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The ERα membrane pool modulates the proliferation of pituitary tumours

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ABSTRACT

The molecular mechanisms underlying the ERα nuclear/cytoplasmic pool that modulates pituitary cell proliferation have been widely described, but it is still not clear how ERα is targeted to the plasma membrane. The aim of this study was to analyse ERα palmitoylation and the plasma membrane ERα (mERα) pool, and their participation in E2-triggered membrane-initiated signalling in normal and pituitary tumour cell growth. Cell cultures were prepared from anterior pituitaries of female Wistar rats and tumour GH3 cells, and treated with 10nM of estradiol (E2). The basal expression of ERα was higher in tumour GH3 than in normal pituitary cells. Full-length palmitoylated ERα was observed in normal and pituitary tumour cells, demonstrating that E2 stimulation increased both, ERα in plasma membrane and ERα and caveolin-1 interaction after short-term-treatment. In addition, the *Dhhc7* and *Dhhc21* palmitoylases were negatively regulated after sustained stimulation of E2 for 3h. Although the uptake of BrdU into the nucleus in normal pituitary cells was not modified by E2, a significant increase in the GH3 tumoral cell, as well as ERK1/2 activation, with this effect being mimicked by PPT, a selective antagonist of ERα. These proliferative effects were blocked by ICI 182780 and the global inhibitor of palmitoylation. These findings indicate that ERα palmitoylation modulated the mERα pool and consequently the ERK1/2 pathway, thereby contributing to pituitary tumour cell proliferation. These results suggest that the plasma membrane ERα pool might be related to the proliferative behaviour of prolactinoma, and may be a marker of pituitary tumour growth.

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INTRODUCTION

Oestrogens act as important regulators of cell proliferation, cell survival, and differentiation in a variety of organ systems and tissues and have been implicated in the aetiology of a variety of malignant cancers and benign tumours, such as pituitary adenomas (Spady, et al. 1999). Most of the effects of oestrogen are mediated through its two receptors: oestrogen receptor alpha (ERα) and beta (ERβ) (Mitchner, et al. 1998). ERα expression has been detected in both normal and tumour cells secreting PRL and gonadotropin (Friend, et al. 1994), and at higher levels in macroadenomas than in microadenomas, and in non-invasive tumours than in invasive ones (Meitzen, et al. 2013). It has been demonstrated that an oestrogen receptor antagonist inhibited pituitary tumour growth in a prolactinoma experimental model (Heaney, et al. 2002), thereby making ERα a potential target for the treatment of high ERα-expressing pituitary adenomas (Gao, et al. 2017).

In addition to the classic nuclear genomic action, oestrogens have been found to induce rapid effects within minutes of administration, which are mediated through a subpopulation of oestrogen receptors associated with the plasma membrane, a process usually termed “membrane-initiated steroid signalling” (MISS), “nongenomic” or “extranuclear” effects (Ueda and Karas 2013; Watson, et al. 2005). Related to this, we previously demonstrated that 17β-estradiol (E2) and FGF2 exerted a cooperative effect on lactotroph proliferation, principally by signalling initiated at the plasma membrane and mediated by the MEK/ERK1/2 pathway (Sosa Ldel, et al. 2013).

The molecular mechanisms underlying the ERα nuclear/cytoplasmic pool modulating adenohypophyseal cell activity have been widely described. Although, it is still not clear how ERα is targeted to the plasma membrane in normal and pituitary tumour cells, it has been reported that one of the requirements for ER to be located at the plasma membrane is the presence of a hydrophobic segment as part of the receptor structure (Marino, et al. 2006;

Morrill, et al. 2015). A post-translational modification of ERα has been previously described, which includes the addition of a palmitate molecule (S-acylation, commonly called palmitoylation) in cysteine residues of the ligand binding region of the gonadal steroid receptors (Acconcia, et al. 2005; Pedram, et al. 2007), by the palmitoyl-acyltransferases (PATs) DHHC7 and DHHC21 (Pedram, et al. 2012). Adding lipid residues increases hydrophobicity, promoting steroid receptor translocation to the caveolae regions of the plasma membrane (Peffer, et al. 2014; Razandi, et al. 2002), with the different isoforms of caveolin (caveolin-1, caveolin-2) being involved in this mechanism (Le Lay and Kurzchalia 2005; Totta, et al. 2015). ERα localisation in caveolae regions has been described in ovarian, prostate and breast tumour cells, suggesting an interaction between caveolin-1 and steroid receptors, which contributes to mERα localisation as well as to the activation of extra-nuclear estradiol signalling (Acconcia, et al. 2003; Park, et al. 2009; Pedram, et al. 2002). However, the functional role of palmitoylation in normal and pituitary tumour cell proliferation and signalling has not yet been explored.

The results reported in the literature are related only to total ERα expression, thus it is interesting to evaluate of mERα expression in pituitary tumours. In the present study, we speculated that the mERα pool modulates cell proliferation in pituitary tumours. Thus, we tested the hypothesis that the increase of mERα mediated by palmitoylation triggers ERK1/2 phosphorylation and consequently pituitary tumour cell growth.

MATERIALS AND METHODS

Cell cultures

A pool of three-month-old female Wistar rats, (n=12) , bred and housed at the Animal Research Facility of National University of Cordoba, was assigned to each culture taken at random cycle stages. The protocol for the dissociation of pituitary cells has been described

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previously (De Paul, et al. 2011). The normal pituitary primary culture includes different type of cells, with the lactotroph (54.1%) and somatotroph (21.8%) being the two principal cell populations (data not shown). After 3 days culture, the cells were maintained in DMEM without phenol red and serum for 24h before applying the treatments. The experiments were approved by the Institutional Animal Care Committee of the School of Medicine, University National of Cordoba.

The rat GH3 lactosomatotroph cell line is derived from rat prolacting-secreting pituitary tumors which synthesize both prolactin and growth hormone, and has been used as a prolactinoma model (Boockfor, et al. 1985; Chao, et al. 2014). The cells were cultured in Ham's F-12 medium, supplemented with 2.5% foetal bovine serum and 15% horse serum (Gibco, NY, USA). The cell cultures with a confluence of 80% were maintained in DMEM without phenol red and serum for 24h and then submitted to different experimental protocols

GH3 and primary adenohypophysis cells, were stimulated for 30min with E2 (10nM), a selective ERα agonist: 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol (10 nM, PPT) or EGF (10ng/mL). For some experiments, the cells were pre-incubated with the global inhibitor of palmitoylation, 2-bromohexadecanoic acid (2BP; 10μM) or ER pure antagonist: ICI 182780, for 30 min.

Determination of palmitoylated proteins by acyl-biotin exchange (ABE) assay

The palmitoylated proteins were determined by the ABE assay according to Wan (Wan, et al. 2007) with modifications. The cells were extracted in cold lysis buffer (1.25% Igepal CA-630, 1mM EDTA, and protease and phosphatase inhibitors), and the proteins were concentrated by precipitation in chloroform-methanol and suspended in the SB buffer (50mM Tris-HCl, pH7.4, 5mM EDTA, 4% SDS) with N-methylmaleimide (NEM-10mM). Next, 1mM NEM was added to the LB buffer (50mM Tris-HCl, pH7.4, 5mM EDTA, 150mM NaCl), which was incubated overnight at 4°C. The samples were divided into two equal

portions H and Tris; with the H samples being diluted in HB buffer (1M hydroxylamine, 150mM NaCl, 0.2%Triton and 1mM HPDP-Biotin) and the Tris samples being diluted with the same buffer without hydroxylamine. Purification of the biotinylated proteins was completed by diluting with LB buffer containing streptavidin-agarose beads. Finally, the samples were re-suspended in 35µl LB containing 0.1% SDS, 0.2% TritonX-100 and 1% β-mercaptoethanol and heated to 95°C. The proteins were analysed by western blot using primary antibody anti-ERα (1/200; Santa Cruz-Biotechnology, CA).

The GH3 cell line was transfected with the plasmid encoding SX8-Cherry as a control of palmitoylated proteins. The expression plasmid (1µg) and the transfection reagent PEI (2µl, Sigma Aldrich, USA) were added for 2h and then the GH3 cells were maintained in Ham's F-12 an additional 24h.

Analysis of cell-surface proteins by biotinylation

The cell cultures were washed with PBS buffer and the cell-surface labelled proteins were purified using a cell surface protein isolation kit (Pierce, Rockford, IL). The proteins from the supernatant and pellet fractions were analysed by western blot using the specific primary antibodies (Santa Cruz Biotechnology, CA): anti-phosphorylated ERα (1/200), anti-β-actin (1/1000), anti-FGFR (1/200) and anti-EGFR (1/400).

Immunoprecipitation

The protein extract was subjected to immunoprecipitation using anti-ERα (5µg/mL). The immune complexes were adsorbed and precipitated using protein G-Sepharose beads (Sigma Aldrich-St. Louis, MO, USA), washed and denatured by boiling for 5min in sample buffer. The samples were analysed by western blot using anti-ERα (1/200) and anti-caveolin-1 (1/1000; Cell Signaling Technology, Beverly, MA, USA).

Preparation of cell lysates for western blotting analysis

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The samples were lysed in cold lysis buffer and the total homogenate (50µg) was separated using 12% polyacrylamide gel. The proteins on nitrocellulose membrane were blocked with 5% non-fat dried milk and 0.1% Tween20 at RT and incubated overnight with primary antibodies 1/700 anti-diphosphorylated ERK1/2 (Sigma-Aldrich, St. Louis, USA) and 1/1000 anti-total ERK1 (Santa Cruz Biotechnology, Inc). The blots were incubated with peroxidase-conjugated anti-rabbit (1/5000) or anti-mouse (1/2500 Jackson Immunoresearch Labs Inc, PA, USA) secondary antibodies and then revealed with ECL detection reagents (Inmun-Star HRP-Substrate Kits, Bio-Rad, CA, USA). Finally, the emitted light was captured by the C-DiGit Chemiluminescence Scanner (LI-COR Biosciences), and signals were quantified with ImageJ software.

Gene expression analysis by qPCR

qPCR analysis of cDNA was performed on an ABI Prism 7500 detection system (Applied Biosystem, Foster City, CA) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, MA) and the upper and lower gene-specific primer sequences used were: DHHC-7 (NM_133394.1) 5'-GAGGATGGACCACCACTGTC-3' and 5'-CATGATAGCCAGCTCATGC-3'; DHHC-21 (XM_006238345.1) 5'-GAGGATGGACCACCACTGTC-3' and 5'-TCATGATAGCCAGCTCATGC-3'; DHHC-11 (NM_001039342.2) 5'-AACAACTTGACTTGGCCTACG-3' and 5'-GGCGAAAGAGTAGACAGCA-3'; and β-actin (NM_031144) 5'-CCCACACTGTGCCCATCTA-3' and 5'-CGGAACCGCTCATTGCC-3'.

Immunogold electron microscopy

The subcellular localisation of the ERα and caveolin-1 in normal and GH3 cells was examined by ultrastructural immunocytochemical techniques applying previously standardized protocols (Petiti, et al. 2015). Thin sections in the grids were incubated with anti-ERα (1/200) followed by anti-caveolin-1 (1/500) antibodies overnight at 4°C. Then the

sections were incubated with anti-rabbit or anti-mouse secondary antibodies conjugated to 15nm and 5nm colloidal gold particles (1/30, Electron Microscopy Science, USA) and examined in a Zeiss LEO 906-E transmission EM (TEM) (Zeiss, Oberkochen, Germany).

Immunofluorescence

For mERα staining, non-permeabilised live cells were incubated with ERα/Alexa fluor 594 for 15 min at 4°C prior to fixation. The ERα/Alexa fluor 594 complex was prepared by mixing an adequate dilution of ERα primary antibody and Alexa fluor 594 secondary antibody for 30 min at 4°C before incubation with the cells. Images were obtained using a Confocal Laser Scanning Microscope FluoView FV 300 (Olympus; Tokyo, Japan) and processed using FV10-ASW 1.6 Viewer software.

Correlative Light and Electron Microscopy (CLEM)

CLEM was carried out on ultrathin cryosections by applying the Tokuyasu technique as described by Oorschot (Oorschot, et al. 2014). The cryosections were transferred on formvar-coated 100µm mesh nickel grids and incubated with anti-ERα antibody 1/50 in 1% BSA-PBS, followed by incubation with anti-rabbit Alexa-Fluor594 (1/300, Invitrogen, California, USA) and DAPI (Sigma-Aldrich, St. Louis, USA) for 1h at 37°C.

For fluorescence light microscopy (FLM), grids layered with a 200nm coat of 2% methylcellulose were mounted with 50% glycerol. For TEM observation, grids were unmounted, washed in milli-Q water and incubated in 0.4% uranyl acetate/1.8% methylcellulose. Fluorescence images were obtained using a confocal laser scanning microscope FluoView FV 1200 (Olympus, Tokyo, Japan) and, EM images using a Zeiss LEO 906-E TEM. The analysis was carried out with ImageJ software.

Immunocytochemical detection of bromo-deoxyuridine uptake

Cells at the DNA synthesising stage were identified by immunocytochemical detection of BrdU. After 30min of E2 stimulation, BrdU (100nM) was added for an

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additional 24h. The cells attached to the coverslips were fixed in 4% formaldehyde in PBS for 2h at room temperature and BrdU incorporation detection was performed as described by Ferraris (Ferraris, et al. 2014). A total of 1000 cells were examined using a systematic process on each glass slide to establish the proportion of positive BrdU in the total cells.

Data analysis

A statistical analysis was carried out on three replicates measured from three independent cell cultures, with ANOVA-Tukey using InfoStat software (Grupo InfoStat, Facultad de Ciencias Agropecuarias, UNC). The results are given as the means ±SEM, and the significance levels were set at $p< 0.05$.

RESULTS

ERa expression in normal and pituitary tumour cells

First, we analysed the expression of total ERα in normal and pituitary tumour cells. The expression of ERα was higher in tumour GH3 than in normal pituitary cells and this did not change with the 30 min E2 treatment (Fig.1A). Next, to determine whether ERα was palmitoylated in normal adenohypophysis and in GH3 pituitary tumour cells, the ABE assay was carried out followed by western blot. ERα full-length expression was observed as bands at around 66 kDa, and this protein was detected as palmitoylated in line H of ABE, in normal adenohypophysis cells (Fig. 1B-top) and in the GH3 cell line (Fig. 1B-bottom). Furthermore, an additional ERα-immunoreactive band around 50kDa was detected in the palmitoylated proteins line in GH3 cells, possibly corresponding to the splicing variant of ERα. The SX8-Cher transfection in GH3 tumour cells showed a 70 kDa band in line H of ABE, confirming the presence of this palmitoylated protein (Fig. 1B-bottom).

E2 regulates Dhhc7 and Dhhc21 palmitoylase expression

Considering that the PATs are key to regulating the subcellular localisation of different ERα pools, *Dhhc7* and *Dhhc21* were evaluated by qPCR. As shown in Figure 1 C-

D, the *Dhhc7* and *Dhhc21* mRNA basal levels were higher in tumour than in normal cells. Then, we evaluated whether E2 was able to regulate the mRNA expression levels of these enzymes, with a significant decrease in *Dhhc7* (Fig. 1-E) and *Dhhc21* (Fig. 1-F) mRNA levels being observed in normal and GH3 cells stimulated with E2 for 3h compared to control. However, this reduction was transient, as *Dhhc7* and *Dhhc21* mRNA levels returned to baseline values after 6 or 9h of E2 treatment. The expression of *Dhhc11* mRNA levels, used as a negative control, did not vary after E2 treatment either cell type (Fig.1-G).

Membrane ERα expression is regulated by palmitoylation

To explore whether palmitoylation could promote changes in mERα expression, pituitary cells were pre-treated with 2BP, the global inhibitor of palmitoylation, and then stimulated with E2 for 30min. In unstimulated and nonpermeabilized pituitary cells, endogenous ERα specific immunostaining was observed at the plasma membrane in some normal pituitary and pituitary tumour cells. However, E2 treatment for 30 min increased ERα expression at the plasma membrane, which was more frequently observed in tumour cells, and was reverted when the cells were pre-treated with 2BP (Fig. 2A-C). We visualised the expression of ERα by CLEM (Fig.2B and D), which enabled simultaneous observation of a given subcellular structure. In normal (Fig.2B) and tumour GH3 (Fig.2D) cells, the ERα was localised at the plasma membrane when the cells were treated with E2 for 30 min.

The changes in mERα expression in pituitary cells were analysed by cell surface biotinylation. As shown in Figure 2, western blot analysis revealed the presence of ERα in the pellet fraction (containing the cell surface biotinylated proteins) and in the supernatant fraction (with the intracellular unbiotinylated proteins). In the pellet fraction, under baseline conditions, lower ERα protein expression was observed in both normal (E) and tumour (F) cells, whereas E2 treatment for 30 min significantly increased mERα expression, which was completely reversed by the 2BP pre-treatment.

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E2 induces ERα and caveolin-1 association

As interaction between ERα and caveolin-1 has benn described in different tissues (Peffer et al. 2014; Wang, et al. 2011), we evaluated whether E2 could promote any interaction in pituitary cells by using a co-immunoprecipitation assay. As shown in Figure 3, in normal (A) and GH3 (C) unstimulated cells, a basal interaction was observed between both proteins, which was significantly increased by E2 treatment. Interestingly, 2BP treatment was able to reverse the E2-induced ERα/caveolin-1 interaction, revealing similar expression levels as the controls.

In addition, we analysed the fine localisation of ERα with caveolin-1 by means of TEM immunogold labelling in normal (B) and tumour GH3 (C) cells. As shown in Figures 4B and C, the immunoreactivity for ERα (15nm gold particle) was distributed in the cytoplasm and occasionally in the plasma membrane in normal and tumour control cells, whereas ERα localisation was frequently observed the plasma membrane, with caveolin-1 (5nm gold particles) being close to each other in E2-treated cells.

The involvement of palmitoylation and mERα in cell proliferation

To analyse the contribution of mERα to cell proliferation, we determined the BrdU uptake into the nucleus of normal and pituitary tumour cells incubated with a palmitoylation inhibitor. The percentage of control normal BrdU positive cells was 2.6%, with no changes observed after the different treatments (Fig. 4 A-B). However, in non-stimulated GH3 cells, the proliferation was around 30%, showing a significant increase after E2 stimulation that was mimicked for PPT respect to control. The E2 effect was blocked partially by ICI 182780 and the global inhibitor of palmitoylation, 2BP (Fig. 4 C-D).

Considering previous results from our laboratory concerning the involvement of the MEK/ERK1/2 and PI3K/AKT pathways in pituitary tumour cell proliferation (Petiti, et al. 2010; Petiti et al. 2015), we determined the phosphorylation of ERK1/2 and AKT in pituitary

tumour cells. Figure 4E shows the significant increase in phosphorylated ERK1/2 after E2 and PPT treatments observed for 30 min, which was blocked when the cells were pre-incubated with ICI 182780 or 2BP, suggesting that ERα palmitoylation may be required to activate these kinases. The expression of phosphorylated AKT increased after E2 or PPT treatments for 30 min, while pre-incubation with ICI 182780 or 2BP did not revert this activation, suggesting that AKT may contribute to the pituitary tumour proliferation induced by E2 in a mERα-independent manner.

Additionally, we tested if the effect of the inhibitor of palmitoylation could affect the cell response to different growth stimulatory factors. With this aim, we analyzed the proliferation and activation of ERK1/2 in GH3 cells stimulated with the epidermal growth factor (EGF) for 30 min, in the presence or absence of the pre-incubation with 2BP. As shown in figure 4, the EGF treatment significantly increased the uptake of BrdU and ERK1/2 phosphorylation (Fig. 4 F-G), effects that were not reverted when the cells were pre-treated with 2BP, suggesting that the cell response to palmitoyation inhibitor is ER specific.

The above results indicate that the plasma membrane ERα localisation mediated by ERα palmitoylation triggers ERK1/2 phosphorylation and consequently pituitary tumour cell growth.

DISCUSSION

This study, found that the subpopulation of ERα localised in the plasma membrane induced pituitary tumour proliferation by the mREα/cavelin1/ERK1/2 pathway. The E2 stimuli significantly increased mERα expression, ERα interaction with caveolin-1, ERK1/2 phosphorylation, and finally led to pituitary tumour proliferation, which were partially reversed by the PAT inhibitor.

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We previously identified the presence of ERα in the plasma membrane in normal pituitary cells (Gutierrez, et al. 2008), and it has also been demonstrated that E2 stimulates the translocation of endogenous ERα and the activation of the PKCα/ERK1/2 pathway (Gutierrez, et al. 2012; Watson, et al. 2012; Zarate, et al. 2012), without any effect on cell proliferation (Sosa Ldel et al. 2013). Considering that lactotroph cells represent the main phenotype in adult female rat pituitaries that express ERα, and that GH3 cells have been employed as a prolactinoma model, we compare the ERα expression in GH3 vs. normal pituitary cells. The analysis of mERα expression by western blot and immunofluorescence, reveled an increased level of this receptor in tumour cells compared to normal pituitary cells. The involvement of mERα in the rapid pro-apoptotic action of oestradiol in normal pituitary cells has been previously demonstrated (Zarate et al. 2012). In contrast, in pituitary tumour GH3B6/F10 cells, high levels of mERα mediated rapid signalling responses to oestrogens, which culminated in functional changes such as prolactin release, cell proliferation, apoptosis, and changes in cell shape (Jeng, et al. 2009; Jeng and Watson 2011). However, these studies in both normal and pituitary tumour cell, did not reveal the contribution of palmitoylation to ERα translocation to the plasma membrane. In the present investigation, we detected palmitoylated ERα in normal and GH3 pituitary tumour cells for the first time, and demonstrated that E2 stimulated ERα expression in the plasma membrane, which was reverted by the palmitoylation inhibitor. Thus, palmitoylation (a reversible posttranscriptional modification) should be considered to be more than just a simple membrane association of soluble proteins. In fact, the palmitoylation status of several proteins has also been linked to their activation and the regulation of the traffic and function of both the nuclear/cytoplasmic and the membrane receptor pool (Fukata and Fukata 2010).

In addition to full-length mER, we detected an ERα palmitoylated variant at around 50kDa in the membrane fraction of pituitary tumour cells. In agreement with this, other

authors have reported that, as well as full-length 66-kDa ERα, truncated forms of this receptor were present in various organs, produced by alternate ER mRNA splicing or specific post-translational processing, often outside the nucleus. In agreement, a 46-kDa truncated variant has been shown to be preferentially palmitoylated and enriched in the cell membranes of endothelial, osteoblast, and breast cancer cells (Denger, et al. 2001; Li, et al. 2003; Marquez and Pietras 2001), and palmitoylation inhibitors were able to block ER-46 membrane localisation (Acconcia et al. 2005). Moreover, variants of lower molecular weights (~39 kDa and ~22 kDa) were detected in the membrane fraction of anterior pituitary cells (Zarate et al. 2012) and breast cancer cells, suggesting that these ERα variants may be considered as a target of palmitoylation and result in their localisation in the plasma membrane (Li et al. 2003; Wang, et al. 2006).

Palmitoyl-acyltransferase isoform expression and localisation is tissue-specific (Ohno, et al. 2006), with at least a dozen of the 23 human DHHC genes having been implicated in tumour growth (Yeste-Velasco, et al. 2015). DHHC7 and DHHC21 are the proteins responsible for the palmitoylation of the sex steroid oestrogen, progesterone and androgen receptors. DHHC-7 and -21 knockdown studies have shown that PATs are required for endogenous ER palmitoylation, membrane trafficking, and rapid signal transduction in cancer cells (Pedram et al. 2012). In the present study, we observed greater *Dhhc7* and *Dhhc21* mRNA expression in tumour cells than in normal pituitary cells, which may be associated with the proliferative behaviour of GH3 cells. It has been reported that the *Dhhc21* gene is significantly overexpressed in human breast cancer compared with normal breast epithelium. It is possible that alterations in the steroid receptor PAT abundance or function contribute to increased ER at the plasma membrane in some situations (Pedram et al. 2012), thereby making the DHHC-7 and -21 proteins attractive novel targets to selectively inhibit membrane sex steroid receptor localisation and function in pituitary tumours.

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Although s-acylation is known to be a major regulator of localisation of cellular protein and pathways, there is still little information about how the dynamics of this process is regulated. It has also been reported that palmitoylation regulation may occur via a regulatory mechanism occurring at the mRNA level of the DHHC enzymes (Chai, et al. 2013). In this study, E2 treatment for 3h reduced both *Dhhc7* and *Dhhc21* mRNA expression, whereas at 30 min the plasma-membrane ERα pool and the interaction ERα/caveolin were increased. The current knowledge of estrogen molecular action includes the ability of the E2–ER complex both to induce gene transcription (Smith and O'Malley 2004) and to evoke the membrane starting activation of specific rapid phosphorylation cascades (ERK/MAPK) (Yang, et al. 2004). Both these processes are integrated and influence the cellular response to estrogen, thus highlighting the ER regulation at genomic and nongenomic levels. The fast action/membrane of E2 (30 min) was not in line with that observed after sustained stimulation of E2 for 3 h, which downregulated the mRNA levels of *Dhhc7* and *Dhhc21*, probably as a compensatory mechanism to regulate the ERα pool at the plasma membrane. These results are in agreement those of an investigation that, demonstrated that E2 stimulation for 1 to 4h decreased by 60% the [3H]-palmitate incorporated into ERα in HeLa cells, suggesting that ERα palmitoylation is negatively modulated by E2 (Acconcia et al. 2005).

The relationship between ERα and caveolin appears to be important for determining E2 effects on different cell types, with it having been demonstrated that caveolin-1 is an essential for joining ERα to the cell membrane, and that this process is facilitated by prior ER palmitoylation (Pedram et al. 2007). Our results revealed that the ERα/caveolin-1 interaction increased after E2 stimulus in normal as well as in GH3 pituitary tumour cells. Concurring with our data, an ERα/caveolin-1 interaction was demonstrated in enriched mERα GH3/B6/F10 pituitary tumour cells (Watson et al. 2012). In addition, it has been reported

that, as oestradiol is highly concentrated in isolated caveolae, it readily engages ERα bound to caveolin-1, which serves as a scaffold for membrane-localised signalling molecules (Peffer et al. 2014). Therefore, caveolin may be a fundamental scaffolding protein whose activation maximises membrane hormone effects and leads to specific biological consequences. This idea is supported by caveolin knockdown rats as they, showed a reduction in membrane ERα functions, thereby suggesting that trafficking of ERα to the plasma membrane is mediated by caveolin (Christensen and Micevych 2012). It has also been demonstated that caveolin-1 protein down-regulation leads to ERα signalling deregulation in mammary epithelia (Wang et al. 2011). We observed that the ERα/caveolin-1 association was palmitoylation-dependent, as indicated by the decrease in this association after palmitoylation inhibition. A non-palmitoylable ERα-Cys477Ala mutant was unable to localise at the plasma membrane, interact with caveolin-1, or generate E2-induced rapid membrane-starting signal pathways to regulate cell proliferation (Acconcia et al. 2005). Moreover, it has been demonstrated that ERα rapid de-palmitoylation and decoupling the ERα action mechanisms impair the activation of the ERK/MAPK and PI3K/AKT signal transduction pathways (Levin 2005; Totta, et al. 2004). These reports are in agreement with our results, where it was observed that pre-incubation with 2BP in GH3 tumoral cells decreased the ERα and caveolin-1 interaction, as well as ERK1/2 phosphorylation, suggesting that palmitoylation is necessary for a mediated E2 effect.

Several members of the MAP kinase signalling pathway, including Src, Shc, and ERKs, are clustered in caveolae-specialized membrane invaginations that are enriched in the caveolin-1 scaffolding protein and compartmentalise signal transduction (Okamoto, et al. 1998). ERα activation may trigger cell proliferation mediated by ERK (Jeng et al. 2009; Watson et al. 2012; Watson, et al. 2010), with the MEK/ERK1/2 pathway being involved in the pathogenesis of several types of tumours including pituitary adenomas (Ebbesen, et al.

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2016; Vlotides, et al. 2008). It has been reported that the inhibition of ERK1/2 signalling reduced cell viability in rat tumour cells after exposure to a general antagonist of ER (Gao et al. 2017). Here, we observed that the estrogen antagonist and the palmitoylation inhibitor prevented the activation of ERK1/2 and resulted in a decrease in ERα expression levels in the plasma membrane as well as in cell pituitary proliferation, indicating that rapid E2-induced signals require ER localisation at the plasma membrane. Pedram et al reported that knockdown of DHHC7 or 21 significantly impaired the ability of E2 to stimulate ERK in breast cancer cells (Pedram et al. 2012). In addition, the expression of ERα without a palmitoylated site interfered with endogenous ER function and inhibited E2-induced ERK activation, cyclin D1 production, cdk4 activity, and G1/S progression, suggesting that the inhibition of mERα expression and its association with the modulation of ERK activity could be put forward as an important therapeutic intervention in breast cancer (Razandi, et al. 2003).

It is generally accepted that oestrogens act as potent mitogens through ERα, exerting a sustained, dose-dependent trophic stimulus on anterior pituitary proliferation (Nolan and Levy 2009). In our study, in primary pituitary cells with a basal proliferation of 2.6%, E2 treatment was unable to modify this mitotic rate. In contrast, in pituitary tumour cells, which exhibited a high basal proliferative activity, E2 stimulation triggered an increase in BrdU uptake with a significant contribution of mERα. Furthermore, palmitoylation inhibition induced a significant decrease in cell proliferation, which was consistent with previous reports showing that mERα contributes, together with the nuclear ERα pool, to the induction of tumour cell proliferation (Razandi et al. 2003). In addition, significantly increased ERα localization in the plasma membrane has been associated with aggressive breast cancer behaviour or resistance to endocrine therapy (Fan, et al. 2007; Yang et al. 2004).

Moreover, these result indicated that oestrogens were able to trigger a proliferative response in the pituitary tumour cell, associated with high levels of ERα and the activation of ERK1/2 signalling (Jeng et al. 2009; Watson, et al. 2008). In breast cancer cells, E2-ERα-induced cell transition through G1 to the S phase of the cell cycle, which significantly blocked by 2BP or by inhibitors of MEK, suggesting that membrane localisation of palmitoylated ERα leads to a signal transduction that contributes to cell cycle progression (Pedram et al. 2007). Additionally, the ERK/MAPK and PI3K/AKT pathways, activated by the E2–ERα complex, cooperatively promote the G1/S transition (Acconcia et al. 2005; Marino, et al. 2002). In our study, we observed that the palmitoylation inhibitor induced a partial reversion in the tumour proliferation by E2 in a pERK1/2 dependent and pAKT independent manner.

The differences in the proliferation effect observed between normal and pituitary tumour cells under E2 treatment may be explained by the high ERα expression in tumour cells compared to normal cells, as well as by the undetectable subtype REβ expression in GH3 pituitary tumour cells as was previously reported in our laboratory. In addition, we determined the specific role of ERβ in the E2 proliferative effect in normal, hyperplastic and pituitary tumour cells, with this hormone being able to increase pituitary cell proliferation only in cells with a high ERα/β ratio, showing that ERβ exerts an inhibitory role on the mitogenic activity of pituitary cells (Perez, et al. 2015). The reason that different cellular phenotypes can respond to the same hormone in a different manner may be due to the diverse expression patterns of ERα and ERβ (McDonnell and Norris 2002).

In summary, our results showed that E2 modulated ERα palmitoylation, enhancing the mERα pool and consequently activating the ERK pathway, thereby contributing to pituitary tumour cell proliferation. These findings suggest that mERα could be related to the proliferative behaviour of prolactinoma and be a possible marker of pituitary tomour growth.

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The authors would like to make the following declarations about their contributions: Conceived and designed the experiments: LS, JP and AT. Performed the experiments: LS, JP, SC, JN, FP, and PP. Analyzed the data: LS, JP, SC, JN and FP. Manuscript preparation: LS, JP, ADP, JV, SG and AT.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1

A- Basal expression of total REa in normal and pituitary tumour cells and after E2 treatment for 30 min.

B- Protein extracts from pituitary cells were tested for palmitoylated ERα following the ABE assay. Biotinylated proteins were purified, separated by SDS-PAGE and stained with anti-REα antibody. ERα palmytoilation is prominent in protein extracts that were treated with hydroxylamine (H). In addition, in GH3 transfected SX8-Cher tumour cells, palmitoylated SX8 was used as a control of palmitoylated protein. The Input in A and B was 10% of total protein extract and Tris: pull down without H.

Relative mRNA expression levels of *Dhhc7* (C) and *Dhhc21* (D) in normal pituitary and GH3 tumour cells. *Dhhc7* (E), *Dhhc21* (F) and *Dhhc11* (G) mRNA expression levels after treatment with E2 (10nM) at 3, 6 and 9 h. A significant decrease in the *Dhhc7* and *Dhhc21* mRNA levels was observed in normal and GH3 cells stimulated with E2 for 3h compared to control. Expression levels were calculated by quantitative real-time PCR analysis. The β-actin gene was used as the internal reference gene and the ΔΔCT method was used for relative quantification and expressed as fold over control: * $p<0.05$ *Dhhc7* vs C and ^ $p<0.05$ *Dhhc21* vs C.

Figure 2

Cell surface immunostaining of ERα in non-permiabilised normal (A) and tumour GH3 (B) cell cultures treated with E2 (10nM) for 30 min with or without pre-treatment of 2BP. Microphotograph represent the merging of Light transmitted and immunofluorescence field.

CLEM of normal pituitary (C) and GH3 tumour (D) rat cell cultures were treated with E2. [1] Immunofluorescence labelling for ERα, [2] TEM images for the whole section and [3] CLEM

overlay of TEM on the corresponding FLM image. Arrows indicate ERα at plasma membrane. Bar: 10μm

Biotinylation of cell-surface biotinylated proteins in normal pituitary and tumour GH3 rat cells. Surface membrane proteins were biotinylated in normal control (E) and tumour GH3 (F) pituitary cells or in cells treated with E2 (10 nM) for 30 min with or without 2BP pre-incubation. Whole cell lysates were subjected to avidin pull-down using streptavidin-agarose beads, and the recovered cell-surface biotinylated proteins (pellet) and intracellular unbiotinylated proteins (supernatant) were analysed by western blot and stained with anti-REα antibody. In the pellet fraction, ERα expression showed an increase after E2 treatment but a decrease after pre-treatment with 2BP. The ERα expression did not change after treatment in the supernatant. The expression of the FGF and EGF receptors and β-actin confirmed equivalent total protein loading. Images correspond to a representative experiment from a total of three with similar results. Values are expressed as mean ± SEM. * $p<0.05$ E2 vs C and ^ $p<0.05$ E2-2BP vs E2.

Figure 3

Association between ERα and caveolin 1. Normal pituitary (A) and tumour GH3 (C) rat cell cultures were treated with E2 (10nM) for 30 min with or without pre-treatment of 2BP. The total cell extracts were used for immunoprecipitation (IP) using anti-ERα and the immunoprecipitates were then probed with anti-cav-1. *$P<0.01$ E2 vs. C and ^ $p<0.05$ E2-2BP vs E2. In total anterior pituitary cell culture lysates, both antibodies recognised the antigens (input).

Electron micrographs of normal pituitary (B) and tumour GH3 (D) with double immune-labelling of ERα (15 nm gold particle) and cav-1 (5nm gold particle). The black arrows

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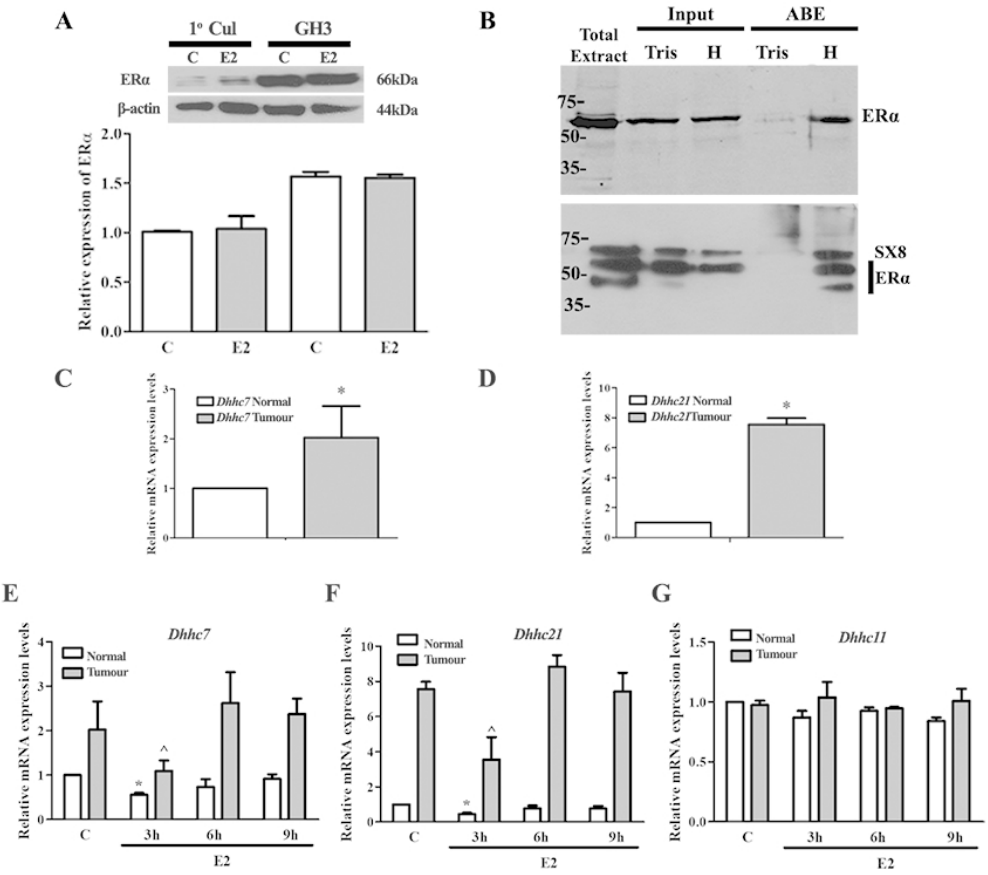
indicate the ERα-cav-1 adhered to the plasma membrane and the white arrows indicate the cytosolic ERα. g=granule; Bar: 100nm

Figure 4

Representative micrographs of immunohistochemical staining for BrdU (red) and quantitative analysis in normal (A-B) and tumour GH3 (C-D) cell cultures. The cells were treated with E2 (10nM) or PPT (10 nM) for 30 min with or without pre-treatment with 2BP or ICI 182780 (I, 100 nM). BrdU was added for an additional 24h and the data represent the proportion of positive BrdU cells in the total cells. Significant differences were found in GH3 cell proliferation: *p<0.01 E2 or PPT vs. C and ^ p<0.05 I+ E2 vs E2. #p<0.05 E2 or PPT vs.E2 or PPT and ~p<0.05 2BP-I+E2 vs.E2. Bar: 100μm. (E) Western blotting of ERK1/2 and Akt in total extract from tumour pituitaries treated with E2 (10nM) or PPT (10 nM) for 30 min with or without pre-treatment of 2BP or ICI 182780 (I, 100 nM). The amount of protein levels was normalised by comparison with total ERK1/2 and AKT expression A representative panel of three independent experiments is shown. (F-G) Quantitative analysis of BrdU staining and western blotting of ERK1/2 in total extract from cells treated with EGF (10ng/mL) for 30min with or without pre-treatment with 2BP (10uM). Significant differences were found in GH3 cell proliferation: *p<0.01 EGF vs. C, while that cell proliferation and ERK1/2 phosphorylation did not change with 2BP treatment.



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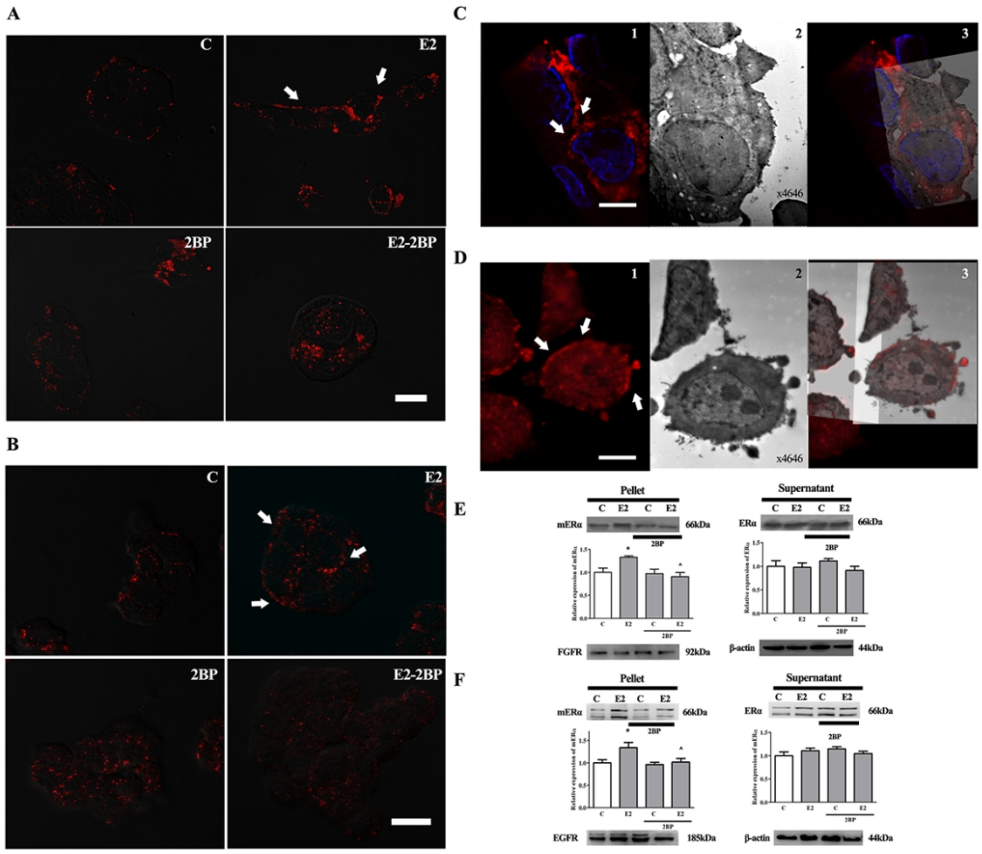

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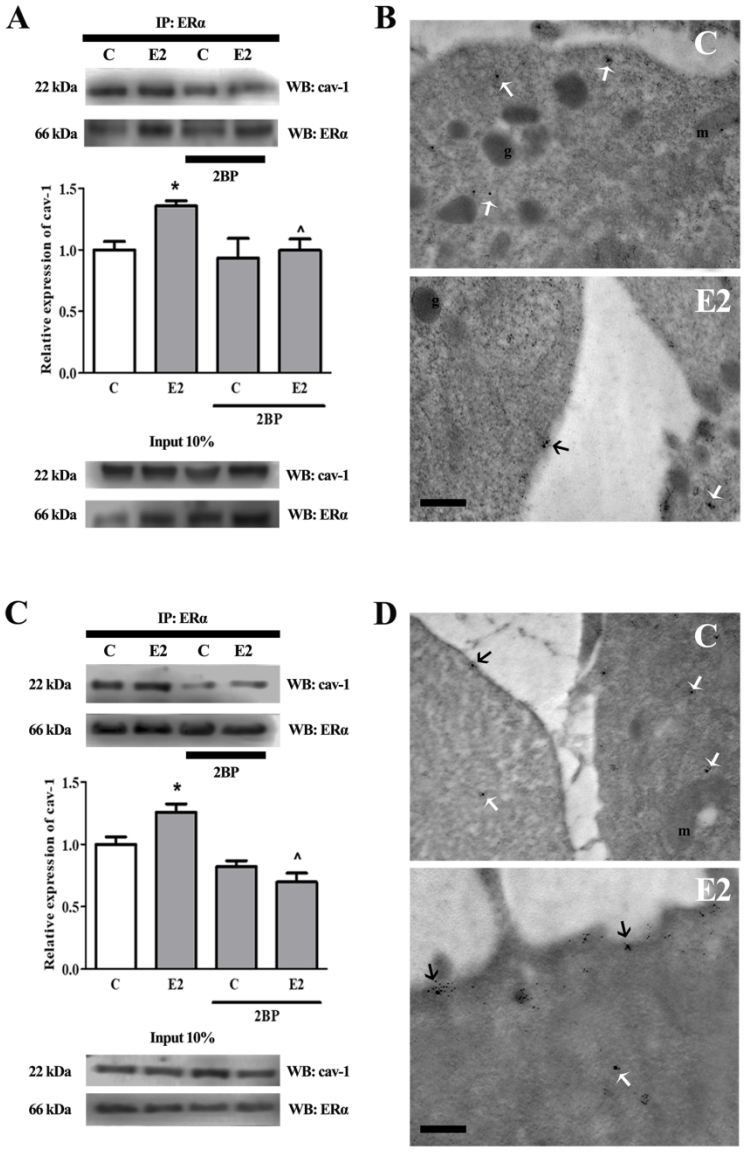

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194x299mm (200 x 200 DPI)

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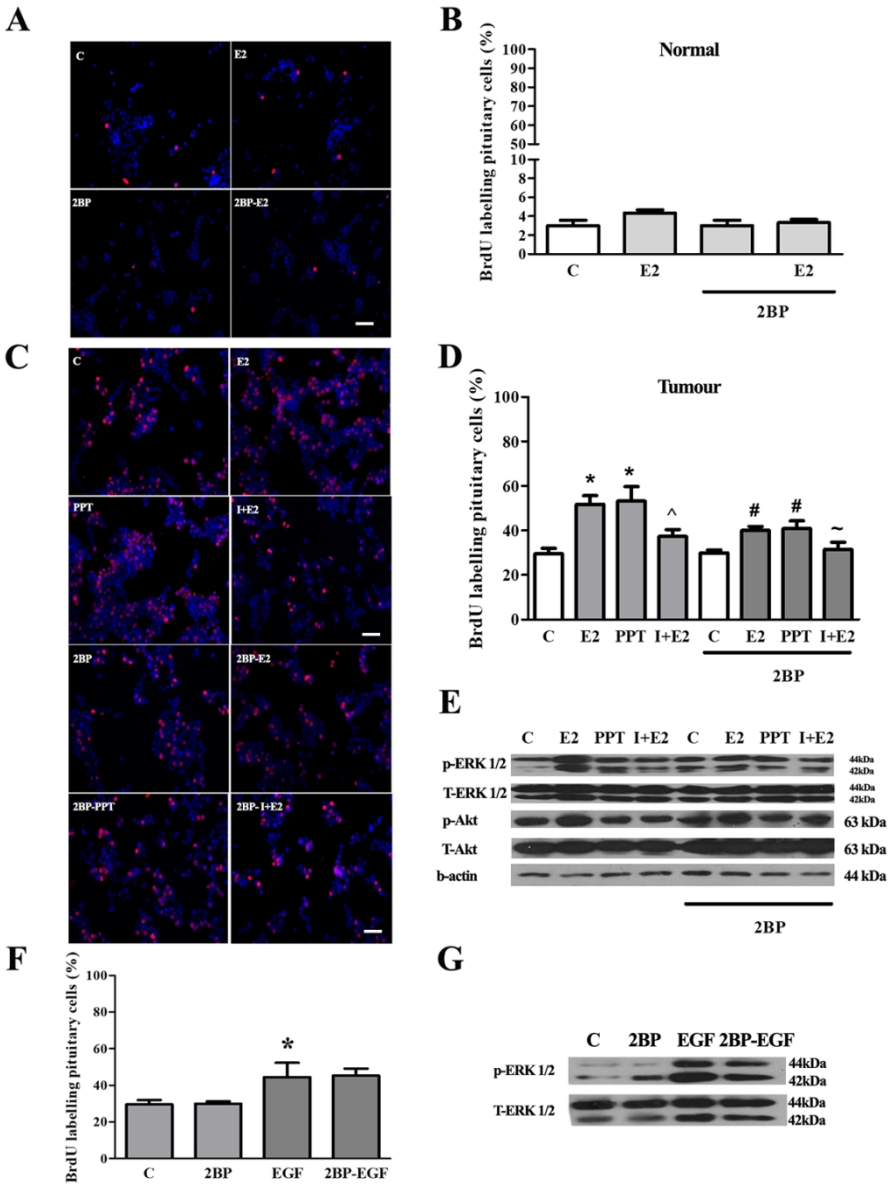

Figure 4: Representative micrographs of immunohistochemical staining for BrdU (red) and quantitative analysis in normal (A-B) and tumour GH3 (C-D) cell cultures. The cells were treated with E2 (10nM) or PPT (10 nM) for 30 min with or without pre-treatment with 2BP or ICI 182780 (I, 100 nM). BrdU was added for an additional 24h and the data represent the proportion of positive BrdU cells in the total cells. Significant differences were found in GH3 cell proliferation: *p<0.01 E2 or PPT vs. C and ^ p<0.05 I+ E2 vs E2. #p<0.05 E2 or PPT vs.E2 or PPT and ~p<0.05 2BP-I+E2 vs.E2. Bar: 100µm. (E) Western blotting of ERK1/2 and Akt in total extract from tumour pituitaries treated with E2 (10nM) or PPT (10 nM) for 30 min with or without pre-treatment of 2BP or ICI 182780 (I, 100 nM). The amount of protein levels was normalised by comparison with total ERK1/2 and AKT expression A representative panel of three independent experiments is shown. (F-G) Quantitative analysis of BrdU staining and western blotting of ERK1/2 in total extract from cells treated with EGF (10ng/mL) for 30min with or without pre-treatment with 2BP (10uM). Significant differences were found in GH3 cell proliferation: *p<0.01 EGF vs. C, while that cell proliferation and ERK1/2 phosphorylation did not change with 2BP treatment.