ORIGINAL RESEARCH ARTICLE

Estrogen receptor β regulates the tumoral suppressor PTEN to modulate pituitary cell growth†

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ABSTRACT

In this study, we focused on ERβ regulation in the adenohypophysis under different estrogenic milieu, by analyzing whether ER modulates the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression and its subcellular localization on anterior pituitary glands from Wistar rats and GH3 lactosomatotroph cells that over-expressed ERβ. ERβ was regulated in a cyclic manner, and underwent dynamic changes throughout the estrous cycle, with decreased ERβ+ cells in estrus and under E2 treatment, but increased in ovariectomized rats. In addition, the ERα/β ratio increased in estrus and under E2 stimulation, but decreased in ovariectomized rats. Double immunofluorescence revealed that lactotroph and somatotroph ERβ+ were significantly decreased in estrus. Also, variations in the PTEN expression was observed, which was diminished with high E2 conditions but augmented with low E2 milieu. The subcellular localization of this phosphatase was cell cycle–dependent, with remarkable changes in the immunostaining pattern: nuclear in arrested pituitary cells but cytoplasmic in stimulated cells, and responding differently to ER agonists, with only DPN being able to increase PTEN expression and retaining it in the nucleus. Finally, ERβ over-expression increased PTEN with a noticeable subcellular redistribution, and with a significant nuclear signal increase in correlation with an increase of cells in G0/G1 phase. These results showed that E2 is able to inhibit ERβ expression and suggests that the tumoral suppressor PTEN might be one of the signaling proteins by which E2, through ERβ, acts to modulate pituitary cell proliferation, thereby adapting endocrine populations in relation with hormonal necessities.

Keywords: PITUITARY; ESTROGEN RECEPTOR β; LACTOTROPH; SOMATOTROPH; PTEN.

1. INTRODUCTION

The anterior pituitary is an endocrine gland with important cellular plasticity, which adapts its populations according to the systemic hormonal requirement. Its proliferative activity varies under different physiological conditions; in the estrous cycle, there is an elevated mitogenic activity in estrus and a decreased activity during diestrus, with the lactotroph and somatotroph cells being those which show the greatest percentage of mitosis (Oishi et al., 1993). The highest cellular proliferative rate in estrus is related to the high ovarian estrogen secretion in proestrus, demonstrating the importance of estrogen as a regulator of this process. Indeed, 17-β estradiol (E2) is able to regulate lactotroph (Sosa Ldel et al., 2013; Toledano et al., 2012) and somatotroph proliferation (Carretero et al., 1997).

The E2 effects are mediated by the specific estrogen receptors (ER) α and β. The ERα in the pituitary gland has been extensively studied (Gutierrez et al., 2012; Zarate and Seilicovich, 2010); whereas, in contrast, hormonal regulation of ERβ expression at the pituitary remains poorly characterized, with the role of this subtype on the pituitary cell growth being far from well understood. Recently, we showed that ERβ exerts an inhibitory role on somatolactotroph GH3 pituitary cells, and reduces the BrdU uptake by modulating cyclin D1 and p21 (Perez et al., 2015). Nevertheless, the mechanisms involved in this effect are not well known. In fact, ERα and β are expressed in all endocrine pituitary cells, including lactotrophs and somatotrophs (Gonzalez et al., 2008), albeit ERβ at a lower level than ERα (Mitchner et al., 1998).

Several studies have analyzed ERβ expression and regulation in the rat pituitary gland with conflicting results. ERβ mRNA has shown constant levels during the estrous cycle (Gonzalez et al., 2008; Vaillant et al., 2002), but decreased ones in the proestrus stage (Schreihofer et al., 2000). Moreover, ERβ mRNA decreased after estrogen therapy (Schreihofer et al., 2000; Tena-Sempere et al., 2004), but was unchanged throughout pregnancy (Vaillant et al., 2002). These findings highlight the increased interest in studying ERβ expression in adenohypophysis in different estrogenic

environments, which have produced new insights concerning the estrogenic effects on the anterior pituitary gland and the regulation of its receptors.

It has been reported that ERβ up-regulates the expression of phosphatases, such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN), in human seminoma cell line (Guido et al., 2012), and in breast cancer (Lindberg et al., 2011). Also, it has been reported that the ERβ expression is lost during the prostate tumorigenesis caused by PTEN deletion (Mak et al., 2015). PTEN has been demonstrated to be an important tumor-suppressor gene (Datta et al., 1999), and is involved in regulation of the PI3K/Akt signaling pathway by suppressing the growth and proliferation of many cell types. It also plays distinct growth-regulatory roles in the cytoplasm and nucleus, being preferentially localized to the nucleus in resting cells, and to the cytoplasm in proliferating cells. Thus, the regulation of PTEN subcellular localization seems to be critical to its functions (Ginn-Pease and Eng, 2003).

In the pituitary gland, PI3K/Akt plays a role in maintaining cellular viability (Vender et al., 2008; Xu et al., 2009), PRL secretion (Sosa et al., 2012), with enhanced levels of phosphorylated Akt being responsible for cell cycle dysregulation in pituitary cells (Musat et al., 2005), and the PTEN/Akt pathway regulating the growth of AtT-20 pituitary cells (Palumbo et al., 2013). However, the PTEN role in this gland is less clear, with the PTEN involvement in the ERβ effects on pituitary cell proliferation not having been explored yet.

Considering this background, we aimed to study the expression of ERβ in adenohypophysis under different estrogenic milieu, by analyzing whether ER regulates PTEN expression and its subcellular localization in the mechanisms that control pituitary cell growth.

2. MATERIALS AND METHODS

2.1 Animals and experimental models

Three-month-old female and male Wistar strain rat were bred and housed at the Animal Research Facility of the National University of Cordoba, under controlled temperature (21 ± 3°C) and lighting conditions (14h light/10h 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 Institute of Health (1996), and the experiments were approved by the Institutional Animal Care Committee of the School of Medicine, National University of Cordoba.

The number of rats used in each experimental model was 4-5 animals for group and the protocols were repeated three times in order to estimate the average for each group. Cycling rats were monitored by daily vaginal smears and rats with three or more normal consecutive 4-5 days (d) estrous cycles were sacrificed between 7:30 am and 9 am of proestrus, estrus or diestrus II. For steroid treatments *in vivo*, slow-releasing capsules made of Silastic tubing (Dow Corning; medical grade) filled with 30 mg estradiol benzoate (Sigma Aldrich, St. Louis, MO, USA) were implanted subcutaneously for 20d and 40d. Other pools of female rats were ovariectomized (OVX) 45d and 60d before experiments. The 0d correspond to other group of female rats in estrus stage. Finally, rats were decapitated within 10s after removal from their cage, thus avoiding any stress or external stimuli.

2.2 Anterior pituitary cell cultures

Large pools of anterior pituitary glands from around 25-30 female rats were used for each primary cell culture. The protocol for the dissociation of pituitary cells has been described previously (De Paul et al., 1997). After 3d of culture, the medium was replaced with serum-free and phenol red-

free DMEM supplemented with hydrocortisone (100µg/l), 3,3'-triiodothyronine (400ng/l), transferrin (10mg/l) and sodium selenite (5µg/l). Then, this medium was replaced every day while the cells were submitted to the different experimental protocols.

2.3 Stable transfection of ERβ

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 24h. The medium was then replaced by Opti-MEM medium (Gibco; NY, USA), and the expression plasmid (1µg) and the transfection reagent FuGene 6 (3µl, Roche; In, USA) were added for 24h. 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 200µg/ml of G418, and the ERβ positive cells were identified by flow cytometry. The GH3 cells in which the ERβ gene was successfully transfected were named GH3β+.

2.4 Cell treatments

Culture cells were exposed to 10nM of 17β-estradiol (Sigma St Louis MO, USA) or to the selective ERα agonist: 4,4`,4``-(4-Propyl-[1H]-pyrazole-1,3,5-tryl)trisphenol (PPT, Tocris Cookson), or ERβ agonist: 2,3-bis (4-Hydroxyphenyl)-propionitrile, (DPN, Sigma Aldrich, St. Louis, MO, USA) in serum-free and phenol red-free culture medium for 72h.

2.5 Antibodies

Anti-ERβ: Y-19, directed to the N-terminal of ERβ (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); anti-ERα: MC-20, directed to the C-terminal of ERα (1:500, Santa Cruz Biotechnology,

Santa Cruz, CA); anti-PTEN: 26H9 (1:400, Cell Signaling Technology); anti-phospho-Akt Ser473: D9E (1:1000, Cell Signaling Technology); anti-total Akt: sc-8312 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin: A1978 (1:5000, Sigma-Aldrich, St. Louis, MO); anti-histone deacetylase 1 (HDAC1): H3284 (1:500, Sigma-Aldrich, St. Louis, MO); anti-PRL (1:3000, National Hormone and Pituitary Program, Torrance, CA); anti-GH (1:3500, NIH Hormone Program).

2.6 Immunofluorescence and confocal laser scanning microscopy

For immunodetection, cultured pituitary cells were fixed in 4% formaldehyde and permeabilized with 0.50% Triton X-100/PBS. Additionally, pituitary gland obtained from animals in the different estrous cycle stages were placed in Crioplast (Biopack, Buenos Aires, Argentina), immersed in liquid nitrogen for a few minutes until the solidification of the embedding medium and sectioned using cryostat. Slices were fixed in absolute methanol.

Subsequently, both pituitary cell monolayers and tissue sections were blocked for 1h in 5% PBS-BSA, incubated with primary antibodies for 1h and further incubated with Alexa 488 anti-goat, Alexa 488 anti-mouse, Alexa 594 anti-rabbit, or Alexa 594 anti-mouse secondary antibody (1:1000; Invitrogen; Carlsbad, USA) for 1h and mounted with fluoromount (Sigma) containing DAPI. To validate the specificity of the immunostaining, negative controls were performed replacing primary antibody with 1% PBS-BSA. 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.

For the morphometric analysis, a total of 1500 pituitary cells were examined by confocal microscopy in randomly chosen fields of each glass slide, and quantified in order to establish the percentage of ERβ expression in lactotroph and somatotroph or nuclei immunoreactive for PTEN. Three slides were analyzed for each group.

2.7 Flow Cytometric analysis

Living dispersed cells were fixed for 20min 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 anti-goat or Alexa Fluor 647 anti-rabbit (1:1000; Invitrogen) for 1h 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 x 10^5 events/experimental treatment). The data analysis was carried out using the FlowJo software (Tree Star; Ashland, OR), and the percentage of ERβ or ERα-positive cells was determined.

2.8 Western blot analysis

Proteins were prepared from pituitary tissues and cultured cells, using RIPA buffer containing a cocktail of enzyme inhibitors 2mM PMSF, 10µg/ml leupeptin and 10µg/ml aprotinin. This was followed by homogenization or scraping, and the lysates were transferred to a centrifuge tube placed on ice. After 30min the lysates were centrifuged at 15000 *g* for 20min 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 (10mM HEPES, 5mM MgCl2, and 40mM KCl) containing 1mM PMSF, 10µg/ml aprotinin and 10µg/ml leupeptin. After 30min incubation on ice, cell homogenates were centrifuged at 5000rpm for 45 min at 4°C. The supernatants collected corresponded to the cytosolic fraction. The pellet was resuspended in Hypertonic Hepes buffer (10mM HEPES, 5mM MgCl2, 40mM KCl and 1.5mM NaCl), followed by brief sonication, and cold centrifugation at 5000*rpm* for 10min. 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 12h at 4°C. After washing in PBS-0.05% Tween, blots were incubated for 1h 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).

2.9 Cell cycle analysis

Cells were washed in PBS, fixed with -4ºC ethanol 70%, washed again with citrate-phosphate buffer at pH:7.8, and incubated with RNAse (10 mg/ml, Ribonuclease A, Sigma; St Louis, USA). This was followed by DNA staining with propidium iodide (50mg/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 G0/G1 phase was determined.

2.10 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 results are shown as the means ± SEM, and the significance levels were chosen at $P<0.05$.

3. RESULTS

3.1 The number of anterior pituitary cells expressing ERβ fluctuates according to the E2 levels

As shown in Figure 1A-B, the expression of ERβ was quantified by flow cytometry in pituitary cells from female Wistar rats (at different stages of the estrous cycle, and when chronically stimulated with E2 or OVX) and also in male rats.

Our results revealed that in proestrus 35.4 ± 2.5 % of the anterior pituitary cells were ERβ+; in estrus, the percentage of ERβ+ cells in the pituitary population was significantly lower (18.79 ± 1.1 %); while during the diestrus stage 34.12 ± 1.56 % of cells expressed ERβ.

In pituitary cells of rats stimulated with E2, the flow cytometry analysis showed a significant decrease in ERβ expression, with values of 10.7 ± 2.2 % and 1.57 ± 0.12 % ERβ+ cells at 20d and 40d respectively, whereas in OVX rats at 45d or 60d, the percentage of cells expressing ERβ increased to 33.48 ± 2.68 and 56.72 ± 5.32 %, respectively, with significant differences respect to 0d. Finally, the percentage of ERβ+ pituitary cells from male rats was found to be the highest (66.48 ± 3.73 %) (P<0.0001 vs. female in estrus).

Considering that E2 mediates its effects by acting through ERα and β, and that the final responses are ultimately dependent on the levels of both ER, the expression of ERα was also determined by flow cytometry, (Figure 1C-D) and the ratio ERα/β was calculated for all experimental conditions (Figure 1E).

The number of pituitary cells expressing the ERα protein was similar at different stages of the estrous cycle, with 77.8 ± 4.66 % of ERα+ cells in proestrus, 71.5 ± 3.98 % in estrus and 70.63 ± 3.66 % in diestrus. In addition , the ERα/β ratio was 2.7 ± 0.74 at the proestrus stage, but with a significant increase shown in estrus, with values of 4.32 ± 0.73, and then returning in diestrus to similar levels to proestrus (diestrus: 2.09 ± 0.47).

The ERα+ pituitary cell percentage from rats stimulated with E2 was 84.03 ± 2.03 at 20d and 91.05 ± 0.32 % at 40d, values significantly higher than at 0d, with the ERα/β ratio also being significantly increased (7.51 ± 0.62 and 63.67 ± 4.23 respectively) compared to 0d. In OVX rats, the number of pituitary cells expressing ERα was 83.85 ± 2.22 % at 45 days and 75.41 ± 2.27 % at 60 days, which were not significantly different from 0d, but the ERα/β ratio values of 2.7 ± 0.24 (45d) and 1.56 ± 0.19 (60 days), were both significantly lower than at 0d. In the pituitary cell population from male rats, the percentage of ERα+ cells was 37.56 ± 2.74 %, which was significantly less than that in estrus female rats (71.5 ± 3.98), with the ERα/β ratio in male pituitary cells of 0.52 ± 0.02, being significantly different compared to estrus or 0d.

3.2 Percentage of lactotroph and somatotroph cells expressing ERβ decreases in the estrus stage

The percentage of lactotroph and somatotroph cells that expressed ERβ was determined by double immunofluorescence in the estrous cycle (Figure 2). In proestrus 27.33 ± 2.41% of lactotrophs were ERβ+, which in the estrus stage was significantly decreased to 20.07±2.04%. In the diestrus stage, a high percentage, 36.19±3.19% of lactotrophs expressed ERβ. Furthermore, the percentage of somatotrophs that expressed ERβ was 13.61±0.41% in proestrus, which decreased in estrus to 9.56±1.26 %, but with higher values of 17.81±3.14% in diestrus. These results showed that in estrus both ERβ+ endocrine cells (lactotrophs and somatotrophs) were significantly decreased.

3.3 Pituitary PTEN is regulates by E2

The pituitary PTEN expression and subcellular localization were analyzed, with its regulation being determined by *in vivo* circulating E2 levels. PTEN protein was measured in pituitary cells from female Wistar rats at different stages of the estrous cycle, when chronically stimulated with E2, in OVX and in male rats by western blot technique, and with its subcellular localization being analyzed by immunofluorescence.

These results showed a specific staining for PTEN protein as a unique band at ≈54 kDa, revealing cyclic-related changes with a reduction in the estrus stage (Figure 3A). Then, after 20d or 40d of E2 stimulation, a more significant reduction was observed (Figure 3B). In OVX rats, there was an increase in PTEN expression at 45d, which reached similar values in the adenohyphophysis as in male Wistar rats (Figure 3C), thereby supporting the hypothesis that the levels of E2 contribute to the modulation of pituitary PTEN expression, and suggesting a negative regulation of E2 on this protein expression.

Considering that the PTEN subcellular distribution has an impact on its function and final effect, the PTEN localization was analyzed by confocal microscopy. As shown in Figure 3D, in pituitary cells obtained from female rats in proestrus and diestrus, the PTEN expression was observed as a cytoplasmatic fluorescent signal with a more intense immunostaining occurring in the peripheral zone, next to the plasma membrane. In agreement with the PTEN expression determined by western blot, a reduction in the fluorescence in estrus was evident, maintaining a cytoplasmatic staining pattern. Furthermore, a decreased and diffuse cytoplasmatic immunostaining was observed after *in vivo* E2 treatment, which was undetectable at 40d of stimulation (Figure 3E). Both the pituitary cells obtained from OVX and male rats showed a greater PTEN immunostaining, being diffusely distributed in the cytoplasm with reinforcement in the peripheral zone (Figure 3F). These results demonstrate that pituitary PTEN expression fluctuates with respect to the estrogenic milieu.

To elucidate if pituitary PTEN subcellular localization is cell cycle–dependent, primary pituitary cells were cultured in serum-free conditions or in DMEN supplemented with 10% serum. The immunostaining showed that there was more PTEN in the nucleus of serum starved pituitary cells, with a weak cytoplasmic labeling. In contrast, PTEN was found in the cytoplasm in cells supplemented with serum (Figure 4A). This differential subcellular localization was confirmed with the nuclear PTEN expression quantification, showing a significant increase in the percentage of PTEN

immunofluorescence at nuclear level when cell cultures were grown under serum free conditions (Figure 4 B).

In order to evaluate the ERα and β participation as mediators of the E2 modulator role on PTEN expression, pituitary cultures were treated with the specific agonists for ERα (PPT) or ERβ (DPN) in serum-free conditions. Only ERβ agonist induced an increase in PTEN protein expression (Figure 4C), with a different subcellular distribution pattern. While PTEN immunostaining showed a peripheral cytoplasmic pattern under PPT stimulation, with a weak nuclear signal the immunolabeling was predominantly nuclear after DPN treatment (Figure 4D-E). These data suggest that PTEN subcellular localization is related to the cellular proliferative status, and that ER subtypes are differentially able to modify pituitary PTEN subcellular distribution.

3.4 ERβ over-expression increased PTEN expression in GH3 cells

To further study the specific ERβ effect on pituitary cells, rat GH3 lactosomatotroph pituitary cell line was used. This cell line showed a low endogenous ERβ expression; with 3.43 ± 0.1% ERβ immunolabeled GH3 cells in basal conditions (referred as GH3β-), being the stimulation with E2, PPT or DPN unable to modify ERβ endogenous protein expression, showing values of 2.53 ± 0.46 % with E2, 2.37 ± 0.53 % with PPT, and 3.53 ± 0.26 % ERβ+ GH3 cells with DPN treatment (Figure 5).

These cells were transfected selecting an enriched stable GH3 cell line for ERβ expression, with 26.8 ± 5.45 % ERβ+ GH3 cells (referred as GH3β+). The stimulation with E2, PPT or DPN was unable to modify these percentages; with 23.78 ± 1.71 % of GH3 cell expressing ERβ under E2 stimulation, 18.01 ± 2.1 % with PPT and 19.95 ± 4.19 % with DPN treatment (Figure 5).

Then, in order to analyse the specific ERβ effect on pituitary PTEN, the expression of this protein in the cytoplasmatic and nuclear fractions of GH3 that over-express ERβ (GH3β+) was determined by western blot (Figure 6A). For GH3β+, the PTEN basal expression was significantly higher respect to the levels found in GH3β- cells in both, the nuclear and cytoplasmatic fractions, and

these levels were maintained after E2 or PPT treatment. In addition, stimulus with the specific ERβ agonist, DPN, increased nuclear PTEN expression compared to GH3β+ control.

As illustrated in Figure 6B, when PTEN immunolabeling was analyzed by confocal microscopy, it was revealed that PTEN in GH3β- cells was located predominantly in the cytoplasm, near to the plasmatic membrane, without any nuclear PTEN expression. However, over-expression of ERβ induced a noticeable subcellular redistribution, acquiring a diffuse punctuated pattern in the cytoplasmatic compartment and also with immunolabeling in the nuclei. The E2 and PPT treatments showed similar immunostaining as GH3β+ control, but DPN increased the nuclear immunofluorescence, confirmed by the morphometric analysis (Figure 6C).

To investigate further the ERβ effects on PTEN signaling pathway, the levels of total and phosphorylated Akt were detected in GH3 that over-expresses the ERβ in serum-free medium. The immunoblotting using specific antibodies revealed bands of 56 kDa, corresponding to total and phosphorylated Akt. The GH3β- cells showed a high basal expression of phosphorylated Akt, indicating that this protein is constitutively active in this cellular line. ERβ over-expression was able to reduce the Akt phosphorylation, showing a weak expression level. In fact, only E2 stimulus increased Akt phosphorylation, which attained GH3β- levels. The total Akt level expression was similar in all the analyzed models, and it was used as the loading control (Figure 6D).

With the purpose of analyzing the cellular cycle in pituitary cells with different PTEN subcellular localizations, the pituitary cell population in the G0/G1, S and G2/M phases was analyzed by flow cytometry. As shown in Figure 6E, 63.45 ± 3.08 % of control GH3β- was found in the G0/G1 phase, with this percentage being increased with ERβ over-expression to 79.97 ± 0.16% under basal conditions, and remaining high for the different treatments (83.43 ± 1.35% with E2, 84.57 ± 1.62% with PPT and 82.10 ± 1.65 % with DPN).

Finally, the same experiments were performed in GH3β- cells. In basal conditions, a weak PTEN expression was detected in nuclear and cytoplasmatic fractions, with similar values under E2,

PPT or DPN treatments (Figure 7A). Also, phosphorylated Akt expression was increased with E2 and PPT stimuli, while after DPN treatment, the pAkt protein expression showed similar values to those of basal condition (Figure 7B). GH3 cell population in G0/G1 was significantly decreased by E2 treatment (48.96 ± 0.63 %), being PPT and DPN unable to modify this percentage, with similar values to control (PPT: 52.94 ± 1.06 %, DPN: 54.39 ± 1.09 %) (Figure 7C).

4. DISCUSSION

In the present study, we observed that ERβ protein expression is regulated in a cyclic manner and undergoes dynamic changes throughout the estrous cycle. E2 played an important role, with ERβ inhibition occurring under E2 treatment and increasing in the OVX rats. It has been previously shown that the ERβ mRNA levels are the lowest on the morning of proestrus when *in vivo* steroid is attaining its highest level (Schreihofer et al., 2000), suggesting that E2 may exert an inhibitory effect on ERβ in anterior pituitary cells. Although, we did not observe any changes in the protein expression in proestrus, it could be considered that E2 may reduce ERβ ARNm during this stage. Nevertheless, effects on protein are seen at the next stage of the estrous cycle, when less protein is synthetized *de novo*. Consistent with these results, and supporting our hypothesis of a negative E2 effect on ERβ, other authors have shown that ovariectomy increases pituitary ERβ mRNA whereas estrogen replacement therapy decreases it (Tena-Sempere et al., 2004). Our results showed that the ERα/β ratio changes under different estrogenic contexts, mainly due to ERβ fluctuations, with also a minor impact of ERα variation, which increased only with E2 treatment. In another investigation, an important influence of the ERα/β ratio was shown on E2-induced cell proliferation, with cell-specific actions of estrogens in the rat pituitary resulting from the modulated expression of both ER subtypes (Zarate and Seilicovich, 2010). Thus, quantification of the cells that express both ER subtypes is crucial to have a better understanding of the E2 effect on the pituitary gland. Considering all these results together, it

may be hypothesized that the increased ERα/β ratio in estrus as well as in chronic E2 stimulation facilitates the pituitary cell growth that is characteristic of both situations.

In view of the fact that lactotrophs and somatotrophs are the pituitary cellular subtypes that exhibit the greatest percentage of mitosis in response to different estrogenic environments (Oishi et al., 1993), as well as expressing ERβ at the greater proportions (Gonzalez et al., 2008) and constituting about 72% of the total cells in the anterior pituitary of adult rats (Dada et al., 1984), we believe that the changes in ERβ expression quantified by flow cytometry in this study correspond mainly to the lactotroph and somatotroph cells. Confirming this speculation, we demonstrated by double immunostaining that ERβ fluctuates specifically in lactotroph and somatotroph cells in estrous cycle.

What could be the physiological significance of ERβ inhibition by E2 in pituitary cells? Considering that in a previous report we demonstrated that ERβ inhibited pituitary cell proliferation (Perez et al., 2015), and that ERα is the main mediator of a positive effect of E2 on the pituitary gland, it is possible that the circulating E2 inhibits ERβ and consequently modulates cell proliferation to respond to the cyclic requirements of PRL and GH. Thus, increased mitogenic activity of lactotroph in estrus (Oishi et al., 1993) could respond, at least in part, to the diminished ERβ expression shown in the present work. On the other hand, it has been suggested that the somatotroph response to E2 may depend on the surrounding hormonal environment (Chowen et al., 2004), and our results indicate that the cyclic estrogenic environment modulates ERβ expression on somatotrophs. Taking the above findings into account, we suggest that E2 inhibits ERβ expression in somatotroph and lactotroph cells in order to facilitate mitogenic activity, thereby adapting these cell populations to finally respond appropriately to hormonal requirements.

Our result showed low basal ERβ protein expression in GH3 cells, being E2 or the ERα or β agonists unable to modify the endogenous ERβ expression. It has been demonstrated that in GH3, the ERβ protein was absent (Schreihofer et al., 2000) or scarce and lower than ERα expression (Mitchner et al., 1999), without E2 effect in the ERβ regulation (Avtanski et al., 2014). It is possible that the low

ERβ expression in GH3 could be, in part, responsible of the high proliferative response to E2, due to the absence of anti-mitogenic effect of ERβ, in comparison with normal pituitary cell (Perez et al., 2015). Also, in GH3 that over-express ERβ, we showed that the stimulation with E2, PPT or DPN was unable to modify ERβ protein expression. Considering that exogenous ERβ protein is regulated by a viral promoter, unlike the endogenous proteins that is under the control of its own promoter, this absence of response to the different treatments is expected.

This study revealed variations in PTEN expression in different E2 environments, with PTEN being reduced at high E2 serum conditions, but augmented at low E2 milieu, suggesting that E2 negatively regulates PTEN protein expression. Changes in PTEN expression in both mRNA and the protein levels throughout the menstrual cycle were reported in the endometrium (Mutter et al., 2000), with the estrogen induced down-regulation of PTEN expression being associated with the phosphorylation of Akt in neurons (Smith et al., 2009), and being involved in the control of pituitary cell growth (Palumbo et al., 2013; Zhou et al., 2016). However, whether ERβ could regulate PTEN pituitary expression was still unknown. Here, our results have shown that PTEN was increased by ERβ over-expression in GH3 cells. In estrogen-sensitive tissues, it has been previously reported that ERβ plays an important role in increasing the expression of anti-proliferative genes such as PTEN (Lindberg et al., 2011) with ERβ inhibiting the PI3K/AKT pathway by increasing the PTEN expression (Li et al., 2013). There is also evidence that ERβ can bind to the promoter region of PTEN through Sp1 and subsequently increase PTEN transcription (Guido et al., 2012). In the present study, we observed low PTEN expression in GH3β- cells, being the E2, PPT or DPN unable to increase PTEN levels. In this sense, it has been reported that ERα induces PTEN down-regulation in breast cancer cells (Noh et al., 2011). This could explain the lack of PTEN increase in GH3β- cells, which mainly express ERα, and reinforced the hypothesis of the differential ERα and β effect on PTEN regulation in pituitary cells. Also, our result showed a high basal level of phospho-Akt in GH3 in agreement with its accelerated progression through the cell cycle described in these cells. Therefore,

phospho-Akt augmentation under E2 or PPT treatment could be an ERα-mediated effect. In contrast, the reduction of Akt phosphorylation induced by ERβ over-expression supports the idea of this signaling pathway being involved in the ERβ effects, possibly by inhibiting the pituitary cell proliferation. Taken together, these results and others suggest that up-regulation of PTEN could be an anti-proliferative mechanism for ERβ, and that E2 may inhibit or increase PTEN expression according to the ERα/β balance in pituitary cells, thereby modulating pituitary cell proliferation.

Our *in vitro* studies revealed that pituitary PTEN subcellular localization was cell cycle dependent, with remarkable changes occurring in PTEN subcellular localization in normal pituitary cells, with nuclei being immunostained in arrested cells by serum-free conditions, but cytoplasmic labeling taking place in serum stimulated cells. It has been previously demonstrated that PTEN enters the nucleus and varies throughout the cell cycle, with higher nuclear PTEN levels present in the G0/G1 phase and lower nuclear PTEN expression in the S phase, suggesting that nuclear PTEN activity might directly regulate the cell cycle (Ginn-Pease and Eng, 2003). Furthermore, here PPT and DPN induced PTEN redistribution, which was found mainly in the peripheral cytoplasm with PPT, but in the nucleus with DPN, indicating that the specific activation of ERα might have induced PTEN nuclear export whereas ERβ was able to retain it in the nucleus. These observations support the idea that the ERα/β ratio regulates PTEN import and export to and from the nucleus, possibly contributing to regulating pituitary cell proliferation.

In GH3β- cells, a weak PTEN signal in peripheral cytoplasm was found, suggesting that at least part of the increased phosphorylation of Akt could be secondary to a reduction in cytoplasmic PTEN expression in these cells. Our results also showed that ERβ over-expression induced an increase in PTEN expression, in the cytoplasm, but mainly in the nucleus compartment, with an increase in cells also in the G0/G1 phase. Moreover, it has been reported that nuclear PTEN induces cyclin D1 down regulation, increasing the cell number in arrest (Chung et al., 2006). Interestingly, our results are in agreement with our previous report showing a cyclin D1 reduction by ERβ over expression in GH3

cells (Perez et al., 2015). Thus, all these findings, suggest that PTEN is involved in ERβ-induced pituitary cell growth by two mechanisms: modulating Akt phosphorylation in the cytoplasm, and possibly, inducing G0/G1 arrest in the nuclei.

In conclusion, pituitary ERβ protein expression revealed temporal changes throughout the estrous cycle, related to estrogenic fluctuations, suggesting that E2 was able to inhibit ERβ expression. Furthermore, the E2 effects mediated by ERβ might involve PTEN, possibly as anti-proliferative mechanism. Thus, we propose that PTEN might be one of the signaling proteins by which E2, through ERβ, modulates pituitary cell proliferation by adapting endocrine populations to hormonal necessities.

5. DECLARATION OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.

6. FUNDING

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7. AUTHORS CONTRIBUTIONS

P.A.P. and S.G. contributed to the conception and design of the research; P.A.P., F.P., C.B.G., E.G. and J.H.M. performed the experiments; P.A.P., L.d.V.S. and S.G. analyzed the data; P.A.P., J.P.P., F.P., L.d.V.S., A.L.D.P., and S.G. interpreted the results of the experiments; P.A.P. and S.G. prepared the figures; P.A.P., and S.G. drafted the manuscript; P.A.P., J.P.P., A.L.D.P., A.I.T. and S.G. edited and revised the manuscript; P.A.P, J.P.P., F.P., C.B.G., L.d.V.S., E.G., J.H.M., A.L.D.P., A.I.T and S.G. approved the final version of the manuscript.

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10. FIGURE LEGENDS

Figure 1. ERα and β expression in pituitary cells by flow cytometry. A. Percentage of pituitary cells that expressed ERβ (**P* < 0.05 vs. diestrus, vs. proestrus; ▲ *P* < 0.05 vs. 0d; • *P*<0.0001 vs. female in estrus). **B.** ERβ quantification, representative images of one of the three independent experiments performed. **C**. Pituitary cell percentage that expressed ERα (**P* < 0.05 vs. 0d; • *P* < 0.0001 vs. female in estrus). **D**. ERα quantification, representative images of one of the three independent experiments performed. **E**. ERα/β ratio (**P* < 0.05 vs. diestrus, vs. proestrus, • *P* < 0.05 vs. 0d, ▲ *P* < 0.05 vs. female in estrus). 10^5 events for each experimental condition. Tukey test.

Figure 2. ERβ expression in lactotroph and somatotroph cells. A. Percentage of lactotroph cells that express ERβ in the estrous cycle: proestrus (P), estrus (E) and diestrus (D) (**P* < 0.05 vs. proestrus, vs. diestrus). **B.** Representative images of double immunostaining for PRL and ERβ (arrows)**. C.** Percentage of somatotroph cells expressing ERβ in the estrous cycle (**P* < 0.05 vs. proestrus, vs. diestrus), **D.** Representative double immunostaining GH and ERβ images. Arrows indicate lactotroph and somatotroph ERβ+ cells. *Bar*= 20 µm.

Figure 3. PTEN expression in pituitary glands. A. PTEN protein expression determined by western blot in the estrous cycle (**P* < 0.05 vs. proestrus, vs. diestrus), **B.** and under E2 stimulation (**P* < 0.001 vs. 0d), **C.** OVX female and male (**P* < 0.05 vs. 0d). Protein expression is represented as a percentage relative to estrus (estrus data were set to 100%). The β actin expression was used as a loading control. **D**. Immunolabeling of PTEN in the estrous cycle, **E**. under estrogenic treatments, **F**. and in OVX female and in male rats. Arrows indicate peripheral immunostaining in the cytoplasm. *Bar*= 20 µm. Representative images of three independent experiments are presented.

Figure 4. PTEN expression in cultured normal pituitary cells. **A.** PTEN immunolocalization in cultures with and without serum. **B.** Morphometric analysis of nuclear PTEN expression. (*$P < 0.0001$ vs. 10% serum). **C.** Western blotting analysis of PTEN protein expression (*$P<0.05$ vs. control) is represented as a percentage relative to control (control-data were set to 100%). The β actin expression was used as a loading control. **D.** PTEN immunolabeling in serum free conditions with PPT or DPN stimuli. Arrows: cytoplasmatic PTEN staining, arrowheads: nuclear PTEN signal. *Bar*= 20 µm. Representative images of three independent experiments are presented. **E.** Morphometric analysis of nuclear PTEN expression. (*$P < 0.05$ vs. control, vs DPN).

Figure 5. ERβ expression in GH3β- and β+ cells by flow cytometry. A. Percentage of GH3 cells that expressed ERβ (*$P < 0.05$ vs. control GH3β-). 10^5 events for each experimental condition, Tukey test. **B.** ERβ quantification, representative images of one of the three independent experiments performed.

Figure 6. PTEN expression in GH3β+ cells. A. Western blotting analysis of PTEN expression in nuclear and cytoplasmatic fractions. Protein expression is represented as a percentage relative to the control (control GH3β- data were set to 100%). (*$P < 0.05$ vs. GH3β-, ▲$P < 0.05$ vs. control GH3β+). **B.** PTEN immunolocalization by confocal microscopy. Arrows: cytoplasmatic PTEN staining, arrowheads: nuclear PTEN signal. *Bar*=20 µm. Representative images of three independent experiments are presented. **C.** Morphometric analysis of nuclear PTEN expression. (*$P < 0.05$ vs. GH3β-; ▲$P < 0.05$ vs. control GH3β+). **D.** Western blotting analysis of total and phosphorylated Akt protein expression is represented as a percentage relative to the control (control GH3β- data were set to 100%) (*$P < 0.01$ vs. GH3β-). **E.** GH3β- and GH3β+ cells in the G0/G1 phase (*$P < 0.05$ vs. GH3β-).

Figure 7. PTEN expression in GH3β- cells. A. Western blotting analysis of PTEN expression in nuclear and cytoplasmatic fractions. Protein expression is represented as a percentage relative to the

control. **B.** Western blotting analysis of total and phosphorylated Akt protein expression is represented as a percentage relative to the control (*P < 0.05 vs. control). **C.** GH3 cells in the G0/G1 phase (*P < 0.05 vs. control).

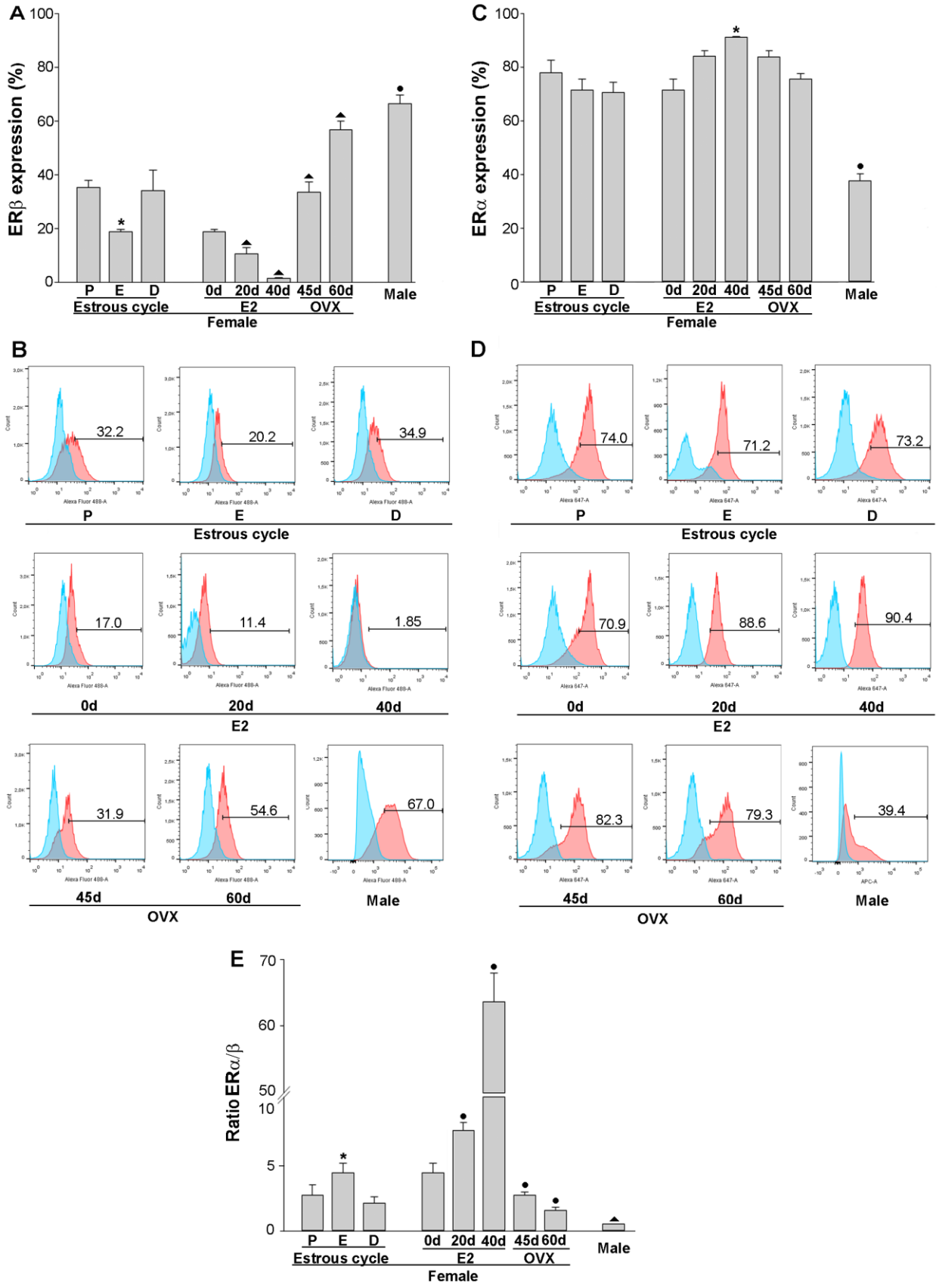
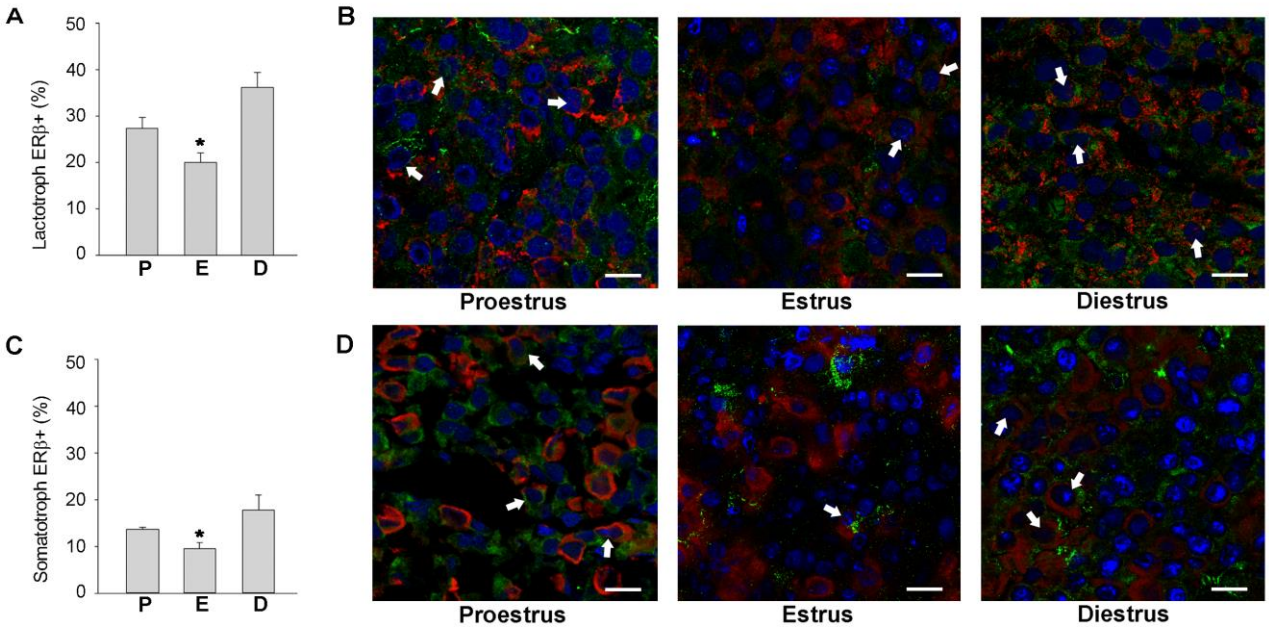


Figure 1



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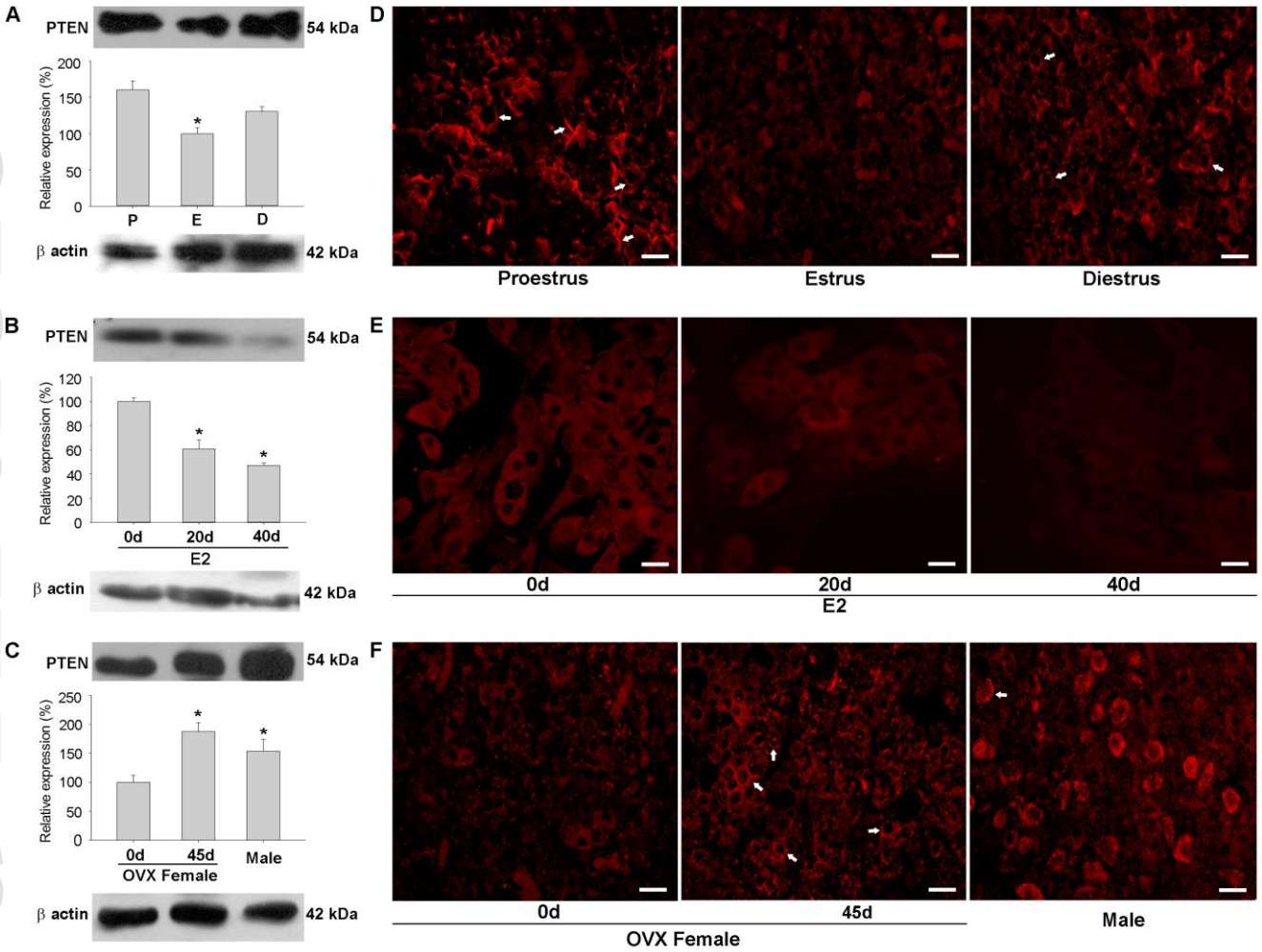

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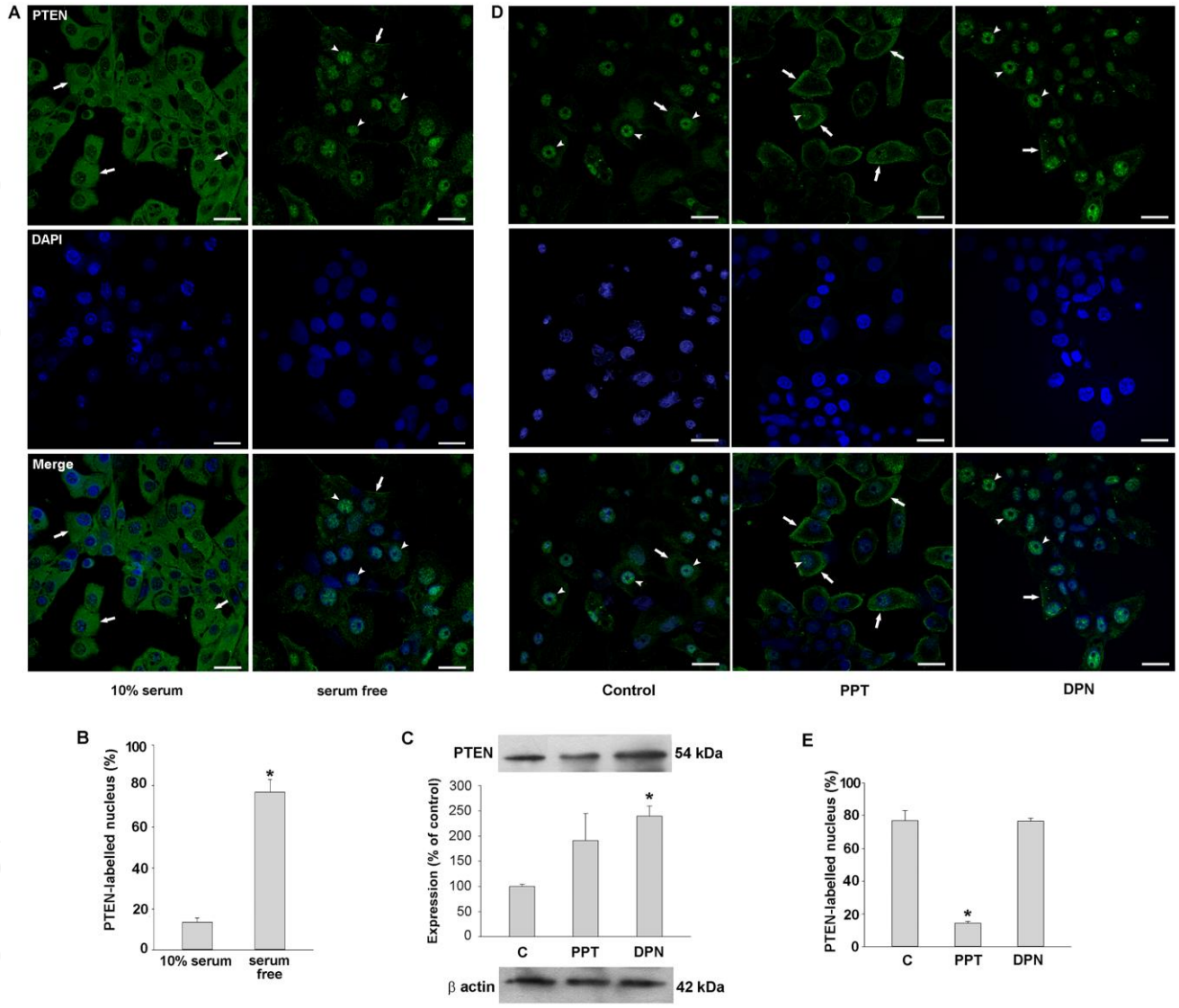

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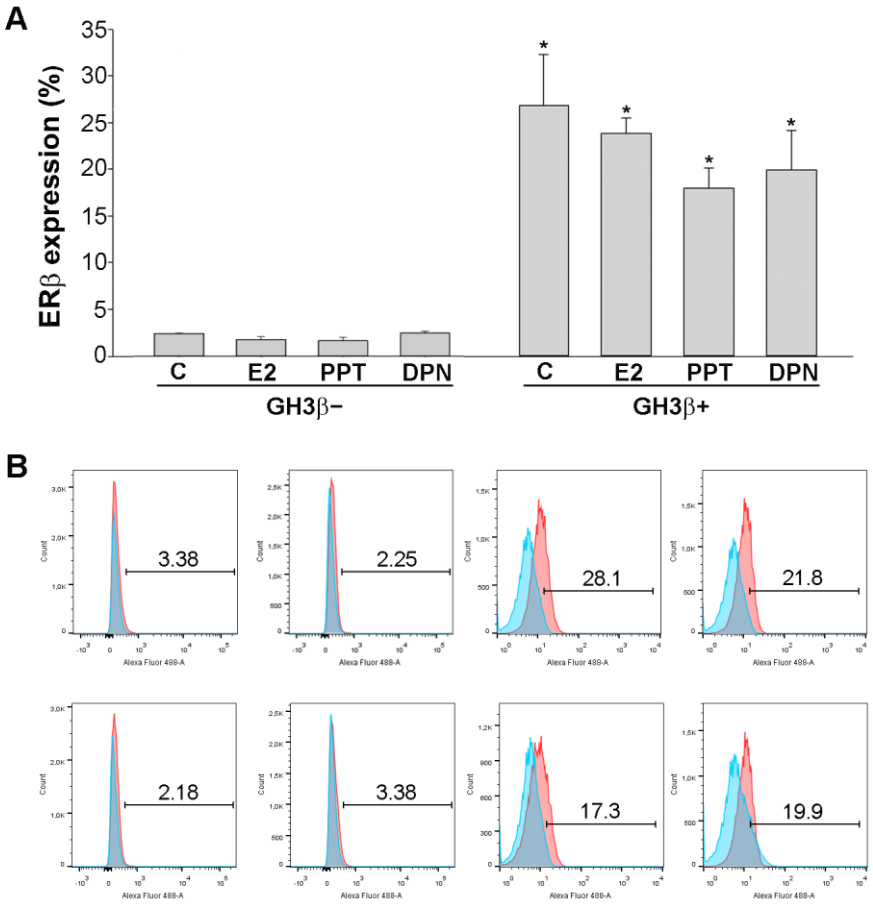

Figure 5

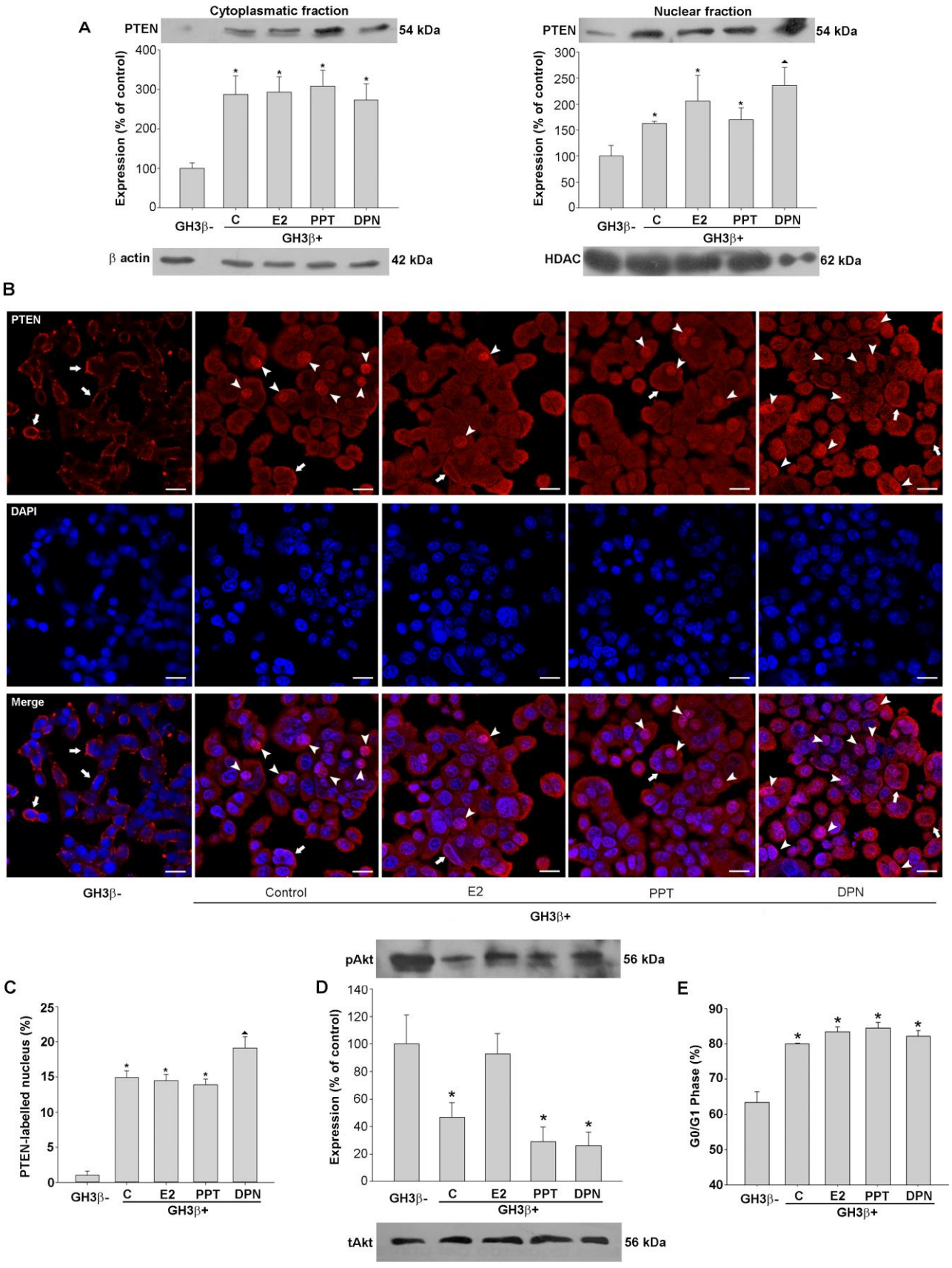

Figure 6

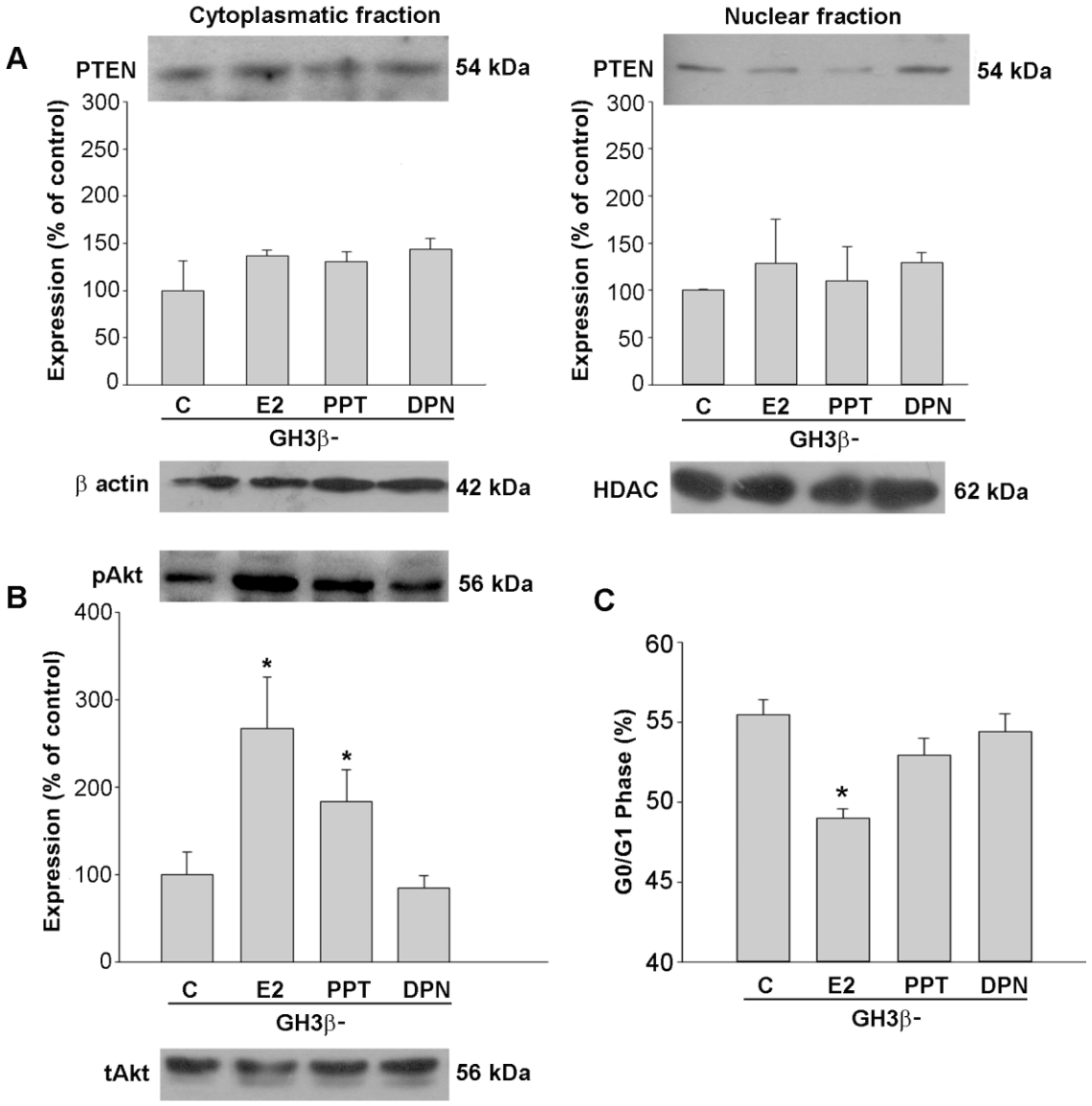

Figure 7