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PRECLINICAL STUDY

Inhibition of mammary tumor growth by estrogens: is there a specific role for estrogen receptors alpha and beta?

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Abstract To evaluate the extent to which each estrogen receptor (ER) subtype contributes to the stimulation or to the inhibition of mammary tumor growth, we evaluated the effects of specific agonists in MC4-L2 cells, which are stimulated by 17β-estradiol (E_2), and in mammary carcinomas of the MPA mouse breast cancer model, which are inhibited by E_2. Both express ERα and ERβ. In MC4-L2 cells, 4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT; ERα agonist) and (4-hydroxy-phenyl)-propionitrile (DPN; ERβ agonist) stimulated cell proliferation, whereas the opposite occurred in C4-HI primary cultures. The inhibitory effect was associated with a decrease in ERα and cyclin D1 expression and an increase in progesterone receptor (PR) expression as well as in the Bax/Bcl-xl ratio. In vivo, mice carrying C4-HI or 32-2-HI tumors were treated with E_2, PPT or DPN (3 mg/kg/day) or with vehicle. PPT and DPN inhibited tumor size, as did E_2, during the first 72 h. After a few days, DPN-treated tumors started to grow again, while PPT-treated tumors remained quiescent for a longer period of time. A pronounced decrease in the mitotic index and an increase in the apoptotic index was associated with tumor regresion. All treated tumors showed: (a) an increase in integrin α6 and Bax expression, (b) an increased stromal laminin redistribution, and (c) a decrease in ERα, Bcl-xl and Bcl-2 expression ($P < 0.001$). Apoptosis-inducing factor (Aif) expression was increased in DPN-treated tumors, while active caspase 9 was up-regulated in PPT-treated mice, demonstrating the involvement of the intrinsic apoptotic pathway in estrogen-induced regression in this model. In conclusion, our data indicate that although there may be some preferences for activation pathways by the different agonists, the stimulatory or inhibitory effects triggered by estrogens are cell-context dependent rather than ER isoform dependent.

Keywords Aif, apoptosis · Bax · Bcl-xl · Breast cancer · Caspase-9 · DPN · Estrogen receptors alpha · Estrogen receptors beta · Mammary carcinomas · PPT · Progesterone receptors · Tumor regression

List of abbreviations used

Aif	Apoptosis-inducing factor
Ch-FCS	Steroid-stripped fetal calf serum
DPN	(4-hydroxy-phenyl)-propionitrile
E_2	17-β-estradiol

Rocío Soldati and Victoria Wargon had equal participation.

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ER	Estrogen receptor
ERα	ER alpha
ERβ	ER beta
H&E	Hematoxylin and eosin
HPF	High power field
MPA	Medroxyprogesterone acetate
PI	Propidium iodide
PR	Progesterone receptor
PR_A	PR isoform A
PR_B	PR isoform B
PPT	4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol
RU	RU-486
Sc	Subcutaneous

Introduction

Clinically, more than 60% of breast carcinomas are ERα-positive and respond to an endocrine therapy aimed either to block estrogen synthesis or to alter ER signaling pathways [1], supporting a growth stimulatory effect for estrogens in breast cancer. In addition, estrogens stimulate cell growth of most of the human breast cancer lines expressing ERα [2], and they are an absolute requirement (in most cases) for their in vivo growth as xenografts [3]. Their role in the development and progression of breast cancer has been studied for years [4, 5]. Paradoxically, estrogens have also satisfactorily been used to treat breast cancer (Haddow A, cited in Lewis et al. [6, 7]). Inhibitory effects on mammary tumor growth have been observed in several models, including ERα- [8, 9] or ERβ-transfected cells [10], the T61 human tumor line [11], the mouse mammary tumors from the MPA-breast cancer model [12], and human breast cancer cells with acquired tamoxifen resistance or with PKCα over expression [13, 14].

Sustained antiproliferative and proapoptotic effects are necessary to induce complete tumor regression. Estrogen-induced apoptosis and cytostasis has been demonstrated in different studies [6, 15–17]. Apoptosis is a form of programmed cell death that is executed by a family of proteases named caspases, which can be activated either by cell-surface death receptors, as is the case for caspase 8 (i.e., the extrinsic pathway), or by perturbation of the mitochondrial membrane (i.e., the intrinsic pathway), as in the case of caspase 9. The intrinsic pathway centers on the mitochondria, which contain key apoptogenic factors such as cytochrome *c* and apoptosis-inducing factor (Aif). Once cytochrome *c* is released into the cytosol, it interacts with apoptotic protease activating factor-1 (Apaf-1) and procaspase 9, leading to the cleavage of procaspase 9 into caspase 9, which is capable of proteolytically activating downstream caspases that then initiate the apoptotic degradation phase [18]. Aif translocates into the nuclei and induces a caspase-independent chromatin condensation and DNA fragmentation [19, 20]. The integrity of mitochondrial membranes is controlled mainly by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the Bcl-2 family. The Bcl-2 protein family contains antiapoptotic members, including Bcl-2/Bcl-x_L, and proapoptotic members, such as Bax. An increased Bax/Bcl-2 or Bax/Bcl-xl ratio is associated with increased apoptosis [21].

Several key proteins involved in proliferation or apoptosis are regulated by hormones. Cyclin D1 and PCNA, a processivity factor for DNA polymerase [22], are established proliferation markers regulated by estrogens or progesterone [23, 24]. In addition, clusterin, initially described as testosterone repressed message 2 (TRPM-2), has been implicated in tissue remodeling (e.g., in changes secondary following androgen ablation in the prostate [25] or in the involution of the mammary gland following weaning [26]). It is also up-regulated in hormone responsive tumors [27], and its role is likely antiapoptotic, inducing the sequestration of Bax [28]. Bcl-2 and Bcl-xl are up-regulated in progestin- or E_2-stimulated cells [29, 30].

Estrogens control transcriptional responses through their ability to bind to two different nuclear ER isoforms, alpha (ERα) and beta (ERβ), which differentially activate promoter elements, thereby inducing specific biological responses. It has been suggested that ERα mediates proliferative responses, while ERβ modulates the ERα-mediated responses, in the mammary gland in an inhibitory fashion [31]. Moreover, the ratio of ERα/ERβ seems to be important in determining the extent of the proliferative or antiproliferative responsiveness [10, 32]. Similar dual effects have been reported for organs other than the mammary gland. An increase in cell proliferation was observed in papillary carcinoma thyroid cells, while apoptosis was induced in anaplastic carcinoma cells. This differential responsiveness has been ascribed to the different subcellular distribution of ER isoforms [33].

In recent years, the development of ERα- and ERβ-specific agonists has provided much needed pharmacological tools to study the role of individual ER isoforms in growth regulation. PPT (4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol) is an ERα-specific agonist that displays roughly a 400-fold selectivity for ERα as compared with ERβ, and it has no effects on ERβ-mediated transcriptional activity [34]. DPN (4-hydroxy-phenyl)-propionitrile) is an ERβ-selective agonist that displays approximately 80-fold more selectivity for ERβ than for ERα, and its relative potency in transcriptional assays is 170-fold greater for ERβ than for ERα [35]. Both agonists are being extensively used in immunology, physiology, and

neuroscience research, but not as yet in breast cancer research.

We developed an experimental murine model in which metastatic ductal mammary carcinomas express ERα, ERβ, and progesterone receptors (PRs) [36]. In response to estrogen [12] or antiprogestin treatment [15, 37], these tumors may regress completely. Interestingly, tamoxifen and the pure anti-estrogen fulvestrant (ICI 182780) also inhibit tumor growth [38]. Paradoxically, the cell lines developed in this model are growth-stimulated by E_2, and in vitro, they behave similarly to MCF-7 human breast cancer cells [39, 40], thus providing an excellent way to study the dual effect of estrogens.

The aim of this series of experiments was to evaluate the effects of PPT and DPN in the MC4-L2 cell line, in which cell proliferation is stimulated by E_2, and in C4-HI and 32-2-HI tumors, in which proliferation is inhibited by E_2. We found that both agents induced similar stimulatory or inhibitory effects to E_2 in both experimental settings in vitro. However, in vivo, only PPT was able to exert a sustained inhibitory effect. The inhibitory effects of E_2, PPT, and DPN were associated with an increase in the Bax/Bcl-xl or Bax/Bcl-2 ratio, but the post-mitochondrial mechanisms involved in the induction of apoptosis were different for each agonist. While DPN preferentially activated a caspase-independent apoptotic pathway, increasing Aif expression, PPT-induced activation of caspase 9.

Materials and methods

Animals

Two-month-old virgin female BALB/c mice (IBYME Animal Facility) were used. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [41]. The protocols were approved by the Institutional Bioethical Committee.

Tumors

C4-HI is a transplantable ductal mammary tumor derived from C4-HD, which was induced by medroxyprogesterone acetate (MPA) in a BALB/c female mouse. 32-2-HI is the HI variant derived from 32-HD [36]. Both tumors express ERα, ERβ, and PRs and regress with antiprogestin or estrogen treatment [42, 43].

Reagents

E_2, MPA, and RU 38486 (RU; mifepristone) were obtained from Sigma Chem. Co. (St Louis MO, USA), and PPT and DPN were from Tocris (Bristol, UK) and were prepared according to the manufacturer's instructions.

Cell lines

MC4-L2 was developed in our laboratory from the C4-HD tumor [39]. It expresses both ERs and PRs, and it is stimulated by E_2 and MPA. Passages 30–35 were used. The human breast cancer cell line, MCF-7 developed by Soule et al. [44], was obtained from ATCC, and passages 3–8 were used.

Primary cultures

Culture media

DMEM/F12 (Dulbecco's modified Eagle's medium: Ham's F12, 1:1, without phenol red, Sigma Chem. Co.), 100 U/ml penicillin and 100 μg/ml streptomycin with 10% fetal calf serum (FCS; Life Technologies Inc., Gaithersburg, MD, USA or Bioser, Buenos Aires, Argentina) was used. Steroid-stripped FCS (chFCS) was prepared as described previously [45].

Primary cultures

Epithelial cells from the C4-HI tumor were isolated and separated from other cells by differential sedimentation [45, 46] and plated with 10% FCS. After attachment, the medium was replaced with fresh medium with 10% FCS, and thereafter, it was changed every 2–3 days.

Cell proliferation

Primary cultures

This assay was performed as previously described [45]. Briefly, cells were seeded into 96-well microplates. After attachment (24 h), the cells were incubated for 24 h with 1% chFCS and then for 48 h with the experimental solutions to be tested in 1% chFCS. Fifty percent of the medium was replaced with fresh medium every 24 h. The cells were incubated with 0.4 μCi of ^{3}H-thymidine (specific activity: 20 Ci/mmol) for 24 h, trypsinized and harvested in a cell harvester. Filters were counted in a liquid scintillation counter. The assays were performed in octuplicates, and the means and standard deviations were calculated for each solution tested.

Cell counting

Cells were seeded in 12-well plates and treated, as described above, in triplicate. After 6 days of treatment,

the cells were trypsinized and counted in Neubauer chambers. Media were refreshed every 2 days.

FACS analysis

Cells were seeded into 6-well plates and treated as explained above for 24 h. Then cells were trypsinized, fixed in 70% ethanol for 24 h, and resuspended in PBS buffer containing 50 μg/ml of propidium iodide (PI), 0.1% sodium citrate, and 50 μg/ml ribonuclease A (Sigma). After 30 min of incubation at room temperature, the samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences).

In vivo experiments

C4-HI or 32-2-HI tumors were subcutaneously (sc) transplanted into BALB/c female mice. When the tumors reached a size of $\sim$50 mm^2, they were treated sc with daily doses of 3 mg/kg body weight of E_2, PPT, or DPN for 2 weeks. Control mice received vehicle or were implanted sc with 5 mg E_2 pellets. Tumor size was evaluated by daily measurement of their length and width using a Vernier Caliper. Three mice/group were autopsied 24, 48, or 72 h after treatment was initiated, and samples were kept in liquid nitrogen or fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin-eosin (H&E) for histological diagnosis. The rest of the animals (4/group) were followed for 2 weeks. Tumors were weighed at the end of the experiment. Vaginal smears were performed in treated animals for 5 consecutive days. Animals carrying 32-2-HI tumors were also treated with E_2 pellets, and the animals were euthanized at different times (3-48 h).

Morphological studies

The morphology of tumor parenchyma (growth pattern, differentiation) and stroma, as well as mitosis and apoptosis, were evaluated on H&E stained sections. The latter were counted in 10 and 15 high power fields (HPF), respectively, of each section, using 1,000× magnification, and expressed as the mean ± standard error (SE) of the percentage of the ratios between the total number of events (mitosis or apoptosis) and the total cell number per HPF. Mitotic figures were identified morphologically by the condensed "hairy" aspect of the chromosomes. Morphological identification of apoptosis was performed according criteria previously reported, which correlated with the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method [15].

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissue were reacted with various antibodies using the avidin biotin peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Briefly, endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Blocking solution (2% normal goat serum) was used before specific antibody addition. Polyclonal antibodies to ERα (MC-20), PR (C-19), Aif (sc-5586), Bax (N-20, sc-493), Bcl-xl (s-18; sc-634), and Bcl-2 (sc-783) were all from Santa Cruz, activated caspase 9 was from Abcam (ab52298), and the monoclonal PR_A (Ab-7) was from Neomarkers (Lab Vision Corp, Fremont, CA). They were all used, unless otherwise specified, at a 1:200 dilution and were incubated overnight at 4°C. Microwave (750 W Philips M902) antigen retrieval (four cycles of 5 min each in 0.1 M citrate buffer) was used before ERα, Bax and Bcl-xl staining. The reactions were developed with 3-3'diaminobenzidine, 0.30 mg % in PBS and H_20_2 at a final concentration of 0.5%, under microscopic control. Specimens were lightly counterstained with 10% hematoxylin, dehydrated and mounted. For quantification, hematoxylin staining was removed by adding 0.5% periodic acid, and the intensity of staining was recorded and quantified using Image J. Tiff images (RGB-8 bytes) were analyzed. For each image, the ratio between the integration of the histogram of the red channel and the integration histogram of all channels (RGB) was calculated. The integration of each histogram was calculated as the sum of the multiplication of every byte of intensity (1-256) and the corresponding population of pixels. The value that was ascribed for each image was the difference between this value and the one obtained in a white picture.

Immunofluorescence

Frozen sections were treated with primary antibodies to integrin α6 (BD Pharmigen, San Diego, CA), laminin (LY Laboratories, San Mateo, CA) or PR_A (C-19), dissolved in blocking buffer, at a 1:100 dilution overnight at 4°C. They were then incubated with FITC-conjugated secondary antibodies (1:100 dilution) for 1 h at room temperature. The nuclei were counter stained with either PI or 4′,6-diamino-2-phenylindole (DAPI, Sigma). Sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed under a Nikon laser confocal microscope.

C4-HI or MC4-L2 cells grown in chamber slides were starved for 24 h and incubated for 24 h with E_2, PPT or DPN in the presence of 1% chFCS. Then, they were fixed in ethanol for 1 h, air-dried and the slides processed as described for frozen tissues. For ERβ detection, the

monoclonal Antibody from Santa Cruz was used (sc-53494).

Western blots

Cell extracts

Tumors were homogenized and processed to obtain total fractions for western blots, as previously described [47]. In order to prepare cell culture extracts, the cells were lysed using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL), following the manufacturer's instructions.

Electrophoresis and blotting

Western blotting was performed as previously described [47]. The membranes were incubated with β-actin (clone ACTN05, Neomarkers, Lab Vision Corp, Fremont, CA), PR (C-19), ERα (MC-20), Bax (N-20, sc-493), Bcl-xl (s-18; sc-634) and Erk (sc-94), all from Santa Cruz.

Statistical analysis

Western blot band intensities and cell staining was quantified with Image Quant® software. ANOVA and the Tukey multiple post *t* test were used to analyze the differences of means of multiple samples; the Student's *t* test was used to compare the means of two different groups. In all graphs, the mean ± SEM is shown, and experiments were repeated at least three times. Regression analysis was used to compare tumor growth curves.

Results

E_2, PPT, and DPN inhibit the proliferation of primary cultures of C4-HI and stimulate the proliferation of MC4-L2 cells

C4-HI tumors are a hormone-independent variant of the C4-HD carcinoma originated in an MPA-treated BALB/c mouse [36]. E_2 inhibits the growth of the ERα- and ERβ-positive C4-HD and C4-HI mammary carcinomas in vivo and in vitro [45, 46]. In order to establish whether ERα and/or ERβ mediate E_2-induced inhibition of cell proliferation, we studied the effects of two specific agonists: PPT (ERα agonist) and DPN (ERβ agonist). Both agonists inhibited MPA-induced ^{3}H-thymidine uptake ($P < 0.001$). The inhibition was significant starting at 0.01 nM for PPT and at 0.001 nM for DPN. The concomitant addition of both agonists at 1 and 100 nM showed no additive effect

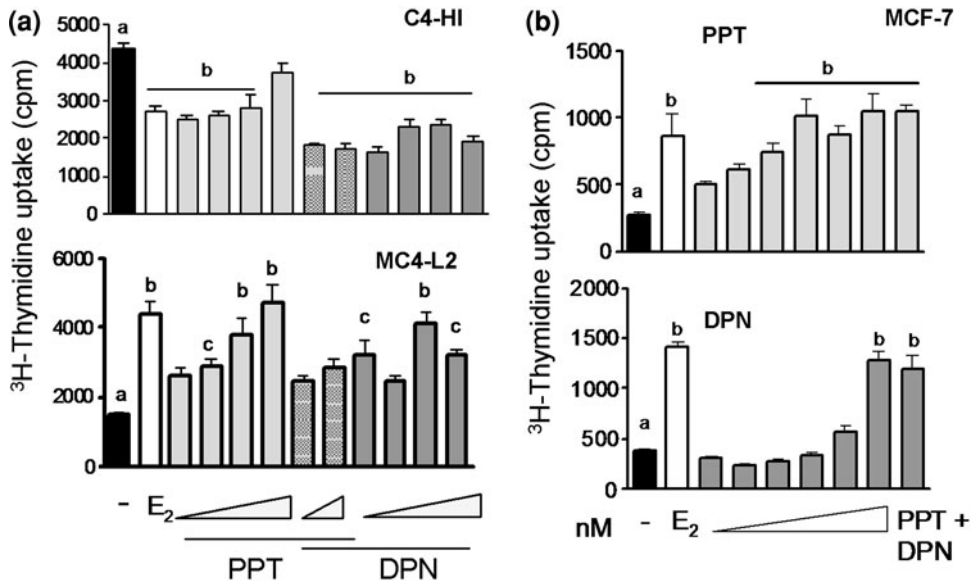

Fig. 1 Effects of E_2, PPT, and DPN on ^{3}H-thymidine uptake. **a** Primary cultures of murine C4-HI tumor cells (*top*) or MC4-L2 cells (*bottom*) were plated in 96-well plates. After 48 h, cells were incubated with 1% chFCS (plus 10 nM MPA for C4-HI cells), 1 nM E_2, or different concentrations (0.001, 0.01, 1, and 100 nM) of PPT, DPN, or (1 or 100 nM) PPT plus DPN, as explained in the "Materials and methods" section. Cells were then incubated for another 48 h. (^{3}H)-thymidine was added in the last 18 h before harvesting. All compounds induced an inhibitory effect in MPA-treated C4-HI cells, while a stimulatory effect was observed in MC4-L2 cells; **a** vs. **b**: $P < 0.001$; **a** vs. **c**: $P < 0.05$. A representative experiment of the other three is shown. **b** MCF-7 cells were similarly treated with 1 nM E_2 or different concentrations (0.001–100 nM) of PPT or DPN or with 1 nM PPT plus 1 nM DPN, as explained in the "Materials and methods" section. PPT stimulated ^{3}H-thymidine uptake at concentrations higher than 0.1 nM and DPN at concentrations higher than 10 nM. PPT plus 1 nM DPN induced a similar stimulatory effect as 1 nM PPT; **a** vs. **b**: $P < 0.001$. A representative experiment of the other three is shown; the mean ± SEM cpm is plotted on the *y* axis

(Fig. 1a). Inhibitory effects were seen in the presence (Fig. 1a, top) or absence (not shown) of MPA.

MC4-L2 is an estrogen-responsive cell line, also derived from C4-HD, which express ERα [39] and ERβ (mRNA measured by qPCR; not shown). Cell proliferation is increased in response to E_2 [39]. Treatment with concentrations as low as 0.01 nM of PPT or DPN induced a stimulation of ^{3}H-thymidine uptake similar to that induced by E_2 ($P < 0.001$) The effects of PPT and DPN were not additive at 1 or 100 nM (Fig. 1a, bottom).

For both experimental systems, the agonists induced changes similar those induced by E_2. To further assess the specificity of the agonists, we used MCF-7 cells, which express almost no ERβ [10, 48], and evaluated the effects of PPT and DPN under the same experimental conditions and concentrations as those used for MC4-L2 cells. PPT induced a dose-dependent increase in ^{3}H-thymidine uptake ($P < 0.001$; Fig. 1b). The ERβ agonist DPN was not stimulatory at concentrations lower than 1 nM, which was expected. However, it did stimulate cell proliferation at concentrations higher than 1 nM, which may be attributed to its ability to bind ERα at higher concentrations. As mentioned previously, DPN displays an 80-fold higher selectivity for ERβ compared to ERα. These data suggest that at concentrations of 1 nM or even lower, DPN exerts a pure ERβ agonistic activity.

E_2, PPT, and DPN, induces proapoptotic gene expression only in C4-HI cells

The cells were counted after 6 days of treatment to confirm the inhibitory effect of DPN and PPT on C4-HI proliferation. E_2, PPT, and DPN inhibited MPA-induced increases in cell number (Fig. 2a, $P < 0.001$). Similarly, the increase in cyclin D1 expression induced by MPA was also blocked by all three compounds at the same concentrations (Fig. 2b).

An increase in Bax expression was detected by western blot with all three compounds, and, while no significant changes were observed in Bcl-xl expression (Fig. 2c), the Bax/Bcl-xl ratio was increased in treated cells ($P < 0.05$). Fas-L expression did not change in treated cells (not shown), and no differences in Bax or Fas-L were seen in similarly treated MC4-L2 cells (not shown). These data

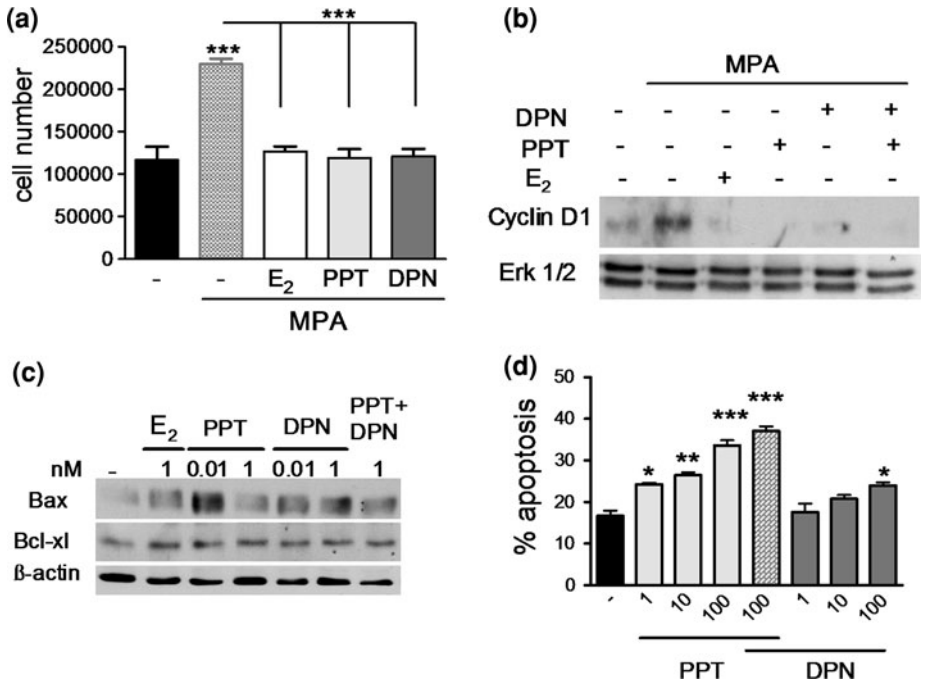

Fig. 2 Effects of E_2, PPT, and DPN on cell proliferation and apoptosis. **a** Primary cultures of C4-HI tumor cells were plated in 12-well plates. After 48 h, cells were starved with 1% chFCS for 24 h and then incubated with 10 nM MPA with or without 1 nM E_2, PPT, or DPN for 6 days. The cell medium was replaced with fresh medium every 2 days. Cells were trypsinized and counted in Neubauer chambers (mean ± SEM). All compounds induced an inhibitory effect in MPA-treated C4-HI cells; *** $P < 0.001$ vs. control. **b** C4-HI cells were seeded in 6-well plates, treated as described in A for 24 h, and processed for western blots. All compounds decreased MPA-induced cyclin D1 expression. **c** C4-HI cells treated with E_2, PPT, and/or DPN were processed for western blot analysis and were immunoblotted using Bax, Bcl-xl, Fas-L, or β-actin antibodies. An increase in Bax (23 kDa) was observed in all treated cells. No changes in Bcl-xl (30 kDa) expression were observed. β-actin was used as a loading control. **d** C4-HI cells were treated with PPT or DPN, and after 24 h, they were processed for apoptosis analysis by FACS. An increase in cells undergoing apoptosis was observed in treated cells (mean ± SEM); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

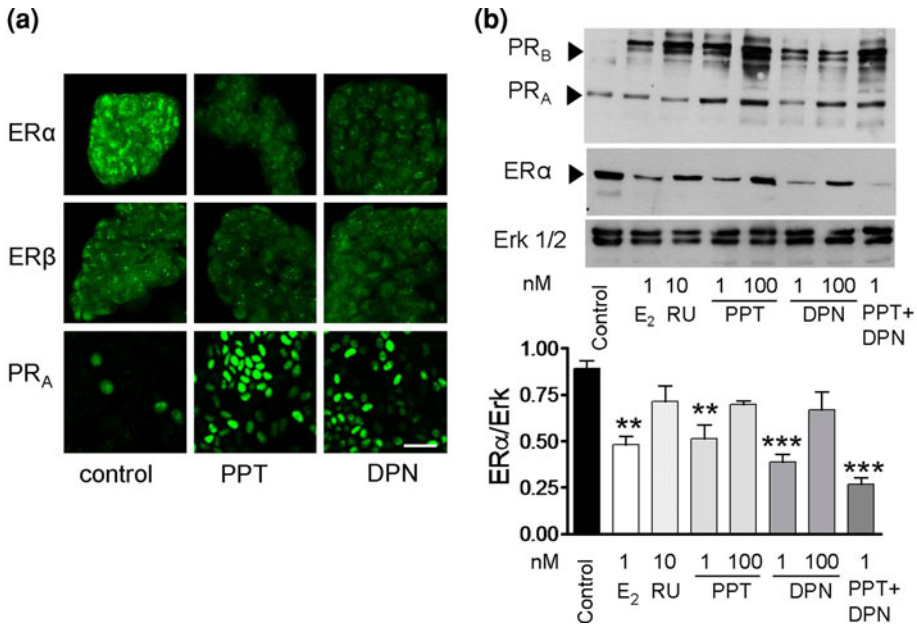

Fig. 3 ER and PR regulation by E_2, PPT and DPN in C4-HI cells. **a** C4-HI cells were grown in chamber slides, and after starving them for 24 h, they were incubated for 24 h with 1 nM PPT or DPN. Immunofluorescence assays were performed using the polyclonal MC-20 antibody for ERα, the monoclonal antibody for ERβ (sc-53494), and the monoclonal Ab-7 for PR_A. FITC-labeled secondary antibodies were used. PPT and DPN induced a similar decrease in ERα expression and an increase in PR_A expression. A slight inhibition was observed for ERβ; *bar*: 50 μm. **b** Western blots: C4-HI cells growing in Petri dishes were treated with 1 nM E_2, 10 nM RU-486 (RU; control) or 1 and 10 nM PPT and DPN, respectively. The C-19 antibody was used to detect both PR isoforms: PR_B (115 kDa) and PR_A (83 kDa). MC-20 was used to detect ERα (66 kDa). Erk was used as a loading control. The intensity of ERα expression was quantified in relation to Erk. It should be noted that 1 nM E_2, PPT, DPN, or PPT + DPN decreased ERα expression. ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control

correlated with an increase in apoptosis observed mainly in PPT-treated cells by FACS analysis ($P < 0.05$: Fig. 2d). These results suggested that both ERα and ERβ are involved in estrogen-induced growth inhibitory signaling in vitro in this model.

E_2, PPT, and DPN down regulate ERα and up-regulate PRs in primary cultures of C4-HI cells

E_2 down regulates ERα and increases PR expression. In order to evaluate possible differential effects between PPT and DPN in the regulation of ERα and PR expression in C4-HI cells, immunofluorescence and western blot studies were performed. By immunofluorescence, it was observed that PPT decreased ERα staining and increased nuclear PR staining after 24 h of incubation (Fig. 3a). DPN induced similar effects in the same line as those induced by PPT, although less pronounced. Western blot data confirmed immunofluorescence data and indicated that 1 nM PPT or DPN was more efficacious in decreasing ERα expression ($P < 0.01$) as compared with higher concentrations (100 nM). All compounds increased PR expression (Fig. 3b). RU 486, which also inhibits C4-HI cell proliferation [46], showed a similar pattern of PR regulation as E_2. Again, in both cases, the agonists induced changes similar to those induced by E_2.

E_2, PPT, and DPN inhibit in vivo tumor growth, inducing cytostasis and apoptosis

C4-HI and 32-2-HI tumors regress upon antiprogestin or estrogen treatment [42, 43, 47]. Having characterized the effects of PPT and DPN in vitro, we were interested in evaluating their effects in vivo, in comparison with those of E_2. Tumors were transplanted sc in BALB/c mice, and when they reached a size of 30 mm^2 (C4-HI) or 70 mm^2 (32-2-HI), the treatments were initiated. We chose a larger size for 32-2-HI tumors because they regress very fast after endocrine treatment [43]. During the first 48–72 h, in both C4-HI and 32-2-HI, PPT and DPN inhibited growth to an extent comparable to that of E_2 (Fig. 4a, top). However, after a few days, DPN-treated tumors started to grow faster, nearly reaching the size of control tumors after 14 days of treatment. PPT-treated tumors remained the same size (32-2-HI; $P < 0.001$) or grew very slowly (C4-HI; $P < 0.001$). E_2-treated tumors experienced a significant reduction in size ($P < 0.001$), which was similar in animals implanted with a 5 mg E_2 pellet or treated

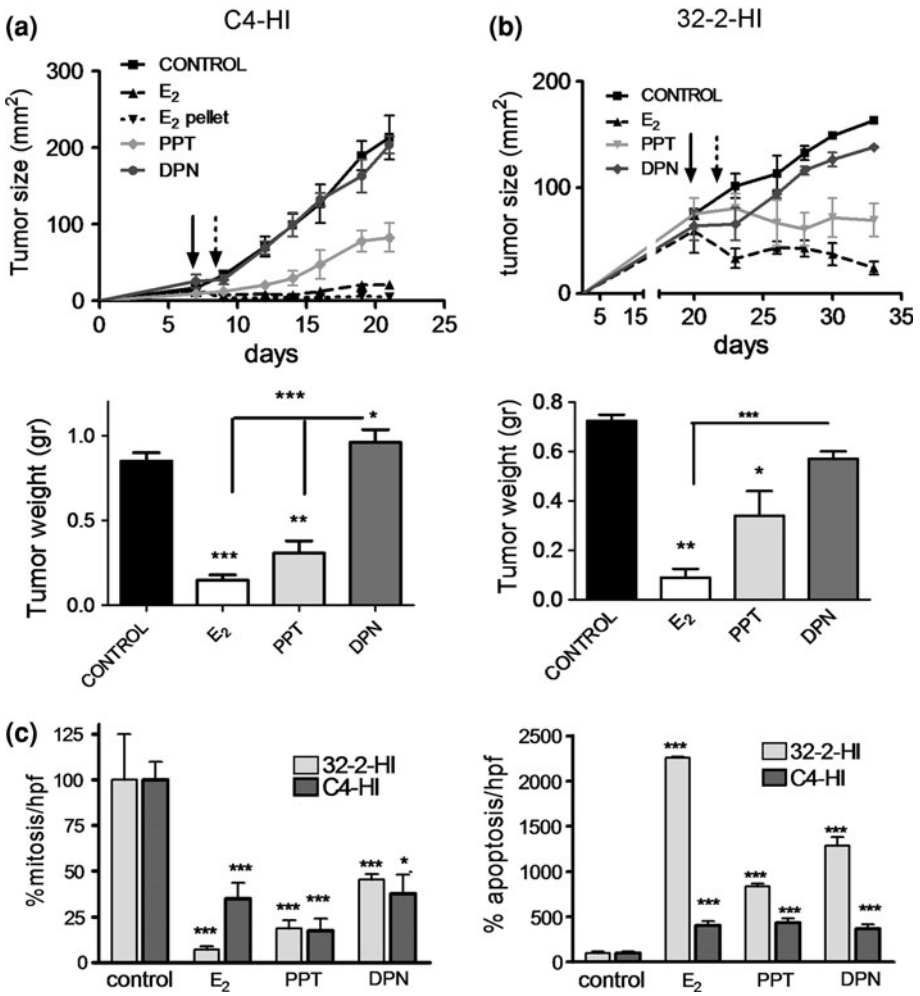

Fig. 4 Effects of E_2, PPT, and DPN on tumor growth. BALB/c mice carrying sc tumors of about 30 mm^2 (C4-HI; **a**) or 70 mm^2 (32-2-HI; **b**) were treated with daily sc doses of PPT, DPN, or E_2 (3 mg/kg body weight). Animals carrying C4-HI tumors were also implanted with 5 mg E_2 silastic pellets. The *full arrow* shows the time point at which treatments were initiated. Three animals per group were euthanized 24, 48, or 72 h after treatment was initiated (*dotted arrow*), and the rest of the animals (4/group) were followed for 2 weeks. Tumors were measured with a Vernier Caliper, and the size (length × width) was plotted (mean ± SEM; *top*). All treatments inhibited tumor growth during the first 72 h of treatments ($P < 0.05$). However, DPN-treated tumors started to grow, while PPT and E_2-treated tumors remained the same size, grew very slowly (PPT), or experienced tumor regression (E_2). Tumors were weighed at the end of the experiment, and the weight plotted (mean ± SEM; *bottom*). Control and DPN-treated tumors were larger than E_2- and PPT-treated tumors; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **c** C4-HI and 32-2-HI tumors from mice treated for 2 or 3 days, respectively, with E_2, PPT, or DPN, were processed for histological evaluation, and the number of mitotic or apoptotic cells was counted as described in the "Materials and methods" section. The mean value ± SEM obtained in control slides was considered as 100%. All compounds increased apoptosis and decreased mitosis; *** $P < 0.001$ experimental vs. control

daily. The results were similar when evaluating tumor size or weight (Fig. 4b, bottom). In summary, DPN exerted inhibitory effects on tumor growth in both C4-HI and 32-2-HI during the first 3-4 days of treatment, whereas PPT continued to be inhibitory during the entire observation period.

Vaginal smears, performed during 5 consecutive days (between day 5–10) after treatment initiation showed that, while E_2-treated mice were at continuous estrous, PPT-treated mice were at proestrous, and DPN- treated mice were at meta-diestrous.

A significant decrease in the mitotic index was observed in both tumors treated with all compounds ($P < 0.001$). However, only in E_2-treated 32-2-HI tumors was there almost a complete absence of mitotic figures; DPN was the least effective in decreasing the mitotic index. The cytostatic effect was higher in 32-2-HI tumors than in C4-HI tumors. A similar pattern was observed in apoptosis: 32-2-HI E_2-treated tumors had a higher apoptotic index than PPT or DPN-treated tumors ($P < 0.001$); and the increase in apoptotic index in C4-HI was not as high as that of 32-2-HI (Fig. 4c). The mitotic and

apoptotic indices reflected the effects of PPT and E_2 on tumor growth. The mechanisms behind the transient inhibitory effects of DPN are now being studied in our lab.

E_2-, PPT-, and DPN-induced tumor morphological changes

Tumors were excised during the growth inhibitory phase. C4-HI is a differentiated mammary carcinoma which regresses after antiprogestin or estrogen treatment; this regression is associated with apoptosis, decreased mitotic index, and increased glandular differentiation [49]. PPT- and DPN-treated tumors maintained the morphology of moderately differentiated carcinomas with glandular and cribiform differentiation and pseudopapillae formation in necrotic areas. Cellularity was lower in PPT- compared to DPN-treated tumors (Fig. 5).

32-2-HI is a poorly differentiated adenocarcinoma. E_2 or PPT treatment induced regression associated with a decrease in mitosis and an increase in apoptosis, which was more evident in E_2 treated tumors, with a noticeable increase in stroma (Fig. 6). This was less conspicuous in DPN-treated tumors.

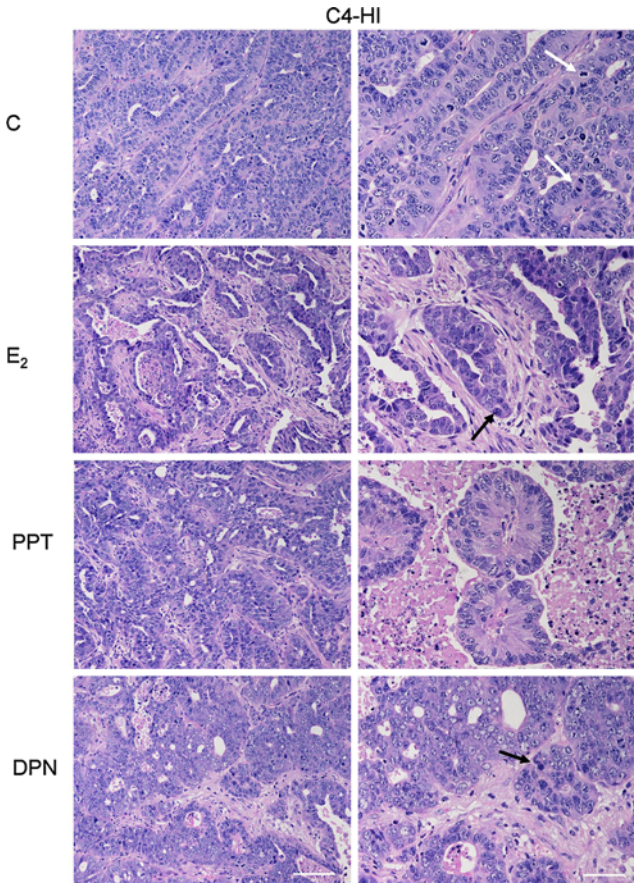

Fig. 5 Changes in C4-HI tumor morphology induced by E_2, PPT, and DPN. Tumors were treated for 72 h with E_2, PPT, or DPN. H&E images show tumor morphology. Controls: *Left*: well differentiated C4-HI adenocarcinomas growing in untreated animals; *Right*, tumor cells differentiate tubular structures, with a high mitotic index (all mitotic figures: *white arrows*). E_2-treated tumors show a high degree of differentiation and an evident increase in the amount of stroma. *Right*: several apoptotic images are evident, as well as the absence of mitotic figures. PPT-treated tumors show a high degree of differentiation. *Right*: pseudopapillary structures (glands) formed by tumor cells surrounded by necrotic tissue. DPN-treated tumors showing cribiform and glandular areas. *Right*: apoptotic cells in differentiated glands (*arrow*). Left images: ×200; *bar*: 100 μm. Right images: ×400; *bar*: 50 μm

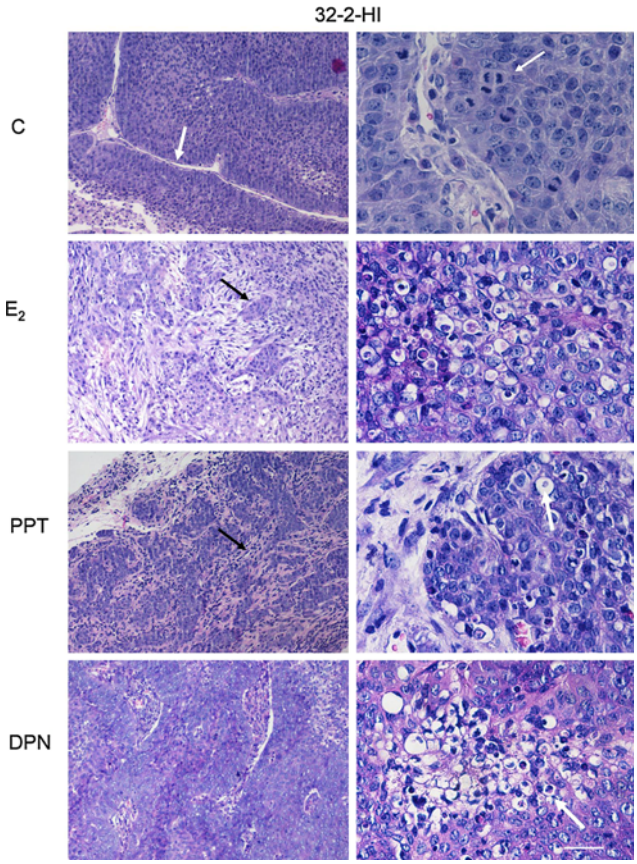

Fig. 6 Changes in 32-2-HI tumor morphology induced by E_2, PPT, and DPN. Tumors were treated for 48 h with E_2, PPT, or DPN. H&E images showing tumor morphology. Controls: *Left*: a poorly differentiated 32-2HI tumor in untreated animals. Tumor cells grow in solid sheets supported by scanty stromal tissue (*arrow*); *Right*: A high number of mitoses are present in control tumors (*white arrow*). E_2-treated tumors: *Left*: increased fibroblastic stromal tissue is intermingled with the epithelial tumor cells. *Arrow* shows an epithelial nest surrounded by stromal tissue. *Right*: Nests of apoptotic cells. PPT-treated tumors show increased fibrous stroma between residual tumor cells arranged in solid nests. *Right*: Epithelial nests showing individual apoptotic cells (*arrow*). DPN-treated tumors: *Left*, the proliferation is almost solid, with scanty stroma. *Right*: nests of apoptotic cells (*arrow*). Left images: ×100; *bar*: 200 μm. Right images: ×400; *bar*: 40 μm

E_2, PPT, and DPN down regulate ERα in vivo

The expression of ERα was evaluated in C4-HI tumors treated for 24 h with PPT or DPN. A decrease ($P < 0.01$) in ERα expression (western blots; Fig. 7a) and an increase in PR-A expression (immunofluorescence; Fig. 7b) was observed. After 3 days of treatment, both ERα ($P < 0.001$) and PR (not shown) were low in all treated tumors (immunohistochemistry; Fig. 7c). These results indicate that both agonists exert the same effects on ERα and PR regulation in vivo. Similarly, in 32-2-HI tumors treated for 48 h with E_2, PPT, or DPN, all treatments induced a significant decrease in ER nuclear staining (Suppl. Fig. 1a). Western blotting performed with extracts from tumors treated for 3-24 h with E_2 confirmed a down regulation of ERα ($P < 0.05$; Suppl. Fig. 1b).

E_2, PPT, and DPN increase the Bax/Bcl-xl ratio and laminin/integrin α6 in vivo, but the activation of caspase 9 is ERα-specific, and the increase in AIF is ERβ-specific

In view of the in vitro data showing an increase in apoptosis and in the Bax/Bcl-xl ratio, we evaluated the expression of members of the intrinsic pathway of apoptosis in treated 32-2-HI and in treated C4-HI tumors. We evaluated the expression of the two pro-apoptotic proteins (Bax and Aif), two antiapoptotic proteins (Bcl-xl and Bcl-2), and the activation of caspase 9. To avoid interference by the high number of stromal elements in western blots, we used immunohistochemistry to identify cell localization. A significant increase in Bax and a decrease in Bcl-xl and Bcl-2 were observed in all treated C4-HI (Fig. 8) and 32-2-HI (Suppl. Fig. 2) tumors ($P < 0.001$). The expression of the proapoptotic protein Aif was increased in all treated mice, with higher levels in DPN-treated tumors compared with E_2- or PPT-treated tumors (Fig. 9; $P < 0.001$). The opposite occurred with activated caspase 9, which was significantly higher in PPT-treated tumors compared to E_2- ($P < 0.001$) and DPN-treated ($P < 0.01$) tumors (Fig. 9). Interestingly, polarized staining was observed in the epithelial cells lying at the interface with the stroma (arrows and insets in Suppl. Fig. 2 and Fig. 9), suggesting that, in vivo, stromal components are playing an important role in apoptotic signaling. Thus, we evaluated the expression of the extracellular matrix protein laminin and one of its receptors, integrin α6, in C4-HI tumors because both have been implicated in potentiating apoptosis [50]. An increase in integrin α6 expression and of laminin distribution was observed in all treated tumors. However, DPN effects were stronger than those of PPT and E_2 ($P < 0.05$; Suppl. Fig. 3). These results suggest that tumor stroma participate in estrogen-induced apoptosis or anoikis in vivo.

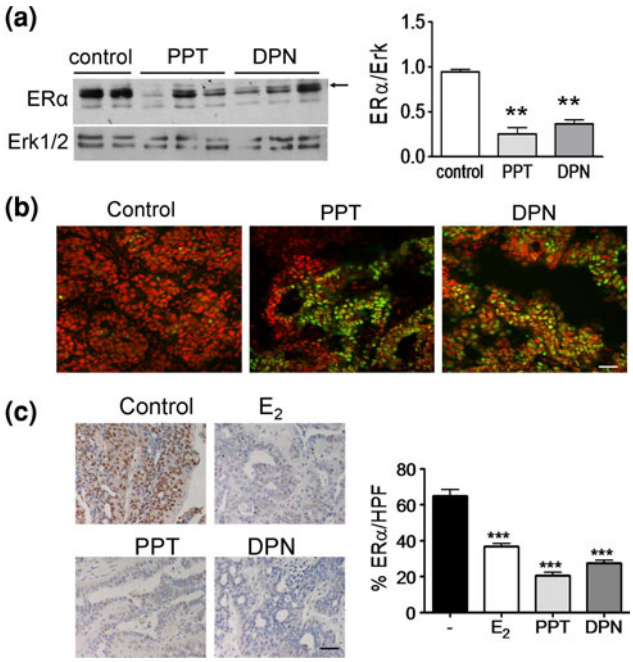

Fig. 7 ER and PR regulation by E_2, PPT, and DPN in C4-HI tumors. **a** *Left*: Western blots showing ERα expression (66 kDa; MC-20, Santa Cruz Antibody) in nuclear extracts from treated (24 h) and untreated C4-HI tumors. *Right*: Quantification of ERα in relation to Erk. A decrease in ERα expression can be observed in treated tumors. **b** Immunofluorescence of PR_A expression (C-19 Antibody, *green*) in control or PPT- or DPN-treated (24 h) tumors. PI was used for nuclear counterstaining. An increase in PR_A staining is observed in PPT- or DPN-treated tumors; *bar*: 60 μm. **c** *Left*: Immunohistochemistry of ERα (MC-20, Santa Cruz) in C4-HI tumors treated with E_2, PPT, or DPN for 72 h. *Right*: Quantification of ERα-positive cells/HPF. A decrease in ERα expression was observed in treated tumors; *bar*: 80 μm

These data indicate that both agonists, at short timepoints, exert similar effects regulating Bax, Bcl-xl, and Bcl-2. DPN behaves as an almost exclusive regulator of Aif in both tumors and PPT as an activator of the caspase 9 pathway, confirming the participation of the intrinsic apoptotic pathway in ERα-mediated tumor regression. The role of ERβ is less clear because the inhibitory effect induced was only transient.

Discussion

In this article, we used specific ER isoform agonists to show that ERα and ERβ may enhance or inhibit cell proliferation, depending on the cell context. In an experimental setting in which estrogens stimulate cell proliferation (e.g., MC4-L2 cells), both agonists exert stimulatory effects; in a scenario in which estrogens inhibit cell proliferation or induce tumor regression (e.g., C4-HI and 32-2-HI tumors), they both exert inhibitory effects, indicating that the final

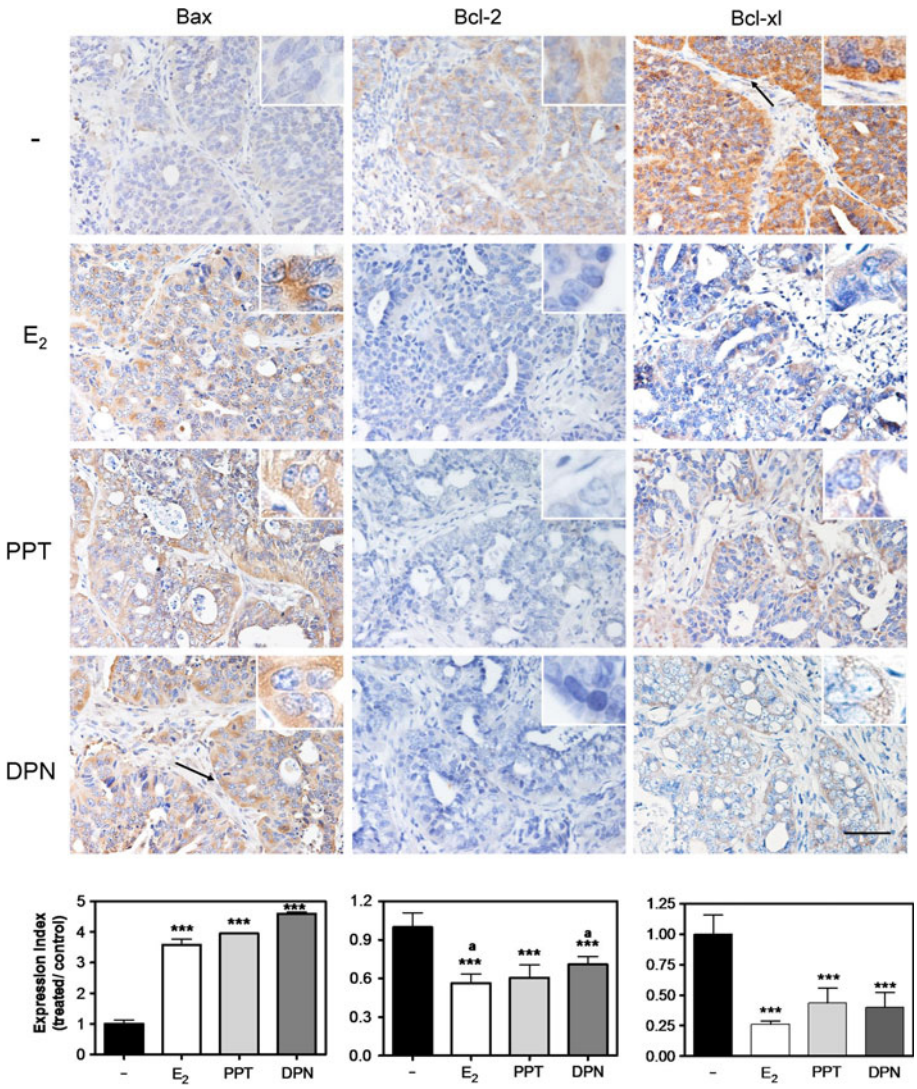

Fig. 8 Bax, Bcl-xl, and Bcl-2 regulation by E_2, PPT, and DPN in C4-HI tumors. Immunohistochemistry showing the expression of the proapoptotic protein Bax and two antiapoptotic proteins, Bcl-2 and Bcl-xl, in C4-HI tumors treated for 72 h with E_2, PPT, or DPN. Immunoreactivity was quantified as described in the "Materials and methods" section, and an increase in Bax staining and a decrease Bcl-2 and Bcl-xl staining was observed in all treated tumors (*bottom*). *** $P < 0.001$. Resolution: ×400; *bar*: 50 μm. *Insets* show, in detail, the cytoplasmic staining which was more intense in the epithelial stromal interface (*arrows*; *bar*: 20 μm)

effect will depend on the cellular context rather than in the estrogen receptor isoform involved.

We were interested in exploring the possibility that the differential response to E_2 between MC4-L2, a cell line derived from C4-HD [39], and C4-HI may be due to a prevalence of one of the ER isoforms with respect to the other. To approach this question, we utilized two widely used specific agonists, PPT and DPN. Considering the reports regarding the inhibitory effects of ERβ in the normal mammary gland [31], we expected to find that PPT stimulated, and that DPN inhibited, MC4-L2 cell proliferation. Conversely, we found that both stimulated cell proliferation, even at concentrations below 1 nM. To critically challenge this unexpected observation, we used MCF-7 cells, which express high levels of ERα and very low levels of ERβ [10, 48]. Regardless, a stimulatory effect was found for DPN at concentrations higher than 10 nM. Because it was possible that these effects were mediated by binding to ERα at high doses [35], we decided to repeat the experiments using concentrations of DPN lower than 10 nM to minimize any possible ERα interference. Additionally, our results showed that the effects of PPT and DPN were not additive, indicating that some of the effects of E_2 in tumor proliferation may be unaccounted for by its direct effect on specific ERs. Interestingly, similar effects by PPT and DPN have recently been reported in MCF-7 cells. Both agonists exerted the same effects as E_2 at 10 nM concentrations, inhibiting MiR-21 microRNA expression [51]. This microRNA has been considered as an oncoMir since it was found to be up-regulated in breast cancer tissues [52].

In in vivo experiments, we used agonist doses that were within the range used by other investigators [53–56] and compared them with the effects of E_2. Although we have previously used 5 mg E_2 silastic pellets to evaluate estrogen effects on tumor growth in this model [12], this was the first time that we tested the effect of this hormone on a daily basis. E_2-induced tumor regression, PPT-inhibited

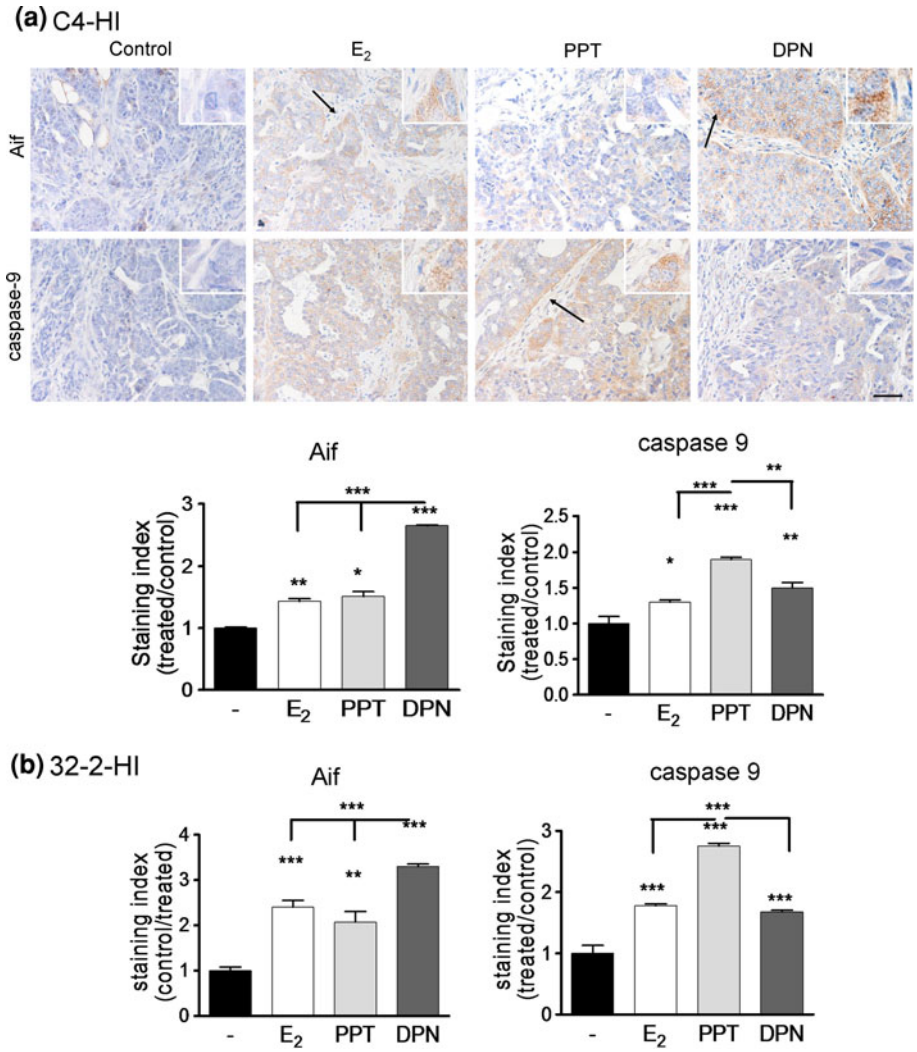

Fig. 9 Aif and activated caspase 9 regulation by E_2, PPT, and DPN in vivo. **a** Immunohistochemistry showing the expression of the proapoptotic protein Aif and activated caspase 9 in C4-HI tumors with and without 72 h treatment with E_2, PPT, and DPN. Staining was quantified as described in the "Materials and methods" section, and a high increase in Aif staining was observed in DPN-treated tumors. An increase in activated caspase 9 was mainly observed in PPT-treated tumors. Resolution: ×400; *bar*: 50 μm. *Insets*, *bar*: 20 μm. **b** Quantification of Aif and activated caspase 9 staining in 32-2-HI tumors with and without 48 h treatment with E_2, PPT, and DPN. The results observed in 32-2-HI tumors reproduced those observed in C4-HI tumors. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

tumor growth, and DPN-induced a transient inhibitory effect. Interestingly, the mitotic and apoptotic indices, registered a few days after treatment, predicted this response because only E_2 completely blocked cell proliferation. The transient effect of DPN could be due to: (a) a lack of drug availability as the tumors become larger, (b) a transient down regulation of ERα, or (c) systemic effects which may be altering the endocrine hormone milieu. In fact, the vaginal smears that were characteristic of pseudopregnancy suggest a progesterone-rich environment in DPN-treated mice. This agrees with the fact that the administration of estrogens to ERKO mice, or an ERβ agonist to a wild type animal, induces high levels of LH [57], which might be associated with increased progesterone. The systemic effects induced by these agonists are currently being studied in this strain of mice.

We have already reported that in some MPA-induced tumors, estrogens induce complete tumor regression through cytostasis, apoptosis, increases in p21, p27, and p53 expression [15], and tissue remodeling, which involves increases in laminin and collagen I and IV redistribution [58]. In some tumors, as in C4-HI, estrogens induce an increase in differentiation, and in this case, tumors may not regress completely [42]. In this study, we have extended our findings to further explore the mechanisms by which tumors regress. Although we observed an increase in apoptosis in vitro, this was negligible when compared with the dramatic changes observed in vivo, stressing the use of in vivo models as the most relevant to study the inhibitory effects of hormones. Changes in tumor parenchyma were accompanied by redistribution of laminin in the stroma and by an increase in integrin-α6 expression in the epithelial cells. This was especially prominent in the epithelial cell layer in direct contact with the stroma, suggesting the involvement of cross-talk between stroma and parenchyma in tumor regression. In cisplatin-treated testicular germ line cells, laminin-integrin-α6 signaling induces the activation of executioner procaspase-3 and -6, as well as Aif

transcription and expression [50], which agrees with our findings that integrin α6 expression was high in tumors with increased Aif expression (Fig. 9 and Suppl. Fig 3).

Both the extrinsic pathways of apoptosis, involving the activation of FasR-FasL, and the intrinsic or mitochondrial pathway, involving an increase in the Bax/Bcl-2 ratio, have been related to estrogen-induced apoptosis in in vitro studies [6, 14]. However, Lewis et al. [6] stressed that the blockage of Bax, Bim, or p53 inhibited estrogen-induced apoptosis in E_2-inhibited MCF-7:5C cells. Our results are in agreement with these data. The increase in p53 observed in E_2-treated tumors in previous studies may be also involved in maintaining a high Bax/Bcl-2 ratio. Indeed, p53 has been involved in the mitochondria-mediated apoptotic cell death by: (1) increasing the transcriptional activation of Bax and Apaf-1 [59], and (2) transcriptionally repressing antiapoptotic proteins such as Bcl-2 [60]. A study by Zhang et al. [16], in which they used T47D:A18/PKCα cells growing in nude mice treated with E_2, highlighted the role of FasR-FasL in E_2-induced tumor regression. This is, to our knowledge, the only report that has evaluated the role of the apoptotic pathways in in vivo estrogen-induced growth inhibition. Although we have not observed a regulation of Fas-L in our primary cultures, and considering the key role of stromal-parenchymal interactions in regulating tumor growth in our tumors, it seems quite possible that other pathways may also be acting in concert with the mitochondrial apoptotic proteins.

Our working hypothesis (Fig. 10) is that in these tumors, ERα is constitutively activated [61], and it participates at least partially in PR expression, which also needs to be activated to induce cell proliferation [46]. Preliminary results of our laboratory suggest that these tumors also express high levels of PCNA and clusterin (not shown). In this setting, Bax is sequestered by Clusterin and Bcl-xl, a PR-regulated gene is up-regulated by activated PRs [29]. In the presence of the estrogenic compounds, there may be a reprogramming of ER by ubiquitination and/or tethering to different promoters, with a consequent down regulation of ER, leading to the decrease in PCNA and in clusterin expression and the release of Bax. The differential expression of Bax and Bcl-xl induces an increase in the Bax/Bcl-xl ratio. Interestingly, Bax and Aif were highly expressed, mainly in the cytoplasm of cells lining the tumor stroma. We have already pointed out the importance of MMP-2 and MMP-9 activation in tissue remodeling at early time points of tumor regression [58]. We now show that integrin α6 expression, one of the components of laminin receptors, is also increased in cells lining tumor stroma, suggesting cross talk between stroma and the epithelial tumor cells, which may be relevant in the induction of apoptosis.

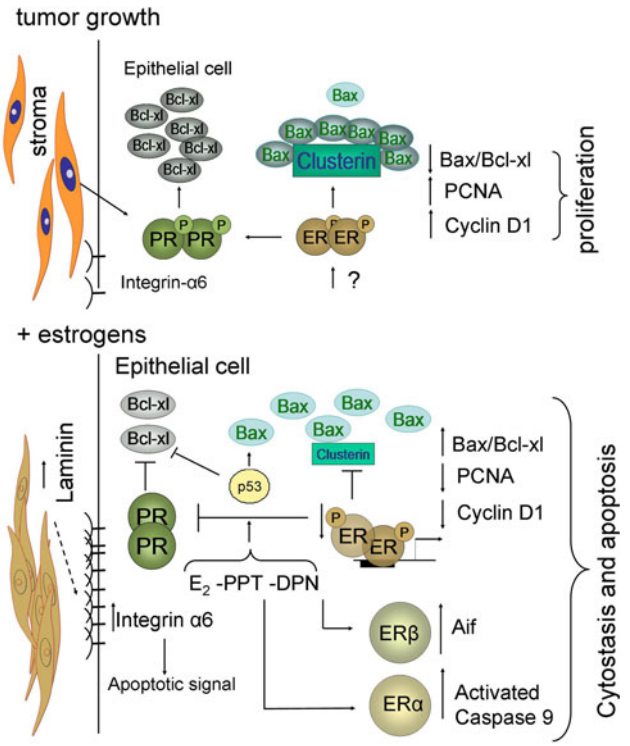

Fig. 10 Working hypothesis. ERα and PRs are activated in C4-HI [32] and in 32-2-HI tumors (ongoing research). Tumors have high levels of two PR-regulated genes, cyclin D1 and Bcl-xl, and preliminary data suggests that the estrogen responsive genes, PCNA and clusterin are also up-regulated in C4-HI tumors. Clusterin decreases Bax such that the Bax/Bcl-xl ratio is low. In the presence of E_2, PPT, or DPN, there is a down regulation of ERα; ERs are possibly tethered to different promoters and/or are ubiquitinated. This interferes with PR-activated genes such that the levels of cyclin D1 and Bcl-xl are lowered. In addition, there is a decrease in clusterin with a consequent increase in Bax release, causing the ratio of Bax/Bcl-xl or Bax/Bcl-2 to increase. E_2 also increases p53 expression in tumors with wt p53 [15]. This increase in p53 may also directly contribute to lower Bcl-2 expression and to increased Bax transcription, leading to apoptosis. ERβ-mediated apoptosis is associated with increased Aif activation, while ERα-mediated apoptosis is associated with activation of caspase 9. These parenchymal changes impact the stromal–parenchymal interactions, and there is an increase in stromal laminin redistribution with a concomitant increase in epithelial integrin α6, which contributes to enhance the apoptotic events

Conclusions

We have demonstrated that both ER isoforms are involved in stimulatory and inhibitory effects in this breast cancer model. This is, to our knowledge, the first report regarding the in vivo effects of DPN and PPT in experimental breast cancer. We have shown that the mitochondrial pathway is involved in estrogen-induced apoptosis, and while at this level no differences between either ER agonist could be detected, the expression of Aif was preferentially increased by the ERβ agonist and activated caspase 9 by the ERα agonist. Our results, together with those of others, indicate

that estrogens might be exploited therapeutically in tumors which are resistant to other endocrine treatments [16, 62], and PPT seems to be a better therapeutic option than DPN. A question that still lingers, and is actively being studied in our lab, is the identification of the tumors that will be stimulated by estrogens or ER agonists and those that will be inhibited.

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Competing interests The author(s) declare that they have no competing interests.

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