Molecular and Cellular Endocrinology 415 (2015) 100–113

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Inhibitory role of ERβ on anterior pituitary cell proliferation by controlling the expression of proteins related to cell cycle progression

Pablo A. Pérez [a], Juan P. Petiti [a], Ignacio A. Wagner [a], Maria E. Sabatino [a], Corina V. Sasso [b], Ana L. De Paul [a], Alicia I. Torres [a], Silvina Gutiérrez [a, *]

[a] Centro de Microscopía Electrónica, Instituto de Investigaciones en Ciencias de la Salud (INICSA-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina
[b] Laboratorio de Hormonas y Biología del Cáncer, Instituto de Medicina y Biología Experimental de Cuyo, (IMBECU-CONICET), CCT-Mendoza, Mendoza, Argentina

ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form
7 August 2015
Accepted 8 August 2015
Available online 14 August 2015

Keywords:
Pituitary
Estrogen receptor β
Estradiol
Cell proliferation
Cyclin D1
p21

ABSTRACT

Considering that the role of ERβ in the growth of pituitary cells is not well known, the aim of this work was to determine the expression of ERβ in normal and tumoral cells and to investigate its implications in the proliferative control of this endocrine gland, by analyzing the participation of cyclin D1, Cdk4 and p21. Our results showed that the expression of ERβ decreased during pituitary tumoral development induced by chronic E2 stimulation. The 20 ± 1.6% of normal adenohypophyseal cells expressed ERβ, with this protein being reduced in the hyperplastic/adenomatous pituitary: at 20 days the ERβ+ population was 10.7 ± 2.2%, while after 40 and 60 days of treatment an almost complete loss in the ERβ expression was observed (40d: 1 ± 0.6%; 60d: 2 ± 0.6%). The ERα/β ratio increased starting from tumors at 40 days, mainly due to the loss of ERβ expression. The cell proliferation was analyzed in normal and hyperplastic pituitary and also in GH3β− and GH3β+ which contained different levels of ERβ expression, and therefore different ERα/β ratios. The over-expression of ERβ inhibited the GH3 cell proliferation and expression of cyclin D1 and ERα. Also, the ERβ activation by its agonist DPN changed the subcellular localization of p21, inducing an increase in the p21 nuclear expression, where it acts as a tumoral suppressor. These results show that ERβ exerts an inhibitory role on pituitary cell proliferation, and that this effect may be partially due to the modulation of some key regulators of the cell cycle, such as cyclin D1 and p21. These data contribute significantly to the understanding of the ER effects in the proliferative control of pituitary gland, specifically related to the ERβ function in the E2 actions on this endocrine gland.

1. Introduction

The pituitary gland is a target of estrogen, which stimulates prolactin secretion and modulates the lactotroph cell population size (Bulayeva et al., 2005; Sosa et al., 2012). Under physiological conditions, the number of prolactin-secreting cells and the serum prolactin content oscillate according to the systemic requirements and the levels of circulating 17β-estradiol (E2), which is the most active estrogen in the body. However, this well-balanced equilibrium can be affected by several not well understood factors, leading to a faster rate of cell division that may eventually result in the development of pituitary tumors, which has generated a great academic interest in the mechanisms that regulate cell proliferation in this gland.

E2 mediates its biological effects by acting through the specific estrogen receptors (ER), α and β, both of which have been identified in different pituitary endocrine cell types (Mitchner et al., 1998). Although an important number of studies have demonstrated the role of ERα in pituitary cell proliferation (Gutierrez et al., 2008; Zarate et al., 2009), the role of ERβ in the growth of pituitary cells is not well known, with the functions of this ER subtype in the

Abbreviations: ER, estrogen receptor; E2, 17β-estradiol; DPN, (4-hydroxyphenyl)-propionitrile; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol.

* Corresponding author. Centro de Microscopía Electrónica, Instituto de Investigaciones en Ciencias de la Salud (INICSA-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Av. Enrique Barros y Enfermera Gordillo, Ciudad Universitaria, CP 5000, Córdoba, Argentina.
E-mail address: silvina@cmefcm.uncor.edu (S. Gutiérrez).

http://dx.doi.org/10.1016/j.mce.2015.08.009
0303-7207/

pathophysiology of this gland still being obscure.

While there is no evidence linking ERβ with the regulation of anterior pituitary cell proliferation, some studies have investigated the expression of ER in pituitary tumors. In male ERα, ERβ, or ERα/ERβ knockout mice, the development of pituitary tumors has not been detected. However, in female mice, the loss of ERβ induced the development of large pituitary tumors (Fan et al., 2010). Moreover, it has been shown that ERα and ERβ are differentially expressed in the various human pituitary adenoma subtypes. A significantly elevated ERα expression was observed in macroadenomas compared with microadenomas and also in non-invasive compared to invasive tumors (Manoranjan et al., 2010). The ERα expression was significantly increased and the ERβ expression decreased in human non-functional pituitary adenomas, suggesting that the balance between ERα and ERβ may affect the invasion of these tumors (Zhou et al., 2011). Also, the ERα values were significantly greater in invasive macroadenomas than in microadenomas (Pereira-Lima et al., 2004).

In other tissues, similar or opposing actions of ERβ compared to ERα in the regulation of cell proliferation have been described, indicating that the effects of both ER subtypes are tissue-dependent (Matthews and Gustafsson, 2003). In breast adenocarcinomas and osteosarcomas, it has been shown that both ER similarly mediate the effects of E2 on gene expression and cellular proliferation, with ERα being a more potent regulator than ERβ in inducing cellular responses (Huang et al., 2011). However, other studies have described that ERα and ERβ have distinct functions and act antagonistically in multiple pathways: with ERα playing an important role in growth and proliferation, while ERβ is linked to increased cell differentiation and inhibition of cell proliferation in breast (Lazennec et al., 2001), prostate (McPherson et al., 2010) and colon cells (Hartman et al., 2009).

The effects of estrogens on cell proliferation are mediated in part by modulation of the expression of cell cycle regulators. In fact, alterations in the level of expression of proteins are critical for cell cycle progression, including the over-expression of cyclins, which are frequent in human pituitary adenomas (Fedele and Fusco, 2010). Furthermore, it has been shown that cyclin D1 expression is regulated by estrogens via ERβ, and that this signaling pathway may influence tumoral development (Nakamura et al., 2013). In fact, cyclin D1 forms a complex with cyclin-dependent kinases (cdk) 4, with it being an essential regulator of the G1/S checkpoint. It was shown that Cdk4-deficient mice exhibited a decrease in lactotroph function and postnatal proliferation, thereby demonstrating that Cdk4 is required for the normal growth of the lactotroph population (Moons et al., 2002). Cdk are modulated by fluctuations in cyclins or cdk inhibitors (CKI) such as p21, which is a regulator of cell cycle progression from the G1 to S phases. Is known that p21 is also involved in cell cycle control in the pituitary homeostasis, thus either preventing an excessive proliferative response (Chesnokova et al., 2008), or conversely, with a pro-proliferative role as suggested by Toledano and co-workers (Toledano et al., 2012). Whereas p21 localization to the cytoplasm promotes cell survival and proliferation, cell growth-inhibiting activity of p21 correlates with nuclear localization, which blocks S phase progression by inhibiting PCNA activity, thus causing cell cycle arrest (Abbas and Dutta, 2009).

Considering the lack of knowledge about the role of ERβ in regulating the growth of the anterior pituitary gland, and taking into account that E2 induces its effects by controlling the proteins critical for cell cycle progression, the aim of this work was to determine the expression of ERβ in normal and tumoral cells and to investigate its implications in the proliferative control of this endocrine gland, by analyzing the participation of cyclin D1, Cdk4 and p21. This research may lead to a better understanding of the mechanisms that underlie the control of pituitary cell growth.

2. Materials and methods

2.1. Animals and experimental models

Three-month-old female Wistar strain rat, were bred and housed at the Animal Research Facility of the National University of Córdoba, under controlled temperature (21 ± 3 °C) and lighting conditions (14 h light/10 h dark), with free access to commercial rodent food and tap water. Animals were kept in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the United States National Institutes of Health (1996), and the experiments were approved by the local Institutional Animal Care Committee.

Considering that exogenous estrogen excess induces pituitary tumors (Mukdsi et al., 2004), intact animals were treated with estradiol benzoate (Sigma Aldrich, St. Louis, MO, USA) for 20, 40, and 60 days. E2 was implanted subcutaneously in slow-releasing capsules made of Silastic tubing (Dow Corning; medical grade) filled with 10 mg estrogen crystals and sealed with Silastic cement. The control group was implanted with empty capsules. Rats were decapitated within 10 s after removal from their cage, thus avoiding any stress or external stimuli. Fresh pituitary glands were photographed *in situ* and weighed immediately after collection. For the quantification of serum estradiol levels, arterial and venous blood was drained from the head and trunk, allowed to clot, and the serum was stored frozen at −20 °C for subsequent electrochemiluminescence analysis. The control rats showed serum E2 values of 829.67 ± 252.7 pg/ml, whereas in the development of the hyperplastic/adenomatous process the values of the serum E2 obtained were always higher than 2000 pg/ml.

The effect of estrogen as an inducer of pituitary tumor development was documented in semithin sections from epoxy resin-embedded glands by high-resolution light microscopy, as described previously (Sabatino et al., 2015). Briefly, pituitaries were fixed in a mixture of 4% v/v formaldehyde and 2% v/v glutaraldehyde in 0.1 M cacodylate buffer, and then treated with 1% OsO_4 before being stained in block with 1% v/v uranyl acetate. Semithin sections (200 nm) were cut using a JEOL ultramicrotome, stained with Toluidine Blue and examined using a Zeiss Axiostar Plus light microscope (Oberkochen, Germany).

2.2. Anterior pituitary cell cultures

The protocol for the dissociation of pituitary cells has been described previously (De Paul et al., 1997). After 3 days of culture, the medium was discarded and replaced with serum-free and phenol red-free DMEM supplemented with hydrocortisone (100 μg/l), 3,3¢-triiodothyronine (400 ng/l), transferrin (10 mg/l) and sodium selenite (5 μg/l). Then, this medium was replaced every day while the cells were submitted to different experimental protocols.

2.3. GH3 cells

The rat GH3 lactosomatotroph pituitary adenoma cell line was used, which secretes high levels of prolactin and is an adequate *in vitro* prolactinoma model. Cells were cultured in HAM-F12 K medium supplemented with 5% fetal calf serum and 12% horse serum (Invitrogen; Carlsbad, USA) in an oven with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, with all cell culture grade reagents being obtained from Sigma (St Louis, USA). After 3 days of culture, there was an observed confluence of 70%, and these cells were exposed to different treatments.

2.4. Stable transfection

Plasmid encoding the full-length sequence of ERβ (pEYFP-C1-ERβ) and the empty plasmid pEYFP-C1 were kindly provided by Dr. Anders Strom, University of Houston, USA. For the stable transfection of ERβ, GH3 cells were initially plated at an 80–90% confluence in a six-well plate in HAM-F12 K medium, and maintained for 24 h. The medium was then discarded, replaced by Optimen medium (Gibco; NY, USA), and the expression plasmid (1 μg) and the transfection reagent FuGene 6 (3 μl, Roche; In, USA) were added for 24 h. Next, the transfected cells were selected in HAM-F12 K medium supplemented with 400 μg/ml of G418 (Invitrogen; Carlsbad, USA) over 3 weeks. Finally, the cells were maintained in HAM F12 K medium with 200 μg/ml of G418, and the ERβ positive cells were identified by flow cytometry. The GH3 cells in which the ERβ gene was successfully transfected (thereby expressing the ERβ protein) were named GH3β+, while GH3, which did not express ERβ, were designated as GH3β−.

2.5. Cell treatments

Culture cells were exposed to 10 nM of 17β-estradiol (Sigma St Louis MO, USA) or to the selective ERα and β agonists: 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-tryl)trisphenol (PPT), and 2,3-bis (4-Hydroxyphenyl)-propionitrile, (DPN) (Tocris Cookson), respectively, in serum-free and phenol red-free culture medium for 72 h. The concentrations were chosen based on preliminary experiments.

2.6. Antibodies

Anti-ERβ: Y-19, directed to the N-terminal of ERβ (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and ab3577 (1:3500, Abcam, Cambridge, UK); anti-ERα: MC-20, directed to the C-terminal of ERα (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); anti-prolactin (1:3000, National Hormone and Pituitary Program, Torrance, CA); anti-β-actin (1:5000, Sigma–Aldrich, St. Louis, MO); anti-p21 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin D1 (1:500, Abcam plc, Cambridge, UK); anti-Cdk4 (1:1000, *Abcam*, *Cambridge*, UK); anti- BrdU (Amersham, Buckinghamshire); anti-histone deacetylase 1 (HDAC1) (1:500, Sigma–Aldrich, St. Louis, MO).

2.7. Light microscopy immuno-labeling

Whole anterior pituitary glands from three rats per experimental group were fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Sections were cut at 3 μm thickness, deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS). Before applying the primary antibody, sections were treated with 3% H_2O_2 for 15 min, followed by treatment with 5% PBS–BSA for 30 min. Thereafter, pituitary sections were incubated overnight in anti-PRL antiserum. Slides were exposed to biotin-labeled antibody against rabbit IgG, before being incubated with avidin–biotin–peroxidase complex (ABC, Vector Labs, Burlingame, CA, USA). Next, the slides were immersed for 7 min in a solution containing 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma; St Louis, USA) in 0.1 M Tris buffer, pH 7.2, with 0.03% H_2O_2, and counterstained with hematoxylin.

2.8. Cell cycle analysis

After gland dissociation, cells were washed in PBS, fixed with −4 °C ethanol 70%, washed again with citrate-phosphate buffer at pH:7.8, and incubated with RNAse (10 μg/μl, Ribonuclease A, Sigma; St Louis, USA). This was followed by DNA staining with propidium iodide (50 μg/ml) (Sigma; St Louis, USA) in order to analyze the cellular DNA content. Cell cycle analysis was performed on a Coulter flow cytometer (BD FACS Canto II), and the percentage of cells in the S and G2/M phase fractions was combined to serve as a proliferative index.

2.9. Confocal laser scanning microscopy

Cultured pituitary cells were fixed in 4% formaldehyde, permeabilized in 0.50% Triton X-100 in PBS, blocked for 1 h in 1% PBS–BSA, incubated with primary antibodies for 1 h and further incubated with an Alexa 488 anti-goat, Alexa 488 anti-mouse, Alexa 555 anti-rabbit, Alexa 594 anti-rabbit, or Alexa 594 anti-mouse secondary antibody (1:1000; Invitrogen; Carlsbad, USA) for 1 h. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1000 (Olympus; Tokyo, Japan). The analysis of confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer.

To validate the specificity of the ERβ immunostaining, both negative and positive controls were performed. The negative controls were carried out by applying the same protocol but replacing primary antibodies with 1% PBS–BSA, or applying the same protocol in GH3 cells, which did not express the ERβ. The absorption control was performed by pre-absorbing the antibody with purified antigens. For this, the antibody dilutions were pre-incubated with a three-fold excess of the antigen peptide, ERβ (Y-19) sc-6821 P, Santa Cruz Biotechnology, overnight at 4 °C. The positive control was performed using MCF7 breast cancer cells.

To determine the cells that co-expressed ERα and ERβ, 1500 pituitary cells of each model were analyzed by counting those cells which showed ERα positive immunofluorescence and/or ERβ positive immunofluorescence. Three slides were analyzed for each group, derived from the different cell preparations.

2.10. Flow cytometric analysis

Living dispersed cells were fixed for 20 min at 4 °C (CITOFIX; BD Biosciences Pharmingen; San Diego, CA), permeabilized with Perm/Wash (BD Biosciences Pharmiogen), incubated with primary antibody overnight at 4 °C and then with secondary antibody Alexa Fluor 488 or 647 (1:1000; Invitrogen) for 1 h at 37 °C. As an isotype control, cells were incubated with Perm/wash instead of the primary antibody. Cells were analyzed on a Coulter flow cytometer (BD FACS Canto II, 1×10^5 events/experimental treatment). A data analysis was carried out using the FlowJo software (Tree Star; Ashland, OR), and the percentage of ERβ or ERα-positive was determined.

2.11. Immunocytochemical detection of cell proliferation by bromodeoxyuridine incorporation

Cells at the DNA-synthesizing stage were individualized using immunocytochemical detection of BrdU, with BrdU (3 mg/ml; Sigma; St Louis, USA) being added to the culture medium for the last 3 h of incubation. Then, cells attached to coverslips were fixed in 4% formaldehyde and permeabilized with 0.5% Triton X-100. Non-specific immunoreactivity was blocked with 1% PBS–BSA. Cells were then incubated overnight with monoclonal antibody to BrdU (Amersham; Buckinghamshire, UK) at 4 °C. Then, cells were incubated in biotinylated anti-mouse IgG, diluted 1:100, for 30 min and incubated in ABC complex. Immunoreactivity of BrdU was visualized using DAB as the chromogen. Controls were also performed by applying the same protocols, but omitting BrdU or the BrdU antibody.

A total of 1000 immunoreactive cells were examined by light

microscopy in randomly chosen fields on each glass slide. Three slides were analyzed for each experimental group.

2.12. Immunoprecipitation

Protein extracts of pituitary glands from normal and hyperplastic/adenomatous pituitaries (750 μg) were obtained according to previous protocols (Petiti et al., 2008) and these were subjected to immunoprecipitation using specific mouse antiserum against p21 (5 μl). The immune complexes were adsorbed and precipitated using Protein G-Sepharose beads (Sigma–Aldrich), washed 3 times with lysis buffer and denatured by boiling for 5 min in the sample buffer.

2.13. Western blot analysis

Proteins were prepared from pituitary tissues and cultured cells, using RIPA buffer containing a cocktail of enzyme inhibitors 2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. This was followed by homogenization or scraping, and the lysates were transferred to a centrifuge tube placed on ice. After 30 min the lysates were centrifuged at 15,000 *g* for 20 min at 4 °C to pellet the insoluble material, and the supernatants were withdrawn and stored in aliquots frozen at −20 °C.

In order to obtain cytosolic and nuclear fractions, the pituitary cells were harvested by scraping with hypotonic Hepes buffer (10 mM HEPES, 5 mM $MgCl_2$, and 40 mM KCl) containing 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. After 30 min incubation on ice, cell homogenates were centrifuged at 5000 rpm for 45 min at 4 °C. The supernatants collected corresponded to the cytosolic fraction. The pellet was resuspended in Hypertonic Hepes buffer (10 mM HEPES, 5 mM $MgCl_2$, 40 mM KCl and 1.5 mM NaCl), followed by brief sonication, and cold centrifugation at 5000 rpm for 10 min. The supernatants collected corresponded to the nuclear fraction.

The protein concentration was determined by the Bradford assay using BSA as a standard and soluble proteins were separated by electrophoresis in 12% SDS-PAGE gels. To estimate the corresponding molecular weights, full range rainbow molecular weight marker was used (Amersham-Life Science; Bucks, England). Proteins were transferred to nitrocellulose membranes (Amersham International) and incubated in 5% non-fat milk in PBS-0.05% Tween solution, which was followed by incubation with the primary antibodies for 12 h at 4 °C. After washing in PBS-0.05% Tween, blots were incubated for 1 h at room temperature with a peroxidase-conjugated (HRP) secondary antibody (Jackson; West Grove, PA, USA, 1:5000).

After further washes, the HRP-coupled secondary antibody was revealed with ECL western blot detection reagents (Amersham; Bucks, UK). Emitted light was captured on Hyperfilm (Amersham; Bucks, UK).

2.14. Electron microscopy immuno-labeling

The subcellular localization of ERβ and p21 was determined by applying a double-labeling post-embedding protocol, which allowed the simultaneous localization of two antigens in the same grid with two gold complexes of different gold particle sizes (De Paul et al., 2012). Briefly, pituitary cells were fixed in a mixture of 4% formaldehyde, 1.5% glutaraldehyde and 0.1 M cacodylate buffer, pH 7.3, at room temperature, with osmiun fixation being omitted. After dehydration and embedding in LR White (London Resin, UK), thin sections were cut using a JEOL ultramicrotome with a diamond knife.

One face of the grids was labeled for ERβ overnight at 4 °C. Then, after washing, sections were incubated with anti-goat secondary antibody conjugated to 15 nm colloidal gold particles (1:50; Electron Microscopy Sciences; Hatfield, USA). The other face of the grids was stained for p21 during 1 h, and then revealed using secondary antibodies conjugated to 5 nm colloidal gold particles (1:50; Sigma–Aldrich, St. Louis, MO). To validate the specificity of the immunostaining, the following controls were performed: (1) replacement of primary antiserum with 1% BSA in PBS; and (2) replacement of primary antiserum with diluted preimmune serum followed by the secondary antibody. Then, sections were stained with an aqueous uranyl acetate saturate solution, examined in a Zeiss LEO 906-E electron microscope, and photographed with a megaview III camera.

2.15. Statistical analysis

Each experiment was repeated in 3 independent studies (performed on different cell preparations). Images of representative experiments are shown. Differences between treatments were determined using an analysis of variance with Tukey post-test. The comparisons with control groups were performed using the Dunnet test, and the comparisons between GH3β− and GH3β+ models were carried out with a two-way analysis of variance (2 × 4). The results are shown as the means ± SEM, and the significance levels were chosen at $P < 0.05$.

3. Results

3.1. The expression of ERβ decreases during the hyperplastic/adenomatous process induced by chronic E2 stimulation

The size of pituitary glands increased directly with time of estrogen administration (Fig. 1A). The glandular weight was of 12.13 ± 0.61 mg in controls (C), 34.93 ± 1.62 mg at 20 days (20 d), 65.17 ± 4.01 mg at 40 days (40 d) and 79.73 ± 2.89 mg at 60 days (60 d) of E2 treatment (Fig. 1B). By high resolution light microscopy, pituitary sections from 60 days of estrogen treatment revealed some endocrine cells with an expanded cytoplasm, evidence of cellular hypertrophy (Fig. 1C). The pituitaries embedded in paraffin and immunolabelled for PRL showed an increase in the lactotrophs (Fig. 1D). Both features are typical characteristic of hyperplastic/adenomatous process induced by chronic E2 stimulation.

The cell cycle progression in the development of the hyperplastic/adenomatous process was analyzed determining the percentage of the pituitary cell population found in the G1/G0 phase and in the proliferative fraction (S + G2/M) by flow cytometry. As shown in Fig. 2, 1.57 ± 0.1% of control pituitary population was found in the proliferative fraction, with this percentage being doubled in hyperplastic glands at 20 days (3.45 ± 0.3%) and maintained at 40 and 60 days (2.86 ± 0.3% and 2.43 ± 0.3%).

In order to analyze the expression and localization of ERβ during pituitary tumor development, we used confocal and transmission electron microscopy, flow cytometry and western blot. Specific staining for ERβ was detected in the cytoplasm, where a punctuated fluorescence signal was obtained, in addition to a slight immunolabelling in the nucleus. Interestingly, during tumoral development, the expression of ERβ showed a gradual decrease, with a very low expression being observed in the cytoplasm of hyperplasic/adenomatous cells at 60 days of E2 stimulation (Fig. 3).

To investigate ERβ specifically in lactotrophs (the main cell subtype that proliferates in this model), a double immunocytochemistry for PRL and ERβ was carried out. The PRL cells revealed expression of ERβ, which was localized in the cytoplasm and showed a remarkable decline in its expression during the development of the hyperplastic/adenomatous model (Fig. 3).

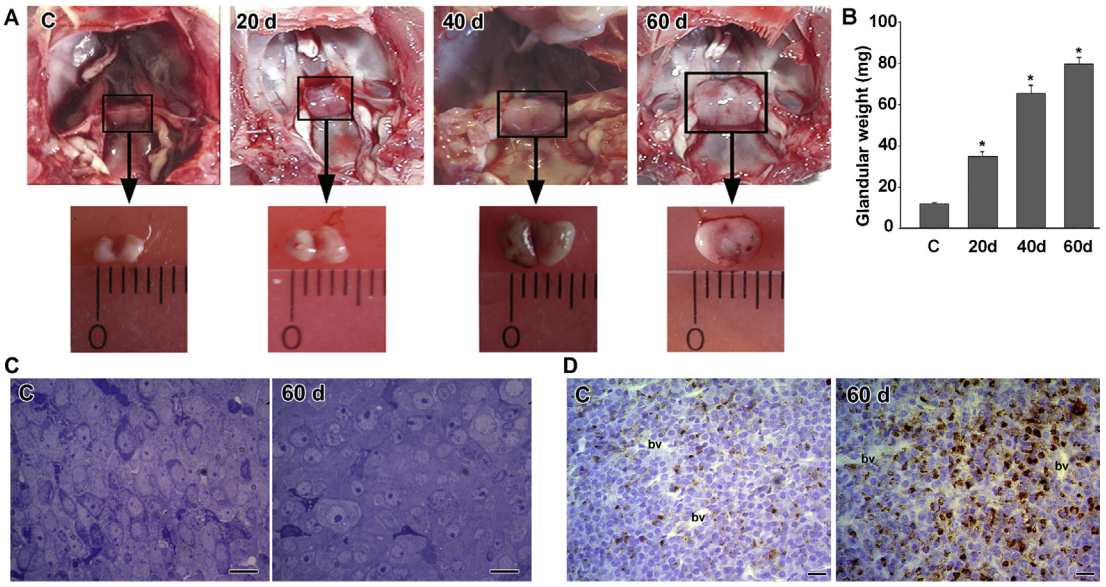

Fig. 1. Hyperplastic/adenomatous pituitary process induced by estradiol. A. Macroscopic observations *in situ* of pituitary glands from control and estrogenized rats at 20, 40 and 60 days. The pituitary gland sizes are depicted by a square. B. The glandular weight was significantly increased under E2 treatment for 20, 40 and 60 days. $^{*}P < 0.01$ vs C (Dunnet's test). C. High resolution light microscopy pituitary sections revealing cellular hypertrophy of some endocrine cells after 60 days of estrogen treatment. *Bar* = 20 μm. D. Sections of the pituitaries embedded in paraffin and immunolabelled for PRL. bv = blood vessel. *Bar* = 20 μm.

The expression of this ER subtype quantified by flow cytometry showed that 20 ± 1.6% of normal adenohypophyseal cells expressed ERβ, with this protein being reduced in the hyperplastic/adenomatous pituitary: at 20 days the ERβ+ population was 10.7 ± 2.2%, while after 40 and 60 days of treatment an almost complete loss in the ERβ expression was observed (40d: 1 ± 0.6%; 60d: 2 ± 0.6%) (Fig. 4A).

The western blot analysis from controls and estrogenized animals at 20, 40 and 60 days showed a band at ≈55 kDa, corresponding to full-length ERβ. This protein showed a significant reduction in its levels during the development of the hyperplastic/adenomatous pituitary process (Fig. 4B).

For the purpose of obtaining the index ERα/β, the expression of ERα was determined. By flow cytometry, the ERα+ population was 68.9 ± 3.64% in controls, in hyperplastic/adenomatous glands at 20 days this population was 84.03 ± 2.03%; at 40 days it was 91.05 ± 0.32%; and at 60 days it was 90.9 ± 5.65% (Fig. 4C). The ERα protein expression detected by western blot was significantly increased at 40 and 60 days of estradiol treatment with respect to the control model (Fig. 4D).

By flow cytometry the ERα/β ratio was 4.73 ± 0.31 in controls, 7.51 ± 0.62 at 20 days, 63.67 ± 4.23 at 40 days and 51.75 ± 10.32 in glands at 60 days (Fig. 4E). The ERα/β ratio by western blot was 1.25 ± 0.69 in controls, 1.46 ± 0.19 at 20 days, 2.06 ± 0.26 at 40 days and 3.99 ± 0.68 in glands at 60 days (Fig. 4F). All these data showed that the ERα/β ratio increased during the hyperplastic/adenomatous process, mainly due to decreased ERβ expression.

The subcellular distribution of ERβ was determined by electron microscopy using an immunolabelling with IgG-colloidal gold technique. All secretory cell types were observed, being identifiable by their secretory granules, which constitutes a distinctive feature. The lactotrophs were recognized by their irregular, large and

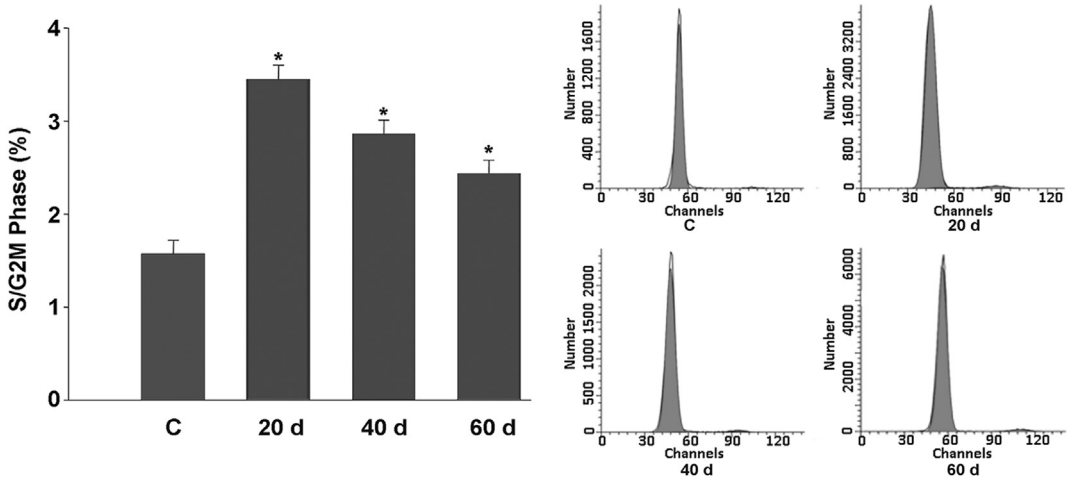

Fig. 2. Cell cycle analysis during pituitary tumoral development. The percentage of the pituitary cell population found in the proliferative fraction (S + G2/M) was significantly increased in hyperplastic/adenomatous glands at 20, 40 and 60 days compared to control. $^{*}P < 0.01$ vs C (Dunnet's test).

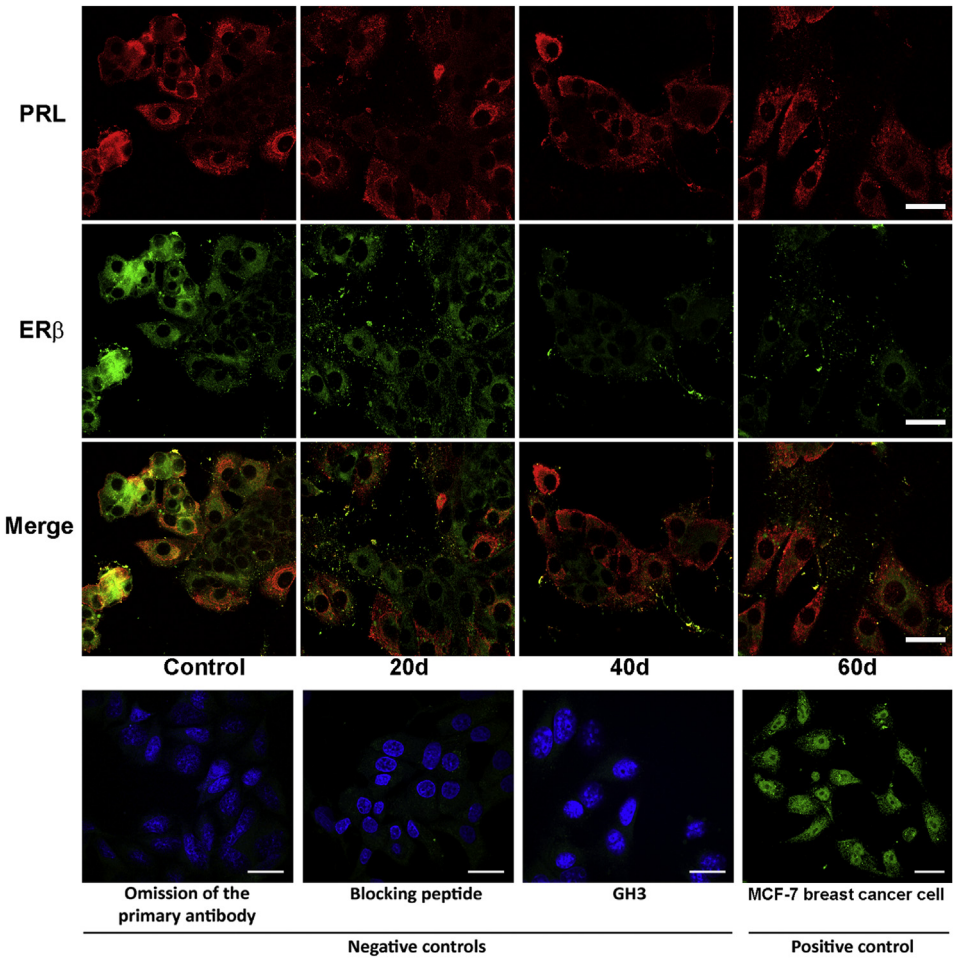

Fig. 3. Double immunostaining for ERβ and PRL in the hyperplastic/adenomatous pituitary process. Expression of ERβ occurred in lactotroph cells, which decreased in the hyperplasic/adenomatous process. Negative controls: replacing primary antibodies with 1% PBS–BSA (omission of the primary antibody), absorption control (blocking peptide), and cells that did not express ERβ (GH3). Positive control: MCF-7 breast cancer cells. *Bar* = 20 μm.

polymorphic secretory granules of sizes ranging between 500 and 900 nm distributed in the cytoplasm. In normal cells, the subcellular localization of the ERβ subtype was mainly cytoplasmatic, with the gold particles appearing to be attached to the cytosol and the rough endoplasmic reticulum, with a few colloidal gold particles being observed in the nucleus (Fig. 5A–B). This distribution pattern was maintained in cells from hyperplastic/adenomatous pituitary at 20 days. No immunostained pituitary cells were observed in hyperplastic/adenomatous glands at 40 or 60 days (Fig. 5C). Immunocytochemical controls evaluated the specificity of the primary antiserum, and no immunolabelling was found after the omission of the primary antibody.

Finally, ERα/β co-expression during the development of the hyperplastic/adenomatous process induced by estradiol was determined by confocal microscopy using a double immunofluorescence staining. As shown Fig. 6, high levels of ERα in the nucleus and cytoplasm were detected in most of the cells. ERβ was also detected, at a minor proportion, with a higher intensity in the cytoplasm than in the nucleus. The morphometric analysis revealed that in controls 16.97 ± 2.3% cells were ERβ+, with co-expression of ERα/β in 15.61 ± 2.42% of the cells. In pituitary glands at 20 days, the percentage significantly decreased for ERβ (9.31 ± 0.34%), with 8.27 ± 0.75% of cells co-expressing ERα/β. For both conditions, almost all ERβ+ cells expressed ERα (91.48 ± 2.64% in controls and 88.52 ± 5.6% at 20 days). In pituitary glands at 40 and 60 days, ERβ expression was not observed.

3.2. The over-expression of ERβ affects the proliferative index and cyclin D1 and ERα expression

The cell proliferation was analyzed in normal and hyperplastic/adenomatous pituitary glands after 40 days of chronic E2 stimulation and also in GH3 cells (GH3β− and GH3β+) which contained different levels of ERβ expression, and therefore different ERα/β ratios. Fig. 7 summarizes the effect of 10 nM of E2 or specific agonists of ERα (PPT) or ERβ (DPN) after 72 h of exposition.

Normal pituitary cells exhibited a similar rate of BrdU incorporation in all models (Fig. 7A). In hyperplastic pituitary cells, E2 and the ERα agonist were able to increase the BrdU-labeled cells, with E2 inducing the highest levels. The treatment with ERβ agonist induced similar values to control (Fig. 7B).

In GH3β− cells, which expressed only ERα, the E2 treatment increased the percentage of BrdU-labeled cells by about 34%, but the ERα or β agonists were unable to induce any changes in the proliferative index, revealing similar values to the control (Fig. 7C). Considering that E2 was able to increase the pituitary cell proliferation only in cells lacking ERβ expression, we suggest that under the present experimental conditions, the absence of ERβ may be implicated in the positive effect of E2 on the pituitary cell proliferation, supporting the idea of the existence of a relationship

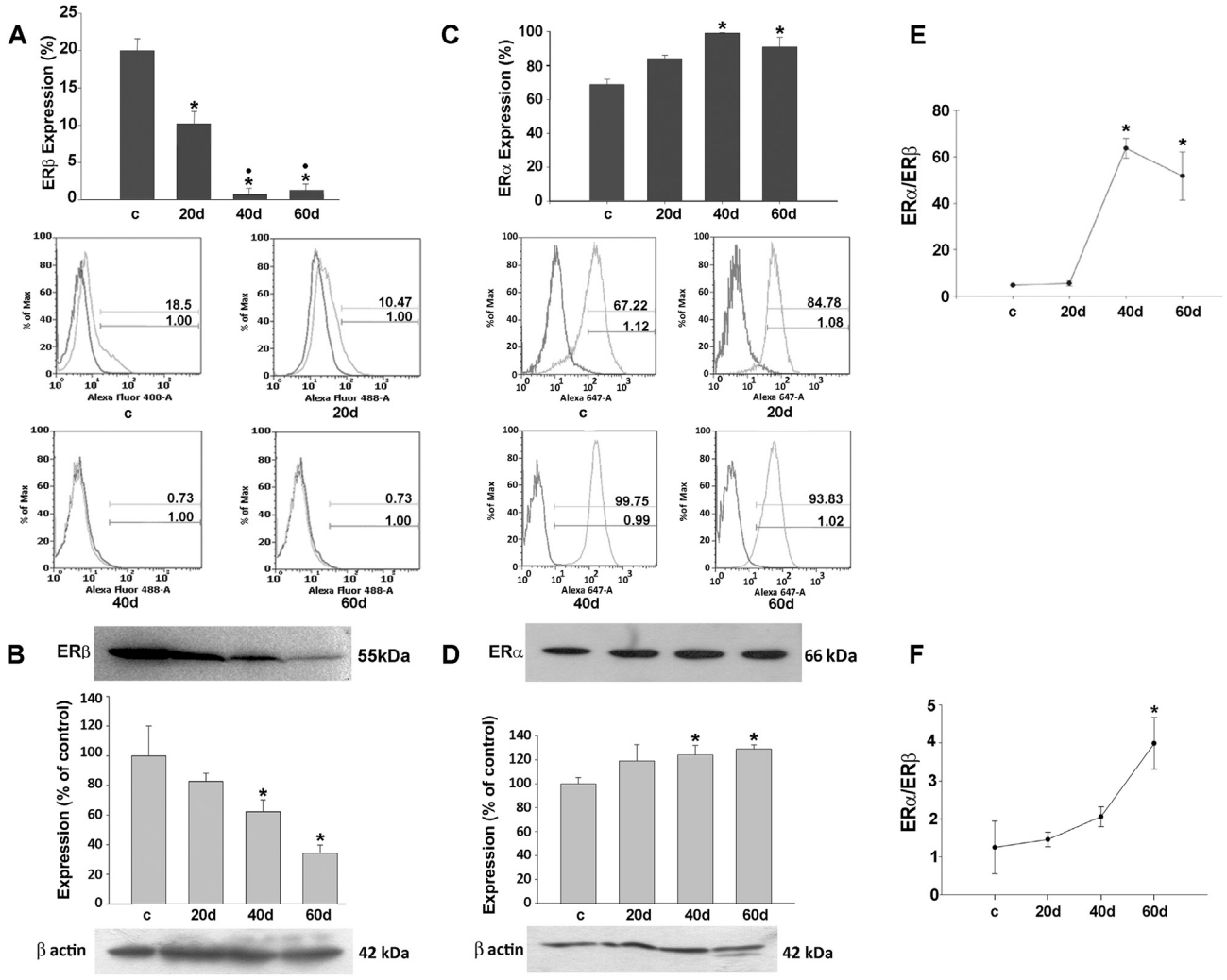

Fig. 4. Expression of ERα and β in the hyperplastic/adenomatous pituitary process. A. ERβ quantification by flow cytometry showed a reduction in its expression during the tumoral process. (10^5 events for each experimental condition, $^*P < 0.01$ vs control, ●$P < 0.01$ vs 20 days, Tukey test). B. The ERβ protein expression detected by western blot decreased in the hyperplastic/adenomatous pituitary process. $^*P < 0.01$ vs control (Dunnet's test). C. ERα expression quantified by flow cytometry showed a significant increase in the number of cells that express ERα starting at 40 days. (10^5 events for each experimental condition, $^*P < 0.05$ vs Control, Dunnet's test). D. The ERα protein expression increased at 40 and 60 days of estradiol treatment. $^*P < 0.05$ vs control (Dunnet's test). The ERα/β ratio obtained by flow cytometry (E) or western blot (F) increased during the hyperplastic/adenomatous pituitary process. $^*P < 0.01$ vs control (Dunnet's test). β-actin expression was used as a loading control. Representative images of three independent experiments are presented. The protein expression is represented as a percentage relative to the control (control data were set to 100%).

between pituitary cell proliferation and ERβ expression (and the ERα/β index).

To try to confirm this finding, GH3 cells were transfected selecting an enriched stable GH3 cell line for the ERβ expression. By flow cytometry, the ERβ expression was found to be 24.87 ± 2.13% in the enriched stable GH3 cell line (GH3β+) and 0.94 ± 0.17% in GH3 cells (GH3β-). Therefore, as shown in Fig. 7C, the ERβ over-expression in GH3 induced a significant inhibition of mitogenic activity in comparison with GH3β−. The BrdU index was reduced by about 40% in control GH3β+ cells with respect to control GH3β− cells, indicating that this was an independent ligand effect. These values did not change after stimulation with E2 or PPT.

Interestingly, the increase in the mitogenic activity induced by E2 in GH3β− cells was reversed when GH3 over-expressed ERβ, thus confirming that the variations in the ERα/β ratio affected the proliferative effect induced by E2, and suggesting an inhibitory role of ERβ in the anterior pituitary cell proliferation.

Next, in order to analyze further the effect of ERβ on anterior pituitary cell growth, we determined the expression of cyclin D1 and Cdk4 in GH3β− and GH3β+ cells. The over-expression of ERβ induced a reduction in cyclin D1 levels, with the treatment with 10 nM of E2 or PPT showing similar levels to that of the control. However, the stimulus with the specific ERβ agonist (DPN) induced an inhibition of cyclin D1 expression (Fig. 8A). In contrast, the Cdk4 levels were similar in GH3β− and GH3β+ cells, without differences resulting from the addition of E2, PPT or DPN (Fig. 8A).

The ERα and ERβ expression levels in GH3β− and GH3β+ cells treated with E2, PPT or DPN were determined. In GH3β+ cells, the basal ERα expression was significantly lower than those found in GH3β− cells. The ERα expression under E2, PPT or DPN stimuli was similar to control in both GH3β− and GH3β+ cells (Fig. 8B). As mentioned above, as GH3 cells did not express ERβ, the changes in ERβ expression were determined in GH3β+ cells and showed similar expression levels for all experimental conditions (Fig. 8C).

3.3. ERβ activation by its agonist DPN changes the subcellular localization of p21

By western blot, a noticeable increase in p21 expression was observed during the tumoral progress, which reached its highest

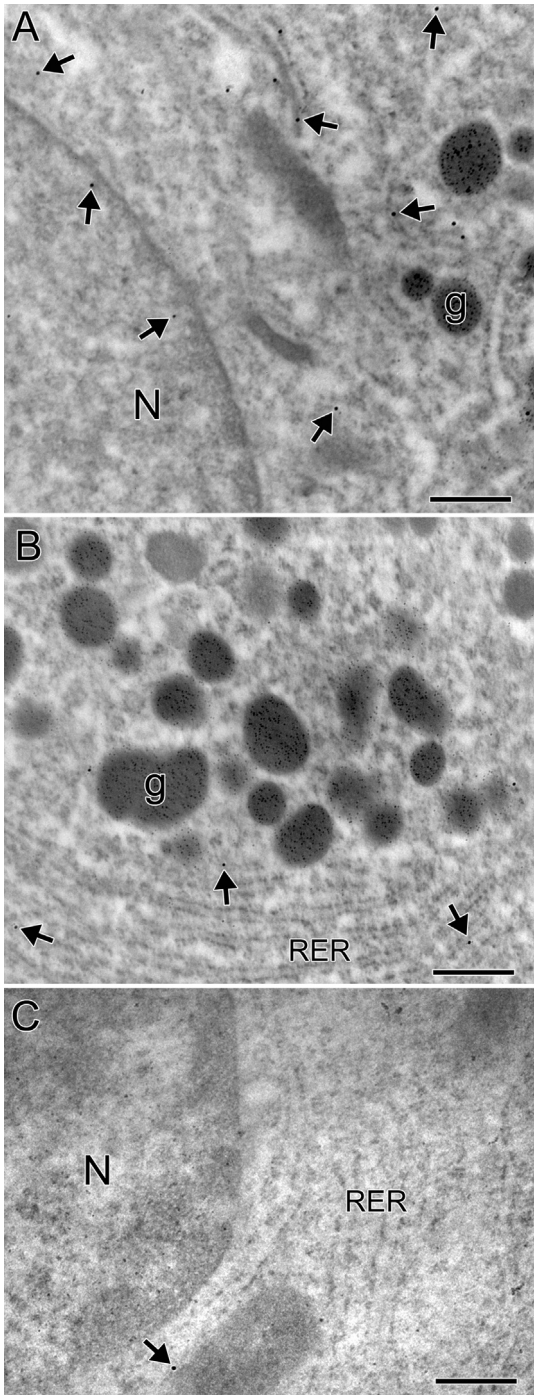

Fig. 5. Immuno-electron-microscopy for ERβ. A-B. Normal lactotroph cells with gold particles of 15 nm indicating the presence of ERβ (arrows) in free cytosol, rough endoplasmic reticulum (RER) and occasionally in the nucleus (N). Secretory granules (g) were specifically identified by immunocytochemistry for PRL (5 nm gold particles). C. Anterior pituitary cell from hyperplastic/adenomatous glands after 60 days of estrogen stimulation showing scarce gold particles expressing ERβ label. Representative images of three independent experiments are presented. *Bar* = 1 μm.

level in cellular extracts from adenomatous/hyperplastic glands at 40 and 60 days (Fig. 9A). This increase was corroborated by confocal microscopy, revealing an intense immunofluorescence signal in tumors of 40 days and cytoplasmatic localization of this protein (Fig. 9B). However, neither GH3β− nor GH3β+ cells expressed p21 (Fig. 9C).

In primary pituitary cells culture a weak p21 expression was shown in the nuclear fraction of control or when treated with E2. In contrast, DPN stimuli were able to induce a notable increase in the p21 nuclear expression with a corresponding decrease in the cytoplasmatic fraction (Fig. 9D). This result was reinforced by confocal microscopy, where p21 was observed in the cytoplasm in controls and under E2 treatment, with DPN being able to increase its nuclear expression (Fig. 9E).

Finally, hyperplastic/adenomatous pituitary glands after 20 days of estrogenization and normal pituitary cells treated with DPN were immunolabelled for p21 or ERβ using secondary antibodies conjugated to colloidal gold particles of 5 nm or 15 nm respectively. As shown in Fig. 10A–D, both proteins were found alone or associated, with colloidal gold-particles of 5 nm and 15 nm visualized at a distance lower than 5 nm. As negative control, the primary antibodies for p21 and ERβ were omitted and no immunolabelling was found.

To validate the results obtained by immuno-electron-microscopy, endogenous p21 was immunoprecipitated from pituitary extracts of controls and pituitary glands treated with E2 for 20 days. As shown in Fig. 10E, both proteins, p21 and ERβ, were showed, suggesting a physical interaction.

4. Discussion

Estrogen is an important regulator of many cellular processes mediated by specific ER. The discovery of a second ER subtype, called β, has caused a paradigm shift in the understanding of the estrogen action and has focused research on evaluating the biological significance of the existence of different ER subtypes in the same tissue or even in the same cell.

In the anterior pituitary gland, estrogen regulates cell proliferation (Spady et al., 1999; Heaney et al., 2002). The chronic stimulation in rats induces pituitary tumors, mainly due to a hyperplasic response of lactotrophs (Toledano et al., 2012; Mukdsi et al., 2004). In this investigation, we observed a loss of ERβ expression and an increase in the ERα/β ratio after chronic E2 treatment *in vivo*, resulting in an increased weight and size of rat pituitary glands and a greater number of cells in the proliferative fraction (S + G2/M). The loss of ERβ expression in the tumoral process shown in this study reflects that chronic E2 stimulus inhibits this ER subtype and may indicate that the imbalance in the ER subtypes, with a subsequent increase in the ERα/β ratio, results in the deregulation of pituitary cell growth, thus contributing to an uncontrolled pituitary cell proliferation. However, there have been no investigations related to this issue in the anterior pituitary gland. In other tissues, the loss of ERβ expression is a common stage in estrogen-dependent tumor progression (Bardin et al., 2004). This ERβ expression decline during breast tumorigenesis (Roger et al., 2001) and this protein is progressively lost in hyperplasia and neoplastic lesions in prostate (Horvath et al., 2001) ovarian (Halon et al., 2011) and colon cancer (Campbell-Thompson et al., 2001).

Contradictory results have been reported concerning ERβ expression and its regulation by estrogen in the pituitary gland. The ovariectomy increases and estrogen replacement decreases the ERβ mRNA expression in this gland (Tena-Sempere et al., 2004). In contrast, estrogen treatment up-regulated the ERβ mRNA levels in pituitary tumor cells, in a time- and dose-dependent manner (Mitchner et al., 1999). Considering that the pituitary responses to E2 are ultimately dependent on the expression levels of the ER protein, in this investigation we analyzed the protein expression of ERβ, revealing that estrogen treatment decreased its expression. This is not in agreement with a previous report showing that the number of pituitary cells that expressed this ER did not significantly change during the estrous cycle (Gonzalez et al., 2008), but concurs

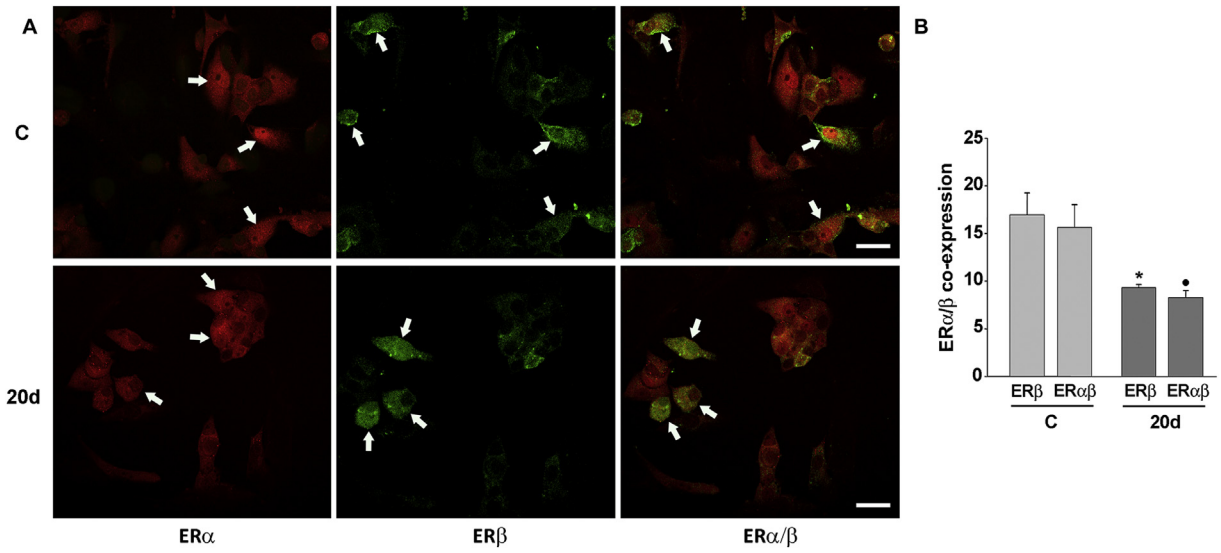

Fig. 6. ERα and ERβ co-expression. A. Representative images showing the ERβ and ERα immunolocalization (arrows) in pituitary cells from controls and hyperplastic/adenomatous pituitary glands of 20 days. *Bar* = 20 μm. B. Quantitative analysis of ERβ expression and ERα/ERβ co-expression in pituitary cells. *$P < 0.05$ vs ERβ control, ●$P < 0.05$ vs ERα/β control, Tukey test).

with others that demonstrated that ERβ mRNA levels were the lowest on the morning of proestrus when *in vivo* steroid levels were at their highest, with E2 treatment of ovariectomized animals suppressing ERβ mRNA expression (Schreihofer et al., 2000). Moreover, the ERβ expression in the female rat pituitary decreased after puberty, again further supporting an effect of increased circulating steroids on ERβ levels (Wilson et al., 1998). Our results also revealed an increase in ERα during pituitary tumoral development. This could be due to the increase in the number of lactotroph cells, which is characteristic of estrogen-induced pituitary tumors, which have a high expression of ERα.

Taking the above findings into account, we suggest that there might be a relation between pituitary cell proliferation and the expression of ERβ (and the ERα/β index), and hypothesize that an adequate maintenance of ERα/β levels could be required for the homeostasis of pituitary cell growth.

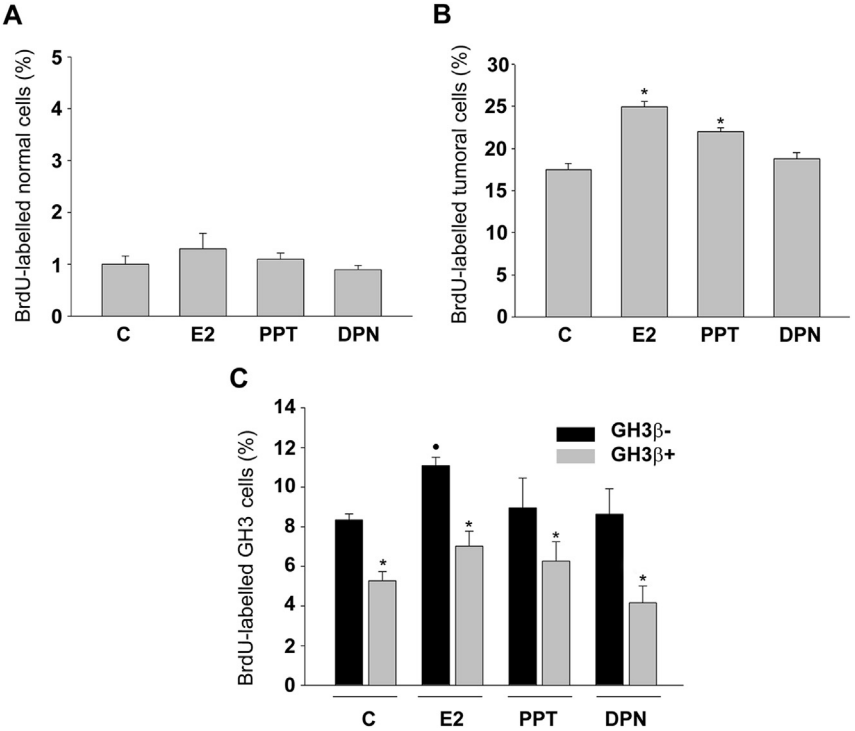

Fig. 7. Effects of E2, PPT or DPN on pituitary cell proliferation. A. E2, PPT or DPN were unable to increase the number of BrdU-labeled normal pituitary cells. B. In tumoral pituitary cells, E2 and PPT increased the percentage of BrdU-labeled cells. *$P < 0.01$ vs Control (Dunnet's test). C. In GH3β+, the BrdU-labeled cells decreased compared to GH3β− cells. A two-way ANOVA and the Dunnet test were used. (No interaction between models and treatments were found P = 0.4372), significant differences between treatments were found, *$P < 0.05$ vs GH3β−, ●$P < 0.05$ vs control GH3β−.

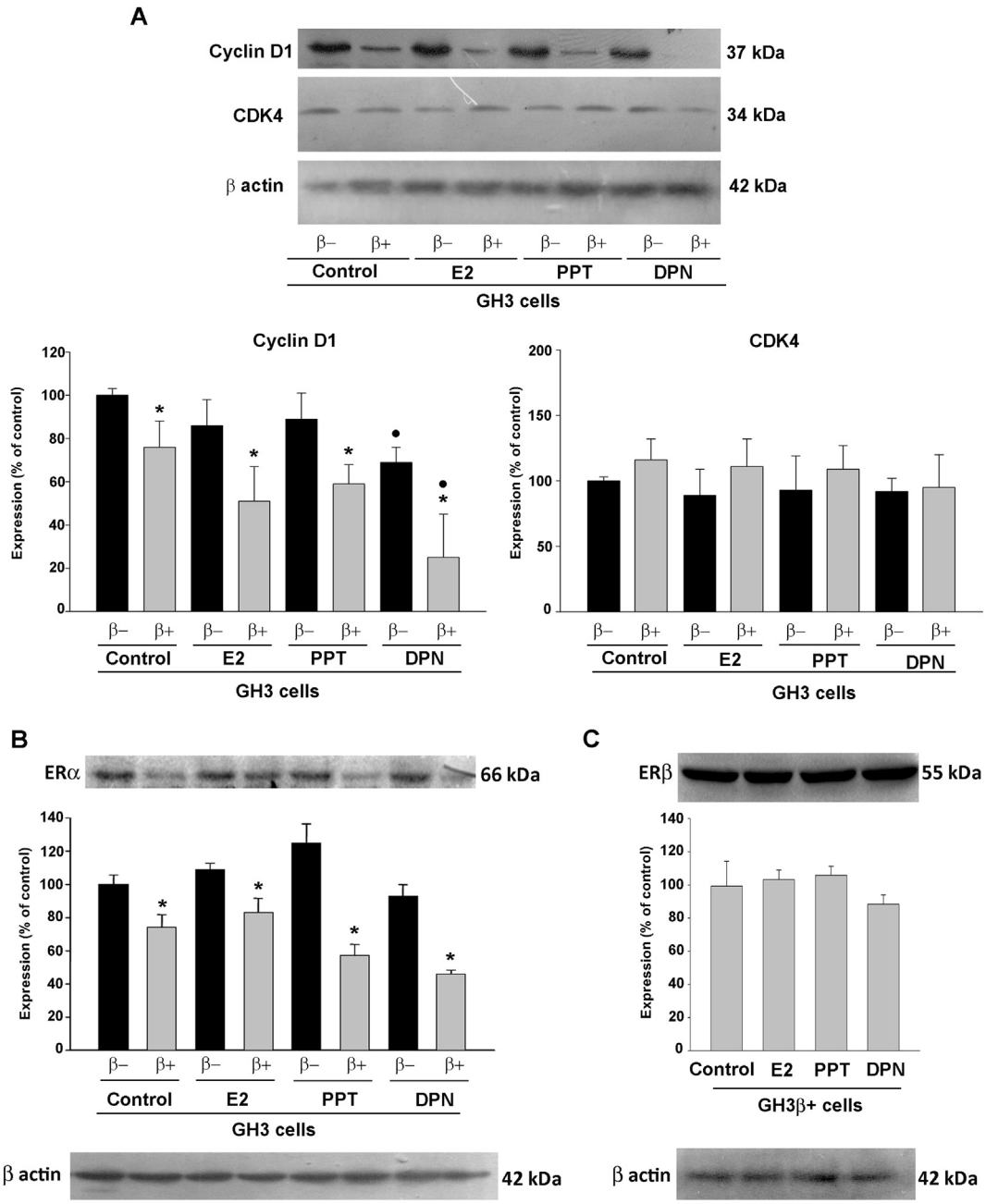

Fig. 8. Western blotting expression of cycline D1, Cdk4 and ERα and β in GH3β– and GH3β+ cells. A. Cyclin D1 decreased with the over-expression of ERβ. A two-way ANOVA and the Dunnet test were used. (No interaction between models and treatments were found P = 0.8185), significant differences between treatments were found, *$P < 0.05$ vs GH3β–, ●$P < 0.05$ vs control. Cdk4 remained unchanged. B. The over-expression of ERβ inhibited ERα expression, but E2, PPT or DPN were unable to modify the expression of this ER subtype. *$P < 0.01$ vs GH3β–. A two-way ANOVA and the Dunnet test were used. C. The ERβ expression in GH3β+ cells was similar in all models. Representative images of three independent experiments are presented. The β-actin expression was used as a loading control. The protein expression is represented as a percentage relative to the control β- (control β-data were set to 100%).

The co-expression of both ER mRNA forms in some pituitary cells has been previously described (Mitchner et al., 1998; Wilson et al., 1998), showing that in the adult gland the ERβ protein is co-expressed with ERα (Nishihara et al., 2000). An interesting finding in this study was that the almost all cells expressing ERβ also expressed ERα, showing co-expression of both proteins in the same pituitary cell in basal conditions as well as in hyperplastic/adenomatous glands at 20 days. This result may indicate an integrated mechanism by which the two receptors may act by mediating the E2 effects on pituitary cell proliferation.

In order to clarify the specific role of ERβ in the E2 proliferative effect, the differential mitogenic activity of normal, hyperplastic and tumoral pituitary cells in response to E2 was evaluated. This hormone was able to increase the pituitary cell proliferation only in cells with a slight amount or absence of ERβ, consequently with a high ERα/β ratio. The reasons that different cells can respond to the same hormone in a different manner may be due to the different expression patterns of ERα and ERβ (McDonnell and Norris, 2002). In the anterior pituitary, it has typically been shown that ERα mediates the major proliferative effects of estrogen on pituitary, with

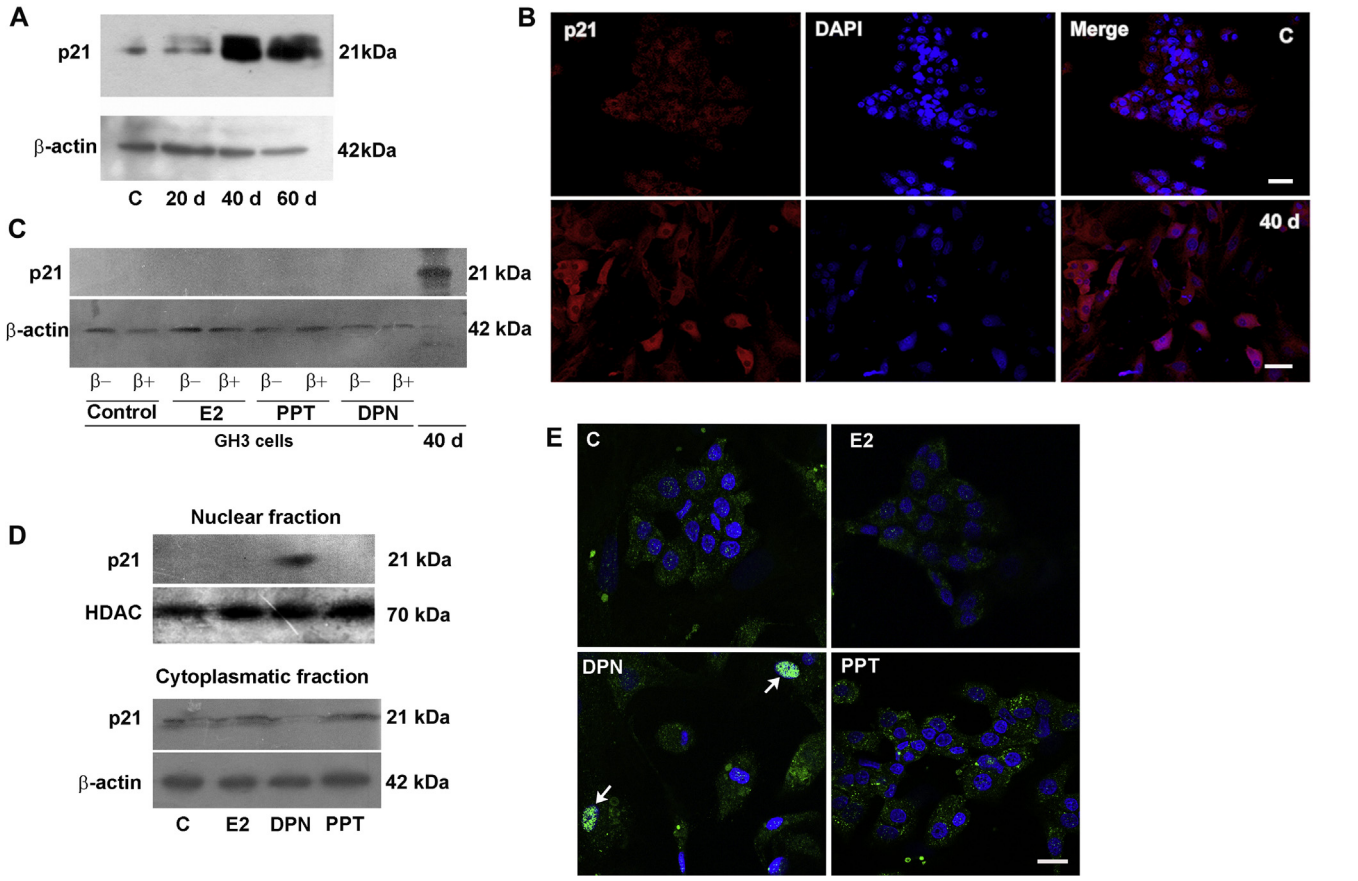

Fig. 9. Expression of p21 in normal, GH3β−, GH3β+ and tumoral pituitary cells. The western blotting expression of p21 increased in pituitary tumoral glands starting at 40 days of stimulation (A), with this protein being cytoplasmatically localized. *Bar* = 20 μm. (B). Neither GH3β− nor GH3β+ cells, expressed p21. Extracts from pituitary tumoral glands from 40 days was used as positive control (40 d) (C). DPN stimuli induced an increase in p21 nuclear expression by western blot (D) and confocal microscopy (arrows). *Bar* = 20 μm. (E). The β-actin expression was used as a loading control. Representative images of three independent experiments are presented.

previous results of our research group demonstrating that ERα modulates lactotroph cell proliferation (Sosa et al., 2013). However, it was not known whether ERβ could cause a proliferative effect in the adenohypophysis. Here, the GH3β+ cells showed a significant reduction of mitogenic activity, suggesting an inhibitory role of this ER subtype on pituitary cell proliferation. For this inhibitory action to occur, the activation of ERβ by E2 or its agonist DPN was not necessary, suggesting that ERβ over-expression is sufficient to inhibit pituitary cell proliferation in a ligand-independent manner. Indeed, it has been previously established that steroid receptors can be activated in the absence of their ligands (Dey et al., 2013). Our observation is in agreement with findings in other tissues, where the inhibition of the cell proliferation induced by ERβ was ligand-independent (Lazennec et al., 2001; Nassa et al., 2014). Moreover, the over-expression of ERβ in the colon adenocarcinoma cell line has an anti-proliferative effect, mostly due to ligand-independent activation of the receptor (Martineti et al., 2005).

Bearing in mind the above findings about ERβ and considering that the estrogen target sites are related to the final effects in the different tissues, we analyzed the subcellular distribution of ERβ by immuno-electron microscopy. Although it is known that ER are members of a nuclear receptors family, our results showed ERβ to be localized in the cytoplasm of lactotroph cells, specifically in the free cytosol and rough endoplasmatic reticulum and scarcely in the nucleus, which was unchanged after E2 stimulus, suggesting that E2 is unable to modify its subcellular localization. In general, ERβ localization reveals cell-type specificity, with ERβ immunoreactivity being localized at the cell nuclei within select regions of the brain (Mitra et al., 2003), as well as in glandular epithelium cells of the uterus and in granulosa cells in the ovary (Hiroi et al., 1999), which induce transcriptional activities through a classical genomic effect (Hiroi et al., 2013). However, several studies on different tissues have shown ERβ in the cytoplasm (Ivanova et al., 2009; De Stefano et al., 2011) failing to translocate to the nucleus in the presence of estrogen, and causing rapid activation of signaling pathways that modulate proliferation by the non-genomic action (Zhang et al., 2009). Gonzalez and co-workers (Gonzalez et al., 2008) described the presence of ERβ in lactotrophs being detected in the cytoplasm, inside the rough endoplasmic reticulum, in secretory vesicles or being free in the cytosol. Although the functional significance of the cytoplasmatic localization of ERβ remains elusive, we consider that the cytoplasmic staining for ERβ is biologically meaningful since it is known that hormone receptors also mediate effects through non-genomic pathways, which usually occur in the cytoplasm, and can lead to cytoplasmic alterations or ultimately to regulation of gene expression (Levin, 2005; Acconcia and Marino, 2003). Moreover, considering that the presence of ERβ in the nucleus was also found in our investigation, it is possible that there was a contribution of the classic genomic mechanisms in the ERβ effects on pituitary cells.

Pituitary cells are particularly sensitive to alterations of the cell cycle machinery. Our results showed that the over-expression of ERβ induced a decrease of cyclin D1 levels, with the specific ERβ agonist being able to induce a complete inhibition of this cyclin. These data support the results observed in the cell proliferation,

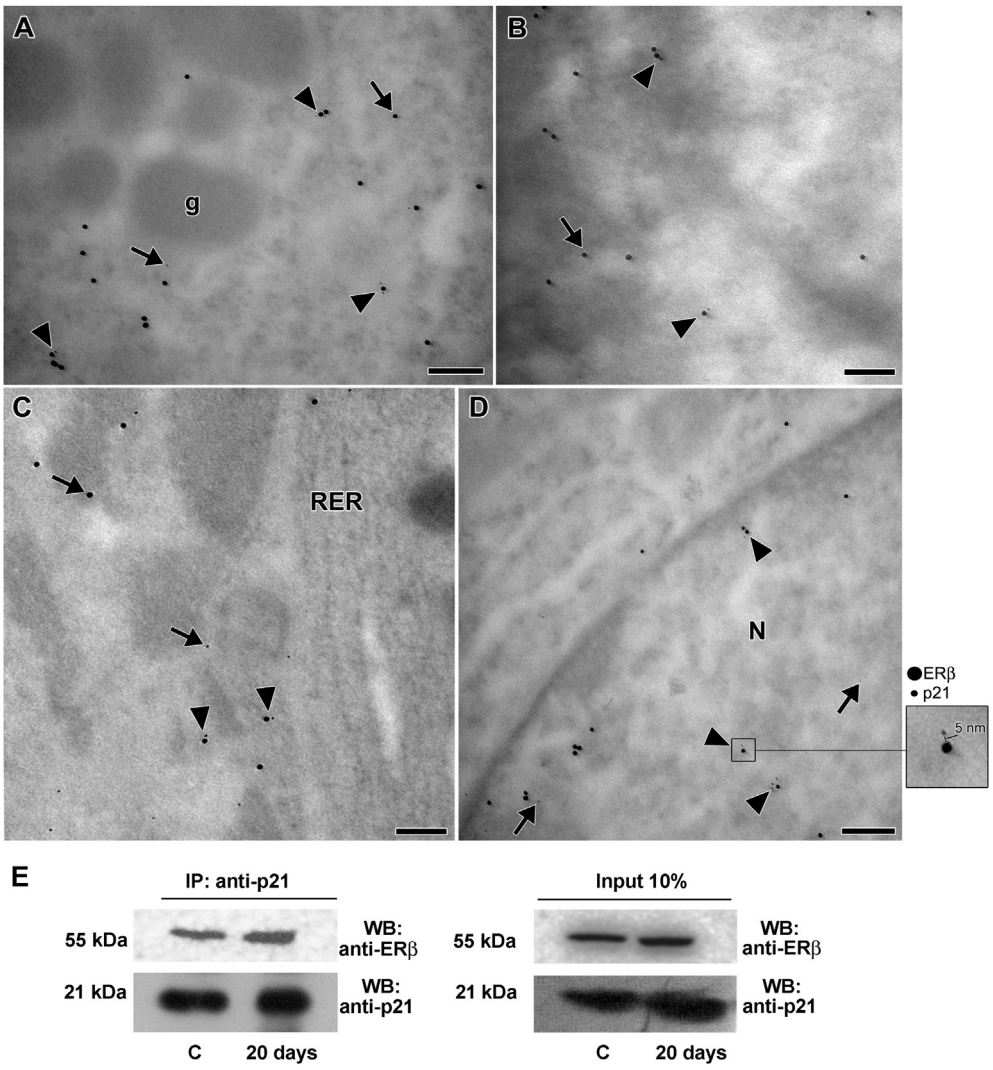

Fig. 10. ERβ and p21 expression. Representative images of immuno-electron-microscopy for ERβ (15 nm gold particles) and p21 (5 nm gold particles) from hyperplastic pituitary cells at 20 days (A–B) and normal pituitary cells treated with DPN (C–D). Both proteins were found alone (arrows) or in the close proximity (arrowheads) in the cytoplasm and in the nucleus (N). The square box in the inset shows the closeness of the two proteins. g: granules, RER: rough endoplasmic reticulum. *Bar* = 0.2 μm. E. Cell lysates were immunoprecipitated with Protein G-Sepharose in combination with the anti-p21 primary antibody. The immunoprecipitated fractions and the whole lysates were analyzed by western blotting using anti-ERβ and anti-p21 antibodies. One representative experiment is shown.

and suggest that the over-expression of ERβ inhibits cell proliferation, with the participation of cyclin D1 occurring in a ligand dependent or independent manner. It has previously been shown that ERα and β exert opposing actions in regulating cyclin D1 gene transcription (Strom et al., 2004; Liu et al., 2002). In fact, ERβ reduced cell proliferation by inhibiting the cyclin D1 gene (Strom et al., 2004; Paruthiyil et al., 2004). A negative correlation between ERβ and cyclin D1 expression was also observed in breast cancer (Bieche et al., 2001), epithelial ovarian cancer (Bossard et al., 2012) and in prostate cancer development (Nakamura et al., 2013), with cyclin D1 having been found to be over-expressed in most pituitary tumor types (Jordan et al., 2000; Turner et al., 2000).

Cdk4 is specifically required for hormonally regulated proliferation of somatotrophs and lactotrophs in postnatal pituitary glands (Jirawatnotai et al., 2004), and pituitary Cdk4 activity may be positively regulated by association with cyclin D1 (Toledano et al., 2012). Moreover, lactotrophs of Cdk4-deficient mice did not proliferate in response to estrogen administration. Our results showed that Cdk4 did not modify its expression by the over-expression of ERβ, which could be explained by considering that the CDK activity is modulated by fluctuations in the cellular concentration of CDK activators (cyclins) or inhibitors (CKI) (Coleman et al., 1997).

To expand the current knowledge about ERβ modulation of pituitary cell proliferation, we investigated whether ERβ affects ERα expression. Our results showed that the over-expression of ERβ in GH3 reduced the baseline ERα expression, with E2, PPT or DPN stimuli being unable to modify its expression. The over-expression of ERβ has a strong effect on ERα levels, thereby diminishing the ERα expression in epithelial ovarian cancer (Bossard et al., 2012), inhibiting the ERα mRNA and protein levels in endometrial stromal cells (Trukhacheva et al., 2009) and down-regulating ERα expression in the presence of high ERβ levels in MCF-7 cells (Chang et al., 2006). The lack of effect of E2 and ERα and β agonists on the ERα expression is consistent with a previous report which shows, in different pituitary cell lines, that ERα and ERβ endogenous protein levels were unchanged after E2 treatment (Avtanski et al., 2014). Also, estrogen stimulation did not alter the expression of ERα mRNA in pituitary tumor cells, but raised the expression of ERβ mRNA (Mitchner et al., 1999).

In our experimental model using GH3β+ cells, the ERβ protein

levels were similar after E2, PPT or DPN treatments. Indeed, endogenous ERβ is under the control of its own promoter, whereas for our GH3β+ cells, exogenous ERβ was controlled by a viral promoter, with this possibly being the reason for the discrepancy when comparing these cells with cells naturally expressing ERβ. Taking all these results into consideration, we suggest that in our model the level expression of ERβ impacted on the ERα expression in a ligand-independent manner, thus emphasizing the importance of the ERα/β radio in the regulation of the pituitary cell proliferation.

The CKI p21 is a decisive component of cell cycle control in pituitary homeostasis (Chesnokova et al., 2008), with E2 being a regulator of this protein (Mandal and Davie, 2010). In our study, pituitary p21 was up-regulated during the development of the pituitary hyperplastic process, coincident with an induced proliferation. Our results showed that p21 was localized in the cytoplasm of the tumoral pituitary glands, with similar results being reported in pituitary tumors from C57/BL6 mice induced by E2 administration (Toledano et al., 2012). In relation to this, we can hypothesise that, for our condition of experimental pituitary tumor, cytosolic p21 may maintain the high proliferative index detected after estrogen treatment, considering that this localization promotes cell survival and proliferation (Abbas and Dutta, 2009). However, in normal pituitary cells, ERβ stimulation with the specific agonist DPN induced an increase in p21 nuclear expression. The fact that nuclear p21 was increased in parallel with the decrease in the cytoplasmatic fraction observed in this work, leads us to suggest that ERβ activation induces nuclear translocation of p21, where it is known that p21 blocks S phase progression by inhibiting PCNA activity. It has been previously demonstrated that ERβ induces p21 expression and cell cycle arrest in colon (Martineti et al., 2005), breast (Paruthiyil et al., 2004) and prostate (Pravettoni et al., 2007). Also, a possible functional interaction between ERβ and p21 was observed in our models. This relationship has been described previously for ERα, thus providing evidence that unliganded ERα acts as a negative regulator of cell growth by physical interaction with p21 via its amino acids 184–283 (Maynadier et al., 2008). Considering that ERβ is highly homologous (95%) to ERα in this site (Kuiper et al., 1996), it is possible that ERβ interacts with p21 in the same position as ERα to induce its translocation into the cell nucleus, where it then blocks S phase progression, as a mechanism for inhibiting pituitary cell proliferation. However, from our results, we may not infer a direct physical interaction between the two proteins, and the presence of the bridging protein in the complex can not be excluded.

In summary, our results have shown that ERβ exerts an inhibitory role on pituitary cell proliferation, and that this effect may be partially due to the modulation of some key regulators of the cell cycle, such as cyclin D1 and p21, with inhibition of ERα expression. These data contribute significantly to the understanding of the ER effects in the proliferative control of pituitary gland, specifically related to the ERβ function in the E2 actions on this endocrine gland, where numerous and intriguing mechanisms are intertwined for maintaining tissue homeostasis.

Acknowledgments

We thank Ms. Elena Pereyra and Ms. Soledad Santa Cruz for their excellent technical assistance. We are also grateful to Anders Strom (University of Houston, USA) and Dimitra Mitsiou (Nacional Hellenic Research Fundation, Greece) for generously providing the plasmids. We also thank Patricia Kunda for the guidance and assistance for the stable transfections. We would also like to thank native speaker Dr. Paul Hobson for revising the English of the manuscript.

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Tecnicas (CONICET), (PIP Nº 464) Fondo para la Investigacion Cientifica y Tecnologica (FONCyT-ANPCyT) (PICT 98/11) and the Secretaria de Ciencia y Tecnologia de la Universidad Nacional de Cordoba (SECyT-UNC) (162/12).

References

Abbas, T., Dutta, A., 2009. p21 in cancer: intricate networks and multiple activities. Nat. Rev. Cancer 9, 400–414.
Acconcia, F., Marino, M., 2003. Synergism between genomic and non genomic estrogen action mechanisms. IUBMB Life 55, 145–150.
Avtanski, D., Novaira, H.J., Wu, S., Romero, C.J., Kineman, R., Luque, R.M., Wondisford, F., Radovick, S., 2014. Both estrogen receptor alpha and beta stimulate pituitary GH gene expression. Mol. Endocrinol. 28, 40–52.
Bardin, A., Boulle, N., Lazennec, G., Vignon, F., Pujol, P., 2004. Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. Endocr. Relat. Cancer 11, 537–551.
Bieche, I., Parfait, B., Laurendeau, I., Girault, I., Vidaud, M., Lidereau, R., 2001. Quantification of estrogen receptor alpha and beta expression in sporadic breast cancer. Oncogene 20, 8109–8115.
Bossard, C., Busson, M., Vindrieux, D., Gaudin, F., Machelon, V., Brigitte, M., Jacquard, C., Pillon, A., Balaguer, P., Balabanian, K., Lazennec, G., 2012. Potential role of estrogen receptor beta as a tumor suppressor of epithelial ovarian cancer. PLoS One 7, e44787.
Bulayeva, N.N., Wozniak, A.L., Lash, L.L., Watson, C.S., 2005. Mechanisms of membrane estrogen receptor-alpha-mediated rapid stimulation of Ca2+ levels and prolactin release in a pituitary cell line. Am. J. Physiol. Endocrinol. Metab. 288, E388–E397.
Campbell-Thompson, M., Lynch, I.J., Bhardwaj, B., 2001. Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. Cancer Res. 61, 632–640.
Chang, E.C., Frasor, J., Komm, B., Katzenellenbogen, B.S., 2006. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 147, 4831–4842.
Chesnokova, V., Zonis, S., Kovacs, K., Ben-Shlomo, A., Wawrowsky, K., Bannykh, S., Melmed, S., 2008. p21(Cip1) restrains pituitary tumor growth. Proc. Natl. Acad. Sci. U. S. A. 105, 17498–17503.
Coleman, K.G., Wautlet, B.S., Morrissey, D., Mulheron, J., Sedman, S.A., Brinkley, P., Price, S., Webster, K.R., 1997. Identification of CDK4 sequences involved in cyclin D1 and p16 binding. J. Biol. Chem. 272, 18869–18874.
De Paul, A., Pons, P., Aoki, A., Torres, A., 1997. Different behavior of lactotroph cell subpopulations in response to angiotensin II and thyrotrophin-releasing hormone. Cell Mol. Neurobiol. 17, 245–258.
De Paul, A., Mukdsi, J., Petiti, J., Gutiérrez, S., Quintar, A., Maldonado, C., Torres, A., 2012. Immunoelectron microscopy: a reliable tool for the analysis of cellular processes. In: Dehghani, D.H. (Ed.), Applications of Immunocytochemistry, Biochemistry, Genetics and Molecular Biology. InTech, Croatia, pp. 65–96.
De Stefano, I., Zannoni, G.F., Prisco, M.G., Fagotti, A., Tortorella, L., Vizzielli, G., Mencaglia, L., Scambia, G., Gallo, D., 2011. Cytoplasmic expression of estrogen receptor beta (ERbeta) predicts poor clinical outcome in advanced serous ovarian cancer. Gynecol. Oncol. 122, 573–579.
Dey, P., Barros, R.P., Warner, M., Strom, A., Gustafsson, J.A., 2013. Insight into the mechanisms of action of estrogen receptor beta in the breast, prostate, colon, and CNS. J. Mol. Endocrinol. 51, T61–T74.
Fan, X., Gabbi, C., Kim, H.J., Cheng, G., Andersson, L.C., Warner, M., Gustafsson, J.A., 2010. Gonadotropin-positive pituitary tumors accompanied by ovarian tumors in aging female ERbeta-/- mice. Proc. Natl. Acad. Sci. U. S. A. 107, 6453–6458.
Fedele, M., Fusco, A., 2010. Role of the high mobility group A proteins in the regulation of pituitary cell cycle. J. Mol. Endocrinol. 44, 309–318.
Gonzalez, M., Reyes, R., Damas, C., Alonso, R., Bello, A.R., 2008. Oestrogen receptor alpha and beta in female rat pituitary cells: an immunochemical study. Gen. Comp. Endocrinol. 155, 857–868.
Gutierrez, S., De Paul, A.L., Petiti, J.P., del Valle Sosa, L., Palmeri, C.M., Soaje, M., Orgnero, E.M., Torres, A.I., 2008. Estradiol interacts with insulin through membrane receptors to induce an antimitogenic effect on lactotroph cells. Steroids 73, 515–527.
Halon, A., Nowak-Markwitz, E., Maciejczyk, A., Pudelko, M., Gansukh, T., Gyorffy, B., Donizy, P., Murawa, D., Matkowski, R., Spaczynski, M., Lage, H., Surowiak, P., 2011. Loss of estrogen receptor beta expression correlates with shorter overall survival and lack of clinical response to chemotherapy in ovarian cancer patients. Anticancer Res. 31, 711–718.
Hartman, J., Edvardsson, K., Lindberg, K., Zhao, C., Williams, C., Strom, A., Gustafsson, J.A., 2009. Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. Cancer Res. 69, 6100–6106.
Heaney, A.P., Fernando, M., Melmed, S., 2002. Functional role of estrogen in pituitary tumor pathogenesis. J. Clin. Investig. 109, 277–283.
Hiroi, H., Inoue, S., Watanabe, T., Goto, W., Orimo, A., Momoeda, M., Tsutsumi, O., Taketani, Y., Muramatsu, M., 1999. Differential immunolocalization of estrogen receptor alpha and beta in rat ovary and uterus. J. Mol. Endocrinol. 22, 37–44.
Hiroi, R., Lacagnina, A.F., Hinds, L.R., Carbone, D.G., Uht, R.M., Handa, R.J., 2013. The androgen metabolite, 5alpha-androstane-3beta,17beta-diol (3beta-diol), activates the oxytocin promoter through an estrogen receptor-beta pathway. Endocrinology 154, 1802–1812.

Horvath, L.G., Henshall, S.M., Lee, C.S., Head, D.R., Quinn, D.I., Makela, S., Delprado, W., Golovsky, D., Brenner, P.C., O'Neill, G., Kooner, R., Stricker, P.D., Grygiel, J.J., Gustafsson, J.A., Sutherland, R.L., 2001. Frequent loss of estrogen receptor-beta expression in prostate cancer. Cancer Res. 61, 5331–5335.
Huang, Y., Li, X., Muyan, M., 2011. Estrogen receptors similarly mediate the effects of 17beta-estradiol on cellular responses but differ in their potencies. Endocrine 39, 48–61.
Ivanova, M.M., Mazhawidza, W., Dougherty, S.M., Minna, J.D., Klinge, C.M., 2009. Activity and intracellular location of estrogen receptors alpha and beta in human bronchial epithelial cells. Mol. Cell Endocrinol. 305, 12–21.
Jirawatnotai, S., Aziyu, A., Osmundson, E.C., Moons, D.S., Zou, X., Kineman, R.D., Kiyokawa, H., 2004. Cdk4 is indispensable for postnatal proliferation of the anterior pituitary. J. Biol. Chem. 279, 51100–51106.
Jordan, S., Lidhar, K., Korbonits, M., Lowe, D.G., Grossman, A.B., 2000. Cyclin D and cyclin E expression in normal and adenomatous pituitary. Eur. J. Endocrinol. 143, R1–R6.
Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.A., 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930.
Lazennec, G., Bresson, D., Lucas, A., Chauveau, C., Vignon, F., 2001. ER beta inhibits proliferation and invasion of breast cancer cells. Endocrinology 142, 4120–4130.
Levin, E.R., 2005. Integration of the extranuclear and nuclear actions of estrogen. Mol. Endocrinol. 19, 1951–1959.
Liu, M.M., Albanese, C., Anderson, C.M., Hilty, K., Webb, P., Uht, R.M., Price Jr., R.H., Pestell, R.G., Kushner, P.J., 2002. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J. Biol. Chem. 277, 24353–24360.
Mandal, S., Davie, J.R., 2010. Estrogen regulated expression of the p21 Waf1/Cip1 gene in estrogen receptor positive human breast cancer cells. J. Cell Physiol. 224, 28–32.
Manoranjan, B., Salehi, F., Scheithauer, B.W., Rotondo, F., Kovacs, K., Cusimano, M.D., 2010. Estrogen receptors alpha and beta immunohistochemical expression: clinicopathological correlations in pituitary adenomas. Anticancer Res. 30, 2897–2904.
Martineti, V., Picariello, L., Tognarini, I., Carbonell Sala, S., Gozzini, A., Azzari, C., Mavilia, C., Tanini, A., Falchetti, A., Fiorelli, G., Tonelli, F., Brandi, M.L., 2005. ERbeta is a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components. Endocr. Relat. Cancer 12, 455–469.
Matthews, J., Gustafsson, J.A., 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol. Interv. 3, 281–292.
Maynadier, M., Ramirez, J.M., Cathiard, A.M., Platet, N., Gras, D., Gleizes, M., Sheikh, M.S., Nirde, P., Garcia, M., 2008. Unliganded estrogen receptor alpha inhibits breast cancer cell growth through interaction with a cyclin-dependent kinase inhibitor (p21(WAF1)). FASEB J. 22, 671–681.
McDonnell, D.P., Norris, J.D., 2002. Connections and regulation of the human estrogen receptor. Science 296, 1642–1644.
McPherson, S.J., Hussain, S., Balanathan, P., Hedwards, S.L., Niranjan, B., Grant, M., Chandrasiri, U.P., Toivanen, R., Wang, Y., Taylor, R.A., Risbridger, G.P., 2010. Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFalpha mediated. Proc. Natl. Acad. Sci. U. S. A. 107, 3123–3128.
Mitchner, N.A., Garlick, C., Ben-Jonathan, N., 1998. Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. Endocrinology 139, 3976–3983.
Mitchner, N.A., Garlick, C., Steinmetz, R.W., Ben-Jonathan, N., 1999. Differential regulation and action of estrogen receptors alpha and beta in GH3 cells. Endocrinology 140, 2651–2658.
Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S., Alves, S.E., 2003. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055–2067.
Moons, D.S., Jirawatnotai, S., Parlow, A.F., Gibori, G., Kineman, R.D., Kiyokawa, H., 2002. Pituitary hypoplasia and lactotroph dysfunction in mice deficient for cyclin-dependent kinase-4. Endocrinology 143, 3001–3008.
Mukdsi, J.H., De Paul, A.L., Munoz, S., Aoki, A., Torres, A.I., 2004. Immunolocalization of Pit-1 in gonadotroph nuclei is indicative of the transdifferentiation of gonadotroph to lactotroph cells in prolactinomas induced by estrogen. Histochem. Cell Biol. 121, 453–462.
Nakamura, Y., Felizola, S.J., Kurotaki, Y., Fujishima, F., McNamara, K.M., Suzuki, T., Arai, Y., Sasano, H., 2013. Cyclin D1 (CCND1) expression is involved in estrogen receptor beta (ERbeta) in human prostate cancer. Prostate 73, 590–595.
Nassa, G., Tarallo, R., Giurato, G., De Filippo, M.R., Ravo, M., Rizzo, F., Stellato, C., Ambrosino, C., Baumann, M., Lietzen, N., Nyman, T.A., Weisz, A., 2014. Post-transcriptional regulation of human breast cancer cell proteome by unliganded estrogen receptor beta via microRNAs. Mol. Cell Proteomics 13, 1076–1090.
Nishihara, E., Nagayama, Y., Inoue, S., Hiroi, H., Muramatsu, M., Yamashita, S., Koji, T., 2000. Ontogenetic changes in the expression of estrogen receptor alpha and beta in rat pituitary gland detected by immunohistochemistry. Endocrinology 141, 615–620.
Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G.R., Firestone, G.L., Leitman, D.C., 2004. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res. 64, 423–428.
Pereira-Lima, J.F., Marroni, C.P., Pizarro, C.B., Barbosa-Coutinho, L.M., Ferreira, N.P., Oliveira, M.C., 2004. Immunohistochemical detection of estrogen receptor alpha in pituitary adenomas and its correlation with cellular replication. Neuroendocrinology 79, 119–124.
Petiti, J.P., De Paul, A.L., Gutierrez, S., Palmeri, C.M., Mukdsi, J.H., Torres, A.I., 2008. Activation of PKC epsilon induces lactotroph proliferation through ERK1/2 in response to phorbol ester. Mol. Cell Endocrinol. 289, 77–84.
Pravettoni, A., Mornati, O., Martini, P.G., Marino, M., Colciago, A., Celotti, F., Motta, M., Negri-Cesi, P., 2007. Estrogen receptor beta (ERbeta) and inhibition of prostate cancer cell proliferation: studies on the possible mechanism of action in DU145 cells. Mol. Cell Endocrinol. 263, 46–54.
Roger, P., Sahla, M.E., Makela, S., Gustafsson, J.A., Baldet, P., Rochefort, H., 2001. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res. 61, 2537–2541.
Sabatino, M.E., Petiti, J.P., Sosa Ldel, V., Perez, P.A., Gutierrez, S., Leimgruber, C., Latini, A., Torres, A.I., De Paul, A.L., 2015. Evidence of cellular senescence during the development of estrogen-induced pituitary tumors. Endocr. Relat. Cancer 22, 299–317.
Schreihofer, D.A., Stoler, M.H., Shupnik, M.A., 2000. Differential expression and regulation of estrogen receptors (ERs) in rat pituitary and cell lines: estrogen decreases ERalpha protein and estrogen responsiveness. Endocrinology 141, 2174–2184.
Sosa, L., Gutierrez, S., Petiti, J.P., Palmeri, C.M., Mascanfroni, I.D., Soaje, M., De Paul, A.L., Torres, A.I., 2012. 17beta-Estradiol modulates the prolactin secretion induced by TRH through membrane estrogen receptors via PI3K/Akt in female rat anterior pituitary cell culture. Am. J. Physiol. Endocrinol. Metab. 302, E1189–E1197.
Sosa, Ldel V., Gutierrez, S., Petiti, J.P., Vaca, A.M., De Paul, A.L., Torres, A.I., 2013. Cooperative effect of E(2) and FGF2 on lactotroph proliferation triggered by signaling initiated at the plasma membrane. Am. J. Physiol. Endocrinol. Metab. 305, E41–E49.
Spady, T.J., McComb, R.D., Shull, J.D., 1999. Estrogen action in the regulation of cell proliferation, cell survival, and tumorigenesis in the rat anterior pituitary gland. Endocrine 11, 217–233.
Strom, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J., Gustafsson, J.A., 2004. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc. Natl. Acad. Sci. U. S. A. 101, 1566–1571.
Tena-Sempere, M., Navarro, V.M., Mayen, A., Bellido, C., Sanchez-Criado, J.E., 2004. Regulation of estrogen receptor (ER) isoform messenger RNA expression by different ER ligands in female rat pituitary. Biol. Reprod. 70, 671–678.
Toledano, Y., Zonis, S., Ren, S.G., Wawrowsky, K., Chesnokova, V., Melmed, S., 2012. Estradiol partially recapitulates murine pituitary cell cycle response to pregnancy. Endocrinology 153, 5011–5022.
Trukhacheva, E., Lin, Z., Reierstad, S., Cheng, Y.H., Milad, M., Bulun, S.E., 2009. Estrogen receptor (ER) beta regulates ERalpha expression in stromal cells derived from ovarian endometriosis. J. Clin. Endocrinol. Metab. 94, 615–622.
Turner, H.E., Nagy, Z., Sullivan, N., Esiri, M.M., Wass, J.A., 2000. Expression analysis of cyclins in pituitary adenomas and the normal pituitary gland. Clin. Endocrinol. Oxf 53, 337–344.
Wilson, M.E., Price Jr., R.H., Handa, R.J., 1998. Estrogen receptor-beta messenger ribonucleic acid expression in the pituitary gland. Endocrinology 139, 5151–5156.
Zarate, S., Jaita, G., Zaldivar, V., Radl, D.B., Eijo, G., Ferraris, J., Pisera, D., Seilicovich, A., 2009. Estrogens exert a rapid apoptotic action in anterior pituitary cells. Am. J. Physiol. Endocrinol. Metab. 296, E664–E671.
Zhang, G., Liu, X., Farkas, A.M., Parwani, A.V., Lathrop, K.L., Lenzner, D., Land, S.R., Srinivas, H., 2009. Estrogen receptor beta functions through nongenomic mechanisms in lung cancer cells. Mol. Endocrinol. 23, 146–156.
Zhou, W., Song, Y., Xu, H., Zhou, K., Zhang, W., Chen, J., Qin, M., Yi, H., Gustafsson, J.A., Yang, H., Fan, X., 2011. In nonfunctional pituitary adenomas, estrogen receptors and slug contribute to development of invasiveness. J. Clin. Endocrinol. Metab. 96, E1237–E1245.