the same DPS buffer. Then the cells were fixed with the Cytofix/Cytoperm-provided buffer, treated with DNase and incubated with the APC labeled-BrdU antibody. The DNA was subsequently stained with 7-amino-actinomycin D (7-ADD) and cells were resuspended in staining buffer for flow cytometric analysis. For cell cycle analysis, 7-ADD and BrdU were measured simultaneously using a BD FACSCalibur flow cytometer.

2.13. Immunoprecipitation of Flag epitope-tagged proteins

LNCaP cells were seeded and transiently transfected with the FLAG-ERβ1 vector as previously described. Cells were treated either with RPMI medium alone or in combination with 1 µM ICI 182,780 for 24 h. Cells were then lysed in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and centrifuged at 19,000g at 4°C for 5 min. For immunoprecipitation of Flag epitope-tagged proteins, 500 mg/ml of protein obtained from each condition were incubated with 40 ml of anti-Flag M2 agarose affinity gel (Sigma, St. Louis, MO), overnight, at 4°C. The agarose beads were washed three times with lysis buffer, extracted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and analyzed by western blotting with c-Jun and SAPK/JNK antibodies.

2.14. Cell sorting of EGFP-ERβ1 transfected cells and western blot analysis of c-Jun, phospho c-Jun and cyclin D1 proteins

EGFP-ERβ1 over-expressing and non over-expressing cells were sorted using the BD FACSAria flow cytometer. Protein extracts obtained from both cellular populations were blotted against the mentioned antibodies as described.

2.15. Statistical analysis

For results shown in Figs 1, 5, 6 and 7, at least three independent-experiments were performed for each group. For results shown in Figs 2, 3 and 4, each experiment was done in duplicate. Analysis of variance was used, when indicated, to assess statistical significance between group means, and groups were considered to be statistically different at p ≤ 0.05 or p ≤ 0.01. Means ± standard deviation are represented in Figs 5–7 and Suppl. Fig. 3: http://www.qub.ac.uk/isco/JCO.
3. Results

3.1. mRNA and protein expression of ERβ1 and ERβ2 in prostate cancer cell lines

A 207 bp product, corresponding to the 3' region of ERβ1, was amplified from LNCaP and PC3 cell lines (Fig. 1(a)) and its identity was confirmed by sequencing. Consistent with the RT-PCR data, the western blot assay confirmed the presence of the ERβ1 protein, using the PA1-313 antibody that recognizes its C-terminal region (Fig. 1(b)). The specificity of the PA1-313 antibody was demonstrated by the disappearance of the specific western blot band when the antibody was preadsorbed with an excess of blocking peptide and the recognition of FLAG-ERβ1 and EGFP-ERβ1 fusion proteins by the PA1-313 antibody in extracts of LNCaP cells transfected with CMV-FLAG-ERβ1 and CMV-EGFP-ERβ1 vectors, respectively (Suppl. Fig. 1: http://www.qub.ac.uk/isco/JCO). LNCaP and PC3 cell lines also expressed ERβ2 mRNA, as detected by RT-PCR (Fig. 1(c)) and confirmed by the sequencing of the amplified 876 bp fragment. Using the antibody generated against the carboxy-terminal region of ERβ2, the expression of this receptor was also demonstrated in both cell lines by western blot (Fig. 1(d)). The specificity of the antibody was demonstrated by ELISA, incubating rabbit sera obtained prior to and following immunization, either with a GST-ERβ2 recombinant protein, a GST purified protein, or without protein. Only the GST-ERβ2 recombinant protein was recognized by the selected hyper-immunized serum and not by the pre-immunization serum of the same rabbit (Suppl. Fig. 2: http://www.qub.ac.uk/isco/JCO). The specificity was also confirmed by the recognition of the HA-ERβ2 fusion protein in LNCaP cells transfected with the CMV-HA-ERβ2 vector (Suppl. Fig. 2: http://www.qub.ac.uk/isco/JCO).

3.2. ERβ expression in cells isolated at specific cell-cycle phases by flow cytometric sorting

Asynchronous cultured LNCaP cells were stained with PI, and the DNA content was measured by flow cytometry. This approach allowed the cell sorting in G0/G1, S and G2/M phases of the cell cycle (Fig. 2(a)). Western blotting of the total protein extracted from each cell population (0.5 × 10^6 sorted cells) with specific antibodies for each phase of the cell cycle (cyclin D1 for G1, cyclin A for S/G2 and cyclin B for G2/M) confirmed that products were efficiently sorted in those phases (Fig. 2(b)). Protein extracts blotted against the ERβ specific antibodies showed that ERβ1 was expressed within the G0/G1 phase of the cell cycle, increased in the S phase and markedly decreased in the G2/M phase (Fig. 2(c)). ERβ2 protein levels decreased in the S phase compared to G0/G1, and increased again in G2/M to levels similar to those observed during the G1 phase (Fig. 2(d)).

Fig. 1. ERβ1 and ERβ2 mRNA and protein expression in the human prostate cancer cell lines LNCaP and PC3. (a) With respect to ERβ1, the expected 207 bp product, corresponding to the 3' region, was amplified by RT-PCR analysis and (b) a band of approximately 64 kDa was detected by western blot assay using the PA1-313 antibody in LNCaP and PC3 cells. (c) The ERβ2 mRNA expression was demonstrated by RT-PCR in both cell lines and (d) its protein expression was confirmed by western blot analysis using the antibody generated against an specific carboxy-terminal peptide.
3.3. ERβ expression during cell cycle in synchronized LNCaP cells

To confirm the above results, LNCaP cells arrested at G0/G1 using the isoleucine-deprived medium, were released into complete medium and harvested at 0 h, 8 h (G0/G1 phase), 15 h (early S phase), 19 h (late S phase), and 22 h (G2/M phase). Synchronization was assessed using flow cytometry (Fig. 3(a)). Analysis of protein expression by western blot showed that ERβ1 protein increased from G1 to the beginning of the S phase (15 h) and decreased to very low levels prior to the entrance into the G2/M phase (Fig. 3(b) and (d)). ERβ2 protein levels decreased from the beginning to the end of the S phase (15–19 h) and increased in G2/M (Fig. 3(c) and (e)). The RNA levels mirrored the protein profile (Fig. 3(b)–(e)). This approach allowed a more precise analysis of the changes in expression through the cell cycle than the former method, since
the results obtained using the flow cytometric sorting of cells growing asynchronously only measured the mean value of each phase. Therefore, the higher variation in the expression of ER/β2 through the S phase (between 15–19 h) was only appreciated after synchronization.
3.4. Subcellular localization of ERβ isoforms

The immunoblotting of nuclear and non-nuclear extracts from the LNCaP cells showed that ERβ1 was localized in both nuclear and non-nuclear fractions, whereas ERβ2 was found exclusively in nuclear fractions (Fig. 4(a)). Retinoblastoma and actin were used as controls for the nuclear and non-nuclear fractions, respectively. The same experiment was performed in cells harvested at 8 h (G0/G1 phase), 17 h (S phase) and 22 h (G2/M phase) after synchronization. ERβ1 was identified within nuclear and non-nuclear fractions in the G0/G1 and S phases, whereas ERβ2 was found exclusively within the nuclear fractions at all phases (Fig. 4(b)).

3.5. Transcriptional activity of the endogenous ERβ in the LNCaP cell line

To elucidate whether endogenous ERβ was able to transactivate ERE sequences or to modulate the transcriptional activity of AP1 or NFκB, we transiently transfected LNCaP cells with a luciferase reporter gene driven by a promoter carrying either a tandem ERE, AP-1 or NFκB binding sequence. Estradiol administration induced a two-fold increase in the luciferase expression in cells transfected with ERE-LUC (Fig. 5(a)). ICI completely abolished the transcriptional effect induced by estradiol, when both chemicals were administered simultaneously, but no transcriptional effect was observed when administered alone. In contrast, estradiol did not induce any significant
change in luciferase expression in cells transfected with AP1-LUC or NFκB-LUC (Fig. 5(a)). To evaluate in which phase of the cell cycle the endogenous ERβ was able to transactivate ERE sequences, cells transfected with the ERE-LUC vector and treated with estradiol were sorted in G0/G1, S and G2/M phases and collected into separate tubes. Relative luciferase activity showed that ERE transactivation was only present during the S phase (Fig. 5(b)).

3.6. Effects on cell cycle progression of ERβ1 over-expression and DPN administration

The EGFP empty vector did not show any effect on cell cycle progression 24 h after its transfection in LNCaP cells (Fig. 6(a)). Flow cytometric analysis of BrdU incorporation in EGFP-expressing and non-expressing cells of the same culture dish showed that ERβ1 transfected cells were arrested at the G1 phase (Fig. 6(b)) and that the number of BrdU-positive cells in the EGFP-expressing population was significantly lower than in non-expressing cells (Fig. 6(c)). The G1 arrest was subsequently confirmed by cell cycle analysis of PI stained cells (Fig. 7(b)). In agreement with these results, immunodetection of BrdU in cells grown in coverslips and transiently transfected with the EGFP-ERβ1 vector, showed that the majority of EGFP-ERβ1 positive cells were BrdU-negative (Fig. 6(d)) and proved that EGFP-ERβ1 expression was nuclear.

LNCaP cells treated with $10^{-6}$ M DPN for 72 h showed a significant decrease in BrdU incorporation (Fig. 6(e)) and a significant increase in the percentage of G1 cells when compared to the vehicle treated cells (Suppl. Fig. 3: http://www.qub.ac.uk/isco/JCO). The administration of a lower concentration of DPN ($10^{-8}$ M) or the same concentration but for a shorter period of time (24 or 48 h) failed to show significant differences (data not shown).

3.7. Mechanism of action of over-expressed ERβ1 on G1 cell cycle phase

In order to test whether the G1 arrest was caused by an ERE-dependent mechanism, LNCaP cells were transiently transfected with the EGFP-ERβ1 EG167/
Fig. 7. Mechanism of action of over-expressed ERβ on G1 cell cycle phase. (a) Flow cytometric analysis of BrdU incorporation in LNCaP cells, transfected with the EGFP-ERβ1 EG167/168AA mutant vector, showed a significant decrease in the number of BrdU positive cells compared to non-transfected cells of the same culture dish. (b) Cell cycle analysis of EGFP-ERβ1 transfected cells incubated with or without ICI confirmed that ERβ1 over-expressing cells were arrested in early G1 (upper left) compared to non-transfected cells of the same culture dish (upper right), and that ICI induced an arrest in late G1 (lower left), compared to control cells (lower right). (c) Luciferase activity in cells co-transfected with AP1-Luc reporter and FLAG-ERβ1 vector and treated with E2, ICI or both, showed that ICI increased significantly endogenous AP-1 transcriptional activity. (d) Immunoprecipitation of FLAG-ERβ1 transfected and non-transfected cells with an anti-FLAG antibody, followed by western blot analysis of c-Jun and JNK1 of the immunoprecipitated (IP) and flow throw (FT) fractions, demonstrated that ERβ1 interacted with JNK1 and that this interaction was abolished by ICI.

168AA construct, which contained two mutations in the DNA-binding domain. Contrarily to what was found in cells transfected with the wild type ERβ1, estradiol administration did not induce the luciferase expression in cells co-transfected with ERE-LUC and EGFP-ERβ1 EG167/168AA (Suppl. Fig. 4: http://www.qub.ac.uk/isco/JCO). BrdU incorporation experiments showed that the over-expression of mutant EGFP-ERβ1 induced the same arrest at G1 as did the wild type protein (Fig. 7(a)).

To find out if the G1 arrest was ligand-dependent, EGFP-ERβ1 transfected LNCaP cells were treated with the specific inhibitor ICI 182,780. Cell cycle analysis of EGFP-ERβ1 positive cells confirmed the arrest in early G1 phase (Fig. 7(b), upper left panel), whereas ICI treated cells were arrested at late G1 phase of the cell cycle (Fig. 7(b), lower left panel), compared to their respective control cells of the same culture dish (Fig. 7(b), upper and lower right panels). To verify whether AP1 activation and cyclin D1 were involved
in the G1 arrest induced by ER\(\beta_1\) over-expression, luciferase activity was analyzed in LNCaP cells co-transfected with ER\(\beta_1\) and AP1 reporter vectors. The results showed that only the ER\(\beta_1\) over-expressing cells treated with ICI were able to significantly increase the endogenous AP1 transcriptional activity (Fig. 7(c)). Co-immunoprecipitation of FLAG-ER\(\beta_1\) with c-Jun and JNK1 in protein extracts of LNCaP cells showed that ER\(\beta_1\) interacted with JNK1 and not with c-Jun, and that this interaction was abolished in the presence of ICI (Fig. 7(d)). Additionally, western blot analysis of phosphorylated c-Jun demonstrated that c-Jun phosphorylation was undetectable in cells over-expressing ER\(\beta_1\), but was present in non-over-expressing cells of the same culture dish as well as in ER\(\beta_1\) over-expressing and non-over-expressing cells after ICI administration (Fig. 7(e)). Finally, analysis of cyclin D1 by western blot showed that, in ER\(\beta_1\) transfected cells treated with the specific inhibitor, cyclin D1 expression was significantly higher than in non-treated cells (Fig. 7(f)).

4. Discussion

The present results demonstrate that ER\(\beta_1\) and ER\(\beta_2\) isoforms are differentially regulated during cell cycle in the human prostate cancer cell line LNCaP and provide evidence that ER\(\beta_1\) controls the G1 progression through non-genomic mechanisms. It has been demonstrated that the epithelial compartment of prostatic carcinoma expresses ER\(\beta\) but not ER\(\alpha\) [18,42]. Prostate cancer cell lines have been reported to exhibit constitutive ER\(\beta\) expression, although results from different groups have proved controversial [29,47,51,56]. To our knowledge, expression studies of the ER\(\beta_2\) protein are lacking. Therefore, an essential prerequisite to our work was to demonstrate ER\(\beta_1\) and ER\(\beta_2\) expression in LNCaP cells, the prostate cell line we selected on the basis of its distinctive profile of ER expression, i.e. ER\(\beta\) but not ER\(\alpha\), similar to that of the epithelial neoplastic compartment (Suppl. Fig. 5: http://www.qub.ac.uk/isco/JCO).

Our results demonstrated that ER\(\beta_1\) and ER\(\beta_2\) mRNA and protein were expressed in LNCaP cells as well as in the human androgen-independent prostate cancer cell line PC3. The mRNA expression of ER\(\beta\) has been previously reported in LNCaP cells, using primers that recognized both ER\(\beta_1\) and ER\(\beta_2\) isoforms [25]. Regarding each specific isoform, ER\(\beta_1\) mRNA levels were found to be low or undetectable in LNCaP cells under basal conditions [29,47,51,56] and increased after exposure to DNA demethylating agents [47,56] or irradiation [52]. In PC3 cells, ER\(\beta_1\) mRNA expression has been also clearly documented [29,47,51,56]. Concerning ER\(\beta_2\) mRNA, there was only one report that demonstrated its expression in prostate cancer cell lines [29]. With regard to the protein expression, ER\(\beta_1\) has been previously detected in LNCaP cells under basal growth conditions whereas it increased after estradiol and DHT administration [32] or irradiation [52]. The size of the ER\(\beta\) western blot bands described in prostate tissue ranged from 63 kDa [27] to 55 kDa [42]. The band we detected in LNCaP and PC3 cells sized approximately 64 kDa. The specificity of the ER\(\beta_1\) antibody used in this study was
demonstrated by the absence of the 64 kDa band when the antibody was preabsorbed with its specific blocking peptide and the detection of the specific band in CMV-FLAG-ER/β1 and CMV-EGFP-ER/β1 transfected cells. The specificity of the antibody generated against ER/β2 was demonstrated because it recognized specifically the GST-ER/β2 recombinant protein by ELISA and the HA-ER/β2 fusion protein by western blot.

Considering that the protein levels of cell-cycle regulators usually fluctuate during cell cycle progression, the next step was to elucidate whether ER/β1 and ER/β2 expression was modulated during the cell cycle in LNCaP cells. To achieve this aim, we used FACS, in addition to the commonly used method of cellular synchronization, for two reasons: first, to increase the purity of the cell populations isolated in each cell phase of the cell cycle, since LNCaP cells are difficult to synchronize, and second, in order to rule out the possibility that changes in ER/β expression were related to the treatment used to arrest the cells. We demonstrate that it was possible to sort viable cells in different cell cycle phases based on their DNA content and to analyze their protein expression by western blot. Changes in the levels of the ER/β1 and ER/β2 proteins were verified by the analysis of the cells harvested at specific time points after G0/G1 synchronization. Using both methods, we demonstrated that each isoform displayed a characteristic expression profile: while ER/β1 increased from G1 to the S phase and decreased to minimum levels before entering the G2/M phase, ER/β2 levels decreased from the beginning to the end of the S phase, and increased in the G2/M phase. Although this is the first demonstration of the fluctuation of ER/β levels during cell cycle, the regulation of other nuclear receptors during cell cycle has been previously reported. It has been shown that the ERα protein is only detectable during the S phase in human osteoblast-like osteosarcoma cells [23], that the androgen receptor loses its transcriptional activity and decrease the level of its protein during the G1/S transition in a fibrosarcoma cell line [34], and that the protein expression of the thyroid receptor β is detected at the late G2 phase, but declines during mitosis in rat pituitary GH3 cells [35]. More recently, it has been demonstrated that the progesterone receptor phosphorylation and function are also cell cycle dependent, with the highest activity taking place during the S phase [38].

The different pattern of expression of ER/β1 and ER/β2 mRNA and protein could be caused by a different promoter usage and/or different mRNA stability. The later hypothesis is highly probable since ER/β1 and ER/β2 contain specific 3’ UTR sequences and it has been demonstrated that the 3’ UTR sequences regulate mRNA stability [9]. However, several data suggest that the different promoter usage hypothesis is also probable. Two promoters, 0K and 0N, have been identified in the 5’-region of the human ER/β gene [22,55,56], and the 0K promoter has been localized 41 kb upstream of the 0N promoter [46]. Since the ER/β2 mRNA human sequence (NCBI #AB006589) contains 5’-UTR exons located upstream of the 0N promoter, its expression must be regulated by an upstream promoter. Therefore, it can be speculated that specific DNA response elements located at each one of these promoters differentially regulate the expression of ER/β1 and ER/β2 in a cell-cycle dependent manner. Additionally, given that each ER/β promoter seems to be associated with a specific exon 8 splicing decision, and that each exon 8 contains a specific 3’ UTR, it is probable that the specific promoter usage in the ER/β gene is associated with a different mRNA stability. The different promoter usage of ER/β1 and ER/β2 would also explain the decrease in the expression of ER/β1 and not of ER/β2 during cancer progression, since it has been demonstrated that the CpG sites located in the 0N promoter are progressively methylated in prostate [39,56] and breast cancer [55].

With regard to sub-cellular localization, we detected ER/β1 in all phases of the cell cycle in both nuclear and non-nuclear fractions, whereas ER/β2 was found exclusively in the nucleus. The nuclear localization of ER/β1 during the G1/S transition may suggest that this receptor modulates the expression of proteins involved in the entry into and the progression through the S phase. The non-nuclear localization of ER/β1 and the changes in its expression during the cell cycle suggest that non-nuclear mechanisms participate in cell cycle regulation. ER/β1 has been previously localized in the cytoplasm, in association with the androgen receptor and the cytoplasmic membrane protein c-Src [36], and also in the mitochondrial fraction [8]. Whether non-nuclear ER/β plays a role in cell cycle progression and whether it acts independently or in coordination with nuclear mechanisms remains to be determined.

In order to decipher the mechanisms of action of ER/β during the cell cycle, we first analyzed the transcriptional activity of the endogenous protein. We demonstrated that estradiol induced the transactivation of ERE-driven reporters, but that it did not significantly change the AP1 or the NFκB-dependent transactivation. Since ERE transactivation was inhibited by ICI, and LNCaP cells do not express ERα, the re-
results suggest that this specific effect is dependent upon the estradiol binding to ER/β. By sorting LNCaP cells transiently transfected with the ERE-LUC vector in G0/G1, S and G2/M phases, we were able to demonstrate that ERE transactivation was only present in S-phase cells. However, the low ER/β levels in LNCaP cells could account for the absence of ERE transactivation in the other cycle phases, as well as the inability of estradiol to indirectly transactivate AP1 and NFκB. Interestingly, the basal transcriptional activity of NFκB in LNCaP cells was about five times higher than that of ERE or AP-1. A robust NFκB transcriptional activity has also been reported in another prostate cancer cell line [30], but the absence of changes after estradiol or ICI administration in our model suggests that endogenous ER/β does not contribute to its induction.

To gain further insight into the mechanisms and effects of the transcriptional activity of ER/β in cell cycle progression, we over-expressed this isoform in LNCaP cells. Our results demonstrated that ER/β over-expression caused a decrease in the number of S phase cells and an arrest in the early G1 phase. A previous study also reported the induction of a G1 phase arrest after restoring the ER/β expression by adenoviral delivery in a prostatic carcinoma cell line devoid of this receptor [10]. Other studies reported the association of ER/β expression with a decreased proliferation in breast [26,49,53] and colon cancer [33].

It is known that LNCaP cells express a mutant androgen receptor (AR) that can be transactivated by estradiol on androgen response elements [32]. Previous studies have analyzed the effects of AR and ER/β on the LNCaP proliferation after estradiol administration using specific ER and AR antagonists, with contradictory results: two reports showed that the anti-estrogen receptor ICI 182,780 decreased the proliferation induced by estradiol in this cell line [4,32], and a recent study proves that this effect is produced by AR antagonists but not ICI 182,780 [1]. Therefore, to confirm that the G1 arrest was caused by ER/β, we treated the cells with the specific inhibitor ICI 182,780 and the specific agonist DPN. The ICI administration caused a double effect: it rescued ER/β over-expressing cells arrested at early G1, but induced a subsequent arrest at late G1. We postulate that the rescue effect is due to the blockade of the inhibitory action of ER/β and that additional players might contribute to the late G1 arrest. The lack of effect of ICI on non-over-expressing cells indicates that levels of ER/β higher than those found in control cells are needed for both, the rescue of early G1 arrest and the induction of late G1 arrest. The administration of the ER/β specific agonist DPN produced a decrease in BrdU incorporation and an accumulation of cells in the G1 phase of the cell cycle. The results obtained with both approaches support that ER/β negatively regulates LNCaP cell proliferation and suggest that the proliferative action of estradiol most likely occurs through AR transactivation. In line with this hypothesis, it has been reported that the administration of estradiol and DPN inhibits proliferation in the DU145 prostate cancer cell line that expresses ER/β but neither ERα nor AR [45].

Another putative player is the progesterone receptor (PR). LNCaP cells express PR [25] and it has been demonstrated that this receptor regulates AP1 activity and cyclin D1 expression in other tissues [7,13]. It has also been shown that ICI 182,780 is able to transactivate a reporter plasmid containing a progesterin responsive element (PRE) in breast cancer cells but this induction is dependent on the presence of estrogen receptor [54]. Therefore, although it is possible that PR is involved in the ability of ICI to rescue the LNCaP cells arrested at G1, the absence of cell cycle changes in control cells in response to ICI attests that ICI action depends chiefly on the levels of ER/β.

The induction of a G1 phase arrest by ER/β over-expression prevents the further analysis of the mechanism of action of the exogenous protein in other cell cycle phases. Our results strongly suggest that the mechanism of induction of the G1 phase arrest is ERE-independent, since the transfection of an ER/β mutated in the DNA-binding domain also results in a G1 phase arrest. The mechanism of induction is probably ligand-dependent, because the administration of the specific inhibitor allows the cells to progress to late G1 phase and the arrest is induced by the specific agonist. It has been previously reported that ER/β is able to regulate the activity of the AP1 transcription factor [41] and that AP1 regulates transcription of cyclin D1 in the presence of anti-estrogens, specifically ICI 182,780 [31]. Analysis of the luciferase activity in LNCaP cells, co-transfected with ER/β and AP1 luciferase reporter vectors, verified that only ER/β over-expressing cells treated with ICI were able to significantly activate AP1, lending support to the above-mentioned hypothesis.

The active role of ER/β in this arrest was demonstrated by the interaction of ER/β with JNK1, the kinase that activates the c-Jun/AP1 transcription factor [15]. Our results suggest that the ER/β-JNK1 interaction interferes with c-Jun phosphorylation and results
in reduced cyclin D1 expression. As expected, c-Jun phosphorylation was not decreased and the levels of cyclin D1 were not reduced in cells of the same culture dish that do not over-express ERβ.

The inhibition of the interaction between c-Jun and JNK as a mechanism to inactivate AP1 has been described for the tumor suppressor gene p16INK4a [11]. These authors propose that the interaction of p16INK4a and JNK occur at the same binding site as for c-Jun, thus interfering with c-Jun phosphorylation and activation. Our data suggest that an analogous mechanism would account for the ERβ inactivation of c-Jun.

With regard to ERβ, there are no studies addressing its potential role in cell cycle. It has been reported that ERα does not bind to ERE [40] and that it has a reduced ability to bind DNA [43]. Recently, Leung and collaborators demonstrated that the only functional ERβ isoform was ERβ1, and that ERβ2 did not form homodimers, but it was able to heterodimerize with ERβ1 and enhance its transactivation [29]. In the present study, we showed that ERβ1 and ERβ2 were both present in the nucleus during the G0/G1 and S phases. In this specific context, it can be hypothesized that ERβ2 is able to heterodimerize with ERβ1 and enhance transcriptional activity on ERE sequences only in the S phase, since during the G1 phase, ERβ1 was not able to activate transcription. In contrast, during the G2/M phase, ERβ2 was abundantly expressed, whereas ERβ1 was almost absent. Interestingly, it has been demonstrated that the hinge domain of ERβ interacts with the mitotic spindle-assembly checkpoint protein MAD2 [44], although the significance of this interaction is not known. In a previous study, we showed that an increased expression of ERβ was associated with an increased expression of MAD2 in the primary spermatocytes of rat ABP transgenic mice, arrested at metaphase [48]. If ERβ plays a role in the mitotic and/or meiotic spindle checkpoint, the results obtained here suggest that this action involves ERβ2, possibly through its interaction with other nuclear proteins.

In conclusion, our data provides evidence that ERβ1 and ERβ2 are differentially regulated during cell cycle in LNCaP prostate cancer cells, and that ERβ1 controls the S phase through ERE-dependent mechanisms in the presence of estradiol. Our results also demonstrate that over-expressed ERβ1 induces a cell cycle arrest in the early G1 phase and reveal a non-genomic mechanism by which ERβ behaves as a tumor suppressor gene.

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A. Hurtado et al. / Estrogen receptor β and the cell cycle

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