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Estrogen receptor-beta agonist diarylpropionitrile counteracts the estrogenic activity of estrogen receptor-alpha agonist propylpyrazole-triol in the mammary gland of ovariectomized Sprague Dawley rats

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ABSTRACT

Although estrogen can bind both types of estrogen receptors, estrogen receptor-alpha (ERα) is dominant in mediating estrogenic activity in the mammary gland and uterus. Excessive estrogenic activity such as estrogen-based postmenopausal hormone replacement therapy increases the risk for breast and endometrial cancers. The adverse effect of estrogen on uterine endometrium can be opposed by progestins; however, estrogen-plus-progestin regimen imposes substantially greater risk for breast cancer than estrogen alone. In this study, we used ERα-selective agonist propylpyrazole-triol (PPT) and ERβ-selective agonist diarylpropionitrile (DPN) to activate ERα and estrogen receptor-beta (ERβ) separately in an ovariectomized rat model and determined whether PPT-activated ERα function in the mammary gland can be suppressed by DPN activated ERβ. Ovariectomized rats were randomly divided into six groups and treated with DMSO (control), DPN, PPT, PPT/DPN, PPT/Progesterone, and PPT/Progesterone/DPN, respectively. In the mammary gland, PPT but not DPN increased cell proliferation and amphiregulin gene expression; importantly, the stimulatory effect of PPT on mammary cell proliferation and amphiregulin gene expression can be suppressed by DPN. In the uterus, the effect of PPT on uterine weight and endometrial cell proliferation was not inhibited by DPN but can be inhibited by progesterone. These data provide *in vivo* evidence that PPT activated ERα activity in the mammary gland can be opposed by ERβ-selective agonist DPN, which may be explored for the development of better hormone replacement therapy regimen with less risk for breast cancer.

1. Introduction

Estrogen has profound effects on a broad range of tissues and organs involved in many physiological processes. Drop in estrogen production after menopause is responsible for many postmenopausal symptoms, thus estrogen or estrogen-plus-progestin can be used for hormone replacement therapy (HRT) to ameliorate postmenopausal symptoms. A major adverse effect associated with estrogen-based HRT is the increased risk for breast cancer and uterine endometrial hyperplasia and malignancy [1,2]. The adverse effect of estrogen on uterine endometrium can be opposed by progestins; however, estrogen-plus-progestin HRT regimen imposes substantially greater risk for breast cancer than estrogen alone [1,3–6]. While the two types of estrogen receptors, ERα and ERβ, bind to natural estrogen with similar affinity, ERα is the dominant receptor that mediates the estrogenic responses in most estrogen regulated tissues including the mammary gland and uterus [7–12]. Deregulation of ERα expression and activity accounts for the majority of breast and endometrial cancers. Approximately 70% of breast tumors and 60% of endometrial tumors are ERα-positive tumors [13,14]. In many breast tumors, the percentage of ERα-positive cells is much higher than that in the normal mammary gland [14–17]. Furthermore, ERα may mediate cell proliferation differently in breast tumors. In the normal mammary gland, $ERα^+/Ki67^+$ cells are very rare and it is believed that ERα acts in a paracrine manner to promote neighboring ERα-negative cell to proliferate [11,15,16,18,19]. In ERα-positive breast tumors or cancer cell lines, the percentage of $ERα^+/Ki67^+$ cells are much higher than that in the normal mammary gland and that ERα may directly stimulate ERα-positive cancer cells to proliferate [15,19,20]. Deregulated expression of ERα in transgenic mice leads to mammary tumorigenesis and makes the uterus more susceptible to estrogen induced uterine tumorigenesis [21–23]. Unlike ERα, ERβ is not required for mammary gland and uterus development

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BW, body weight; DPN, diarylpropionitrile; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; HRT, hormone replacement therapy; IF, immunofluorescent; IHC, immunohistochemical; OVX, ovariectomy or ovariectomized; P4, progesterone; PPT, propylpyrazole triol; UWW, uterine wet weight.

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[8,9,11,24,25]. Epidemiological studies indicate that ERβ expression is lost or decreased in many breast and endometrial tumors, indicating that ERβ may function as a tumor suppressor [26–28].

The precise mechanism(s) by which estrogen promotes tumorigenesis in the mammary gland and uterine endometrium is not fully understood. A major effect of estrogen on the mammary gland and uterus is to stimulate cell proliferation [29–32]. It has been found that estrogen-based HRT significantly increases breast epithelial cell proliferation in postmenopausal women [33]. Deregulation of cell proliferation by oncogenes and tumor suppressors is one of the hallmarks of cancer cells [34,35]. Consistent with its role in breast and endometrial malignancy, ERα is essential and sufficient to mediate estrogen induced cell proliferation [8,11,30,36,37]. In contrast to the positive role of ERα in cell proliferation, ERβ may function as a negative regulator of cell proliferation. Loss of ERβ could lead to increased cell proliferation, whereas overexpression of ERβ has been found to inhibit cell proliferation and xenograft tumor formation in several breast and endometrial cell lines [8,24,38–46]. The molecular mechanism of ERβ action is not fully understood [9,47,48]. Studies using *in vitro* cell lines have demonstrated that ERβ can antagonize ERα in gene expression, cell cycle progression, and cell proliferation [42–45,49–51]. ERα and ERβ may form a subtle balance to regulate estrogen signaling in mammary and endometrial cell proliferation, loss of the balance may lead to tumor initiation and progression [52].

In addition to genetic modification of estrogen receptor expression, ER-selective agonists have been developed to determine the biological functions of ERα and ERβ [9,47,53–55]. These ER-selective agonists may also be used for pharmacological interventions of estrogenic activity [9,53,55]. Despite the significance of estrogenic activity in mammary cell proliferation and tumorigenesis and that ERβ may function as a tumor suppressor, *in vivo* studies of the ERβ-selective agonists in the mammary gland are very limited [30,56,57]. It remains unknown whether endogenous ERβ can be activated to function as a tumor suppressor in the mammary gland *in vivo*. In this study, we used ER-selective agonists propylpyrazole triol (PPT) and diarylpropionitrile (DPN) to separately activate ERα and ERβ in an ovariectomized (OVX) rat model and determined whether ERα-mediated estrogenic activity in the mammary gland can be inhibited by DPN activated ERβ. In receptor competition binding assay for binding affinity relative to estradiol, PPT is an ERα-selective agonist that has a 410 fold higher relative binding affinity to ERα than to ERβ; DPN has a 70 fold higher relative binding affinity to ERβ than to ERα [58,59]. We demonstrated that ERα-mediated estrogenic activity in the mammary gland can be opposed by the ERβ-selective agonist DPN *in vivo*, suggesting that ERβ-selective agonists such as DPN may be explored for the development of better HRT regimens to reduce or eradiate the risk for breast cancer.

2. Methods

2.1. Animals

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont. Ovariectomized (OVX) virgin female Sprague Dawley rats (Charles River - Canada) were housed with a 12-h light and dark cycle and ad libitum access to food and water. Rats were ovariectomized at 5–6 weeks old and rested for two weeks before treatment. Five to six rats were randomly assigned to each group and totally there are six groups, the control group, the DPN group, the PPT group, the PPT/DPN group, the PPT-plus-progesterone (PPT/P4) group, and the PPT/P4/DPN group. PPT, DPN, and progesterone were obtained from Tocris Bioscience and were dissolved in DMSO for stock solution. The drugs were administered by i.p. injection once a day for three consecutive days; the control group rats received the vehicle DMSO only. The dosage of the different drugs used in this study was as follows: PPT at 500 μg/kg BW (body weight), DPN at 1000 μg/kg BW, P4 at 20 mg/kg BW. BrdU (5-bromo-2′-deoxyuridine, from Sigma) solution in PBS was injected (i.p., 20 mg/rat/d) at the same time when the drug(s) was administered. Rats were sacrificed 16 h after the last injection for biopsy sample collection. The timing of treatment and biopsy after the last treatment was chosen based on other studies. In the literature, various lengths of treatment ranging from a couple of hours to several weeks were used for the evaluation of different endpoint parameters [30,31,37,56,57]. The primary endpoint of evaluation in this study was cell proliferation rate, the three day treatment period was chosen as it has been shown in several studies that two to three day treatment significantly increased mammary cell proliferation rate [30,31]. Another reason that we did not choose shorter than three days is the concern that the percentage of proliferating cells in OVX rats induced by shorter treatment period would be too low to allow the detection of any inhibitory effect. The drugs were administered in the afternoon for all three treatments; for the last treatment, drug injections for different animals (with ear tag numbers) were administered at 15 min intervals so that each individual animal was killed at 16 h post the last treatment for biopsy sample collection. Time course studies have shown that estrogen treatment for as short as 4 h significantly increased the percentage of cyclin D-staining cells in the mammary gland; in our previous studies using ERα-positive MCF-7 cell line treated with estrogen, we noticed that the percentage of cells with the Ki-67 proliferation marker started to increase around 12 h [20,30]. Based on these time course studies, we expected that the effect from the last treatment can be detected 16 h later. For mammary gland biopsy, the fourth pair of mammary glands was harvested from each rat and weighed. The right-side was fixed in neutral formalin for 48 h before being processed for paraffin embedding. The left-side was snap-frozen and stored in liquid nitrogen for RNA isolation. The uterus from each rat was first measured for uterine wet weight (UWW) and then fixed in neutral formalin for 24–48 h before being processed for paraffin embedding.

The dosage selection for this study was based on the dosages used by other studies, the relative binding affinity, and the relative transcriptional activity via ERE (estrogen response element) [12,31,36,37,43,56,58–63]. PPT from 50 μg/d/rat to 1000 μg/d/rat was shown with very good response in the uterine endometrium [36]. The body weight of the rats in this study was approximately 200 g, therefore the dose per rat was about 100 μg/d/rat for PPT, 200 μg/d/rat for DPN, and 4 mg/d/rat for progesterone. In the transcriptional activity assay using the U2OS cell system, it was shown that the maximal activity stimulated by PPT was comparable to that by estradiol, and that the EC50 for estradiol via ERα was 8 pM and the EC50 for PPT via ERα was 140 pM [43]. The ratio of 8 pM estradiol/140 pM PPT can be converted as 20 μg/kg BW estradiol/500 μg/kg BW PPT, a dosage that were expected to be functional in the mammary gland as well [30,31,56,64]. The binding affinity of PPT to ERα is approximately 49% of that of estradiol to ERα, or the conversion of 500 μg/kg BW PPT to 176 μg/kg BW estradiol [59]. DPN at 1000 μg/kg BW was within the range used by other studies for its effect on uterus, hot flush, osteoporosis, and cardioprotection [60–62,65–67]. The binding affinity of DPN to ERβ is approximately 18% of that of estradiol to ERβ, or the conversion of 1000 μg/kg BW DPN to 205 μg/kg BW estradiol [58]. Based on these calculations, the theoretically converted PPT and DPN (to estradiol) would have DPN binding to ERβ and PPT binding to ERα at a comparable level. Considering that estradiol may have a two to ten-fold higher binding affinity for ERα than for ERβ, the ratio of DPN-ERβ/PPT-ERα could be lower than the 1:1 ratio [12,43,63]. The binding affinity of PPT to ERβ is 0.12% of that of estradiol to ERβ, or

the conversion of 500 μg/kg BW PPT to 0.42 μg/kg BW estradiol, a concentration that would not interfere much of the DPN binding to ERβ [59]. The binding affinity of DPN to ERα is 0.25% of that of estradiol to ERα, or the conversion of 1000 μg/kg BW DPN to 2.85 μg/kg BW estradiol, a concentration that would not interfere much of the PPT binding to ERα [58]. Progesterone from 15 mg/kg BW to 30 mg/kg BW was shown to have synergistic effect with estrogen or PPT, therefore 20 mg/kg was selected for this study [31,56].

2.2. Immunofluorescent (IF) and immunohistochemical (IHC) staining

IF and IHC assays were used to assess the BrdU-labeled cells and the expression of ERα, ERβ, and cyclin D1 following conventional procedures. The sources for the antibodies were as follows: ERα antibody (MC-20) from Santa Cruz Biotechnology, ERβ (14C8) and BrdU antibodies from Abcam, cyclin D1 antibody (DCS-6) from Fisher Scientific/Pierce. For IF staining, Alexa Fluor 488 (Invitrogen) was used for green fluorescence, Rhodamine Red (Jackson ImmunoResearch) was used for red fluorescence, and DAPI contained in Mounting Medium (Vector Lab) was used for nucleus staining to give blue fluorescence. For IHC staining, VECTASTAIN Elite ABC Kit was used following the manufacturer's procedure. DAB (3,3′-diaminobenzidine) was used as the peroxidase substrate to develop brown color and Hematoxylin QS was used for counterstaining. Antigen retrieval was carried out by microwaving (700 W) slides in 10 mM citrate buffer (pH 6.0), ERα and BrdU for 11 min, ERβ for 25 min, and cyclin D1 for 20 min. ERα antibody was used at 1:200 dilution, ERβ antibody at 1:40 dilution, BrdU antibody at 1:150 dilution, and cyclin D1 antibody at 1:80 dilution. Paraffin sections without incubation with primary antibody were used as negative control of staining; sections from ovary-intact tissues were used as positive control of staining. Immunostaining slides were examined under Olympus BX50 Fluorescence Microscope connected with the Optronics MagnaFire digital camera (Microscopy Imaging Center, UVM). Images were taken with Optronics MagnaFire software and Adobe Photoshop was used for further processing of the digital images.

The NIH software Image J was used for cell counting. For each mammary gland, at least 250 ductal epithelial cells and 500 lobular/alveolar bud epithelial cells were counted. For the uterine endometrium of most rats, at least 400 luminal epithelial cells and 200 glandular epithelial cells were counted. For IHC images of cyclin D1 staining, the staining was assigned at four levels, level 0 is unstained, level 1 is weak staining, level 3 is intensive staining, and level 2 is between level 1 and level 3 for moderate staining. Cells with staining levels 2 and 3 were counted as positive staining cells, and cells with staining levels 0 and 1 were counted as negative staining cells.

2.3. RNA isolation and quantitative real-time PCR assay

Total RNA was extracted with Trizol reagent (Invitrogen) and further purified with RNeasy Kit (Qiagen). Two micrograms of RNA from each sample was used for reverse transcription with SuperScript III (Invitrogen). TaqMan probes for amphiregulin (Areg) and β-actin labeled with FAM dye were purchased from Applied Biosystems (ABI). Quantitative real-time PCR was performed for amphiregulin and β-actin (endogenous control) using TaqMan gene expression assay (ABI) on an ABI 7500 real-time PCR System. PCR for RNA from each sample was performed in duplicate and the average Ct from each sample was used for further calculation. The relative gene expression of amphiregulin transcripts were calculated using the $2^{(-\Delta\Delta Ct)}$ method.

2.4. Statistical analysis

Statistical significance among different groups of animals was assessed by one-way ANOVA. Post hoc Tukey's HSD test was used for pairwise multiple comparisons to determine which groups differ, with the significance level (alpha) set as 0.05. A p value less than 0.05 was considered significant.

3. Results

3.1. Expression of ERα and ERβ in the mammary gland and uterine endometrium of OVX rats

The expression patterns of ERα and ERβ in the mammary gland and uterus of OVX rats were determined by immunofluorescent (IF) and immunohistochemical (IHC) staining, respectively (Fig. 1). In the mammary gland of OVX rats, ERα was detected in more than 50% of luminal epithelial cells in the lobular and ductal structures (Fig. 1). In comparison to that of the ovary-intact rats, the mammary gland of ovariectomized rats showed more cells with ERα expression but the staining intensity was weaker (Fig. 1, and data not shown). ERβ was detected in almost all mammary epithelial cells with strong staining, and ERβ-staining was also detected in some stromal cells in the mammary gland (Fig. 1). In the uterus of OVX rats, ERα was detected in almost all endometrial luminal epithelial cells, glandular epithelial cells, and stromal cells; the staining intensity in the epithelial cells was much stronger than that in the stromal cells (Fig. 1). ERβ expression was detected mainly in the endometrial luminal epithelial cells and glandular epithelial cells (Fig. 1). These data confirmed that both ERα and ERβ were expressed in the mammary gland and uterus of OVX rats.

3.2. DPN counteracts the proliferative effect of PPT in the mammary gland

To determine the effect of ERα and/or ERβ activation on mammary cell proliferation, OVX rats were treated with ERα-selective agonist PPT alone, ERβ-selective agonist DPN alone, or PPT and DPN combined. Mammary cell proliferation rate was determined by the percentage of BrdU-labeled cells in different groups of rats. In comparison to the control group, PPT treatment significantly increased mammary epithelial cell proliferation rate in both ductal and lobular structures (Fig. 2). DPN treatment slightly decreased the percentage of BrdU-labeled mammary epithelial cells; statistically, the percentage of BrdU labeled cells in lobules and total cells (ducts and lobules combined) were significantly different from that of control rats (Fig. 2). In the PPT/DPN group treated with PPT and DPN to activate both ERα and ERβ, mammary cell proliferation rate was significantly lower than that of the PPT group but was not significantly different from that of the control group (Fig. 2).

Cyclin D1 expression was evaluated to confirm the effect of PPT and/or DPN on mammary gland cell proliferation. In the mammary glands of the control group and the DPN group, the percentages of cells with moderate and intensive staining for cyclin D1 were very low (Fig. 3). Consistent with the BrdU-staining data, PPT significantly increased the percentage of cells with moderate and intensive staining for cyclin D1 (Fig. 3). In the mammary glands of the PPT/DPN group, the percentage of cells with moderate and intensive staining for cyclin D1 was similar to that of the control group and the DPN group, indicating that PPT-induced cyclin D1 expression was suppressed by DPN (Fig. 3). Collectively, these data indicate that activation of ERα by PPT but not activation of ERβ by DPN leads to increased mammary cell proliferation, and that the proliferative effect of PPT on mammary cell proliferation can be suppressed by DPN.

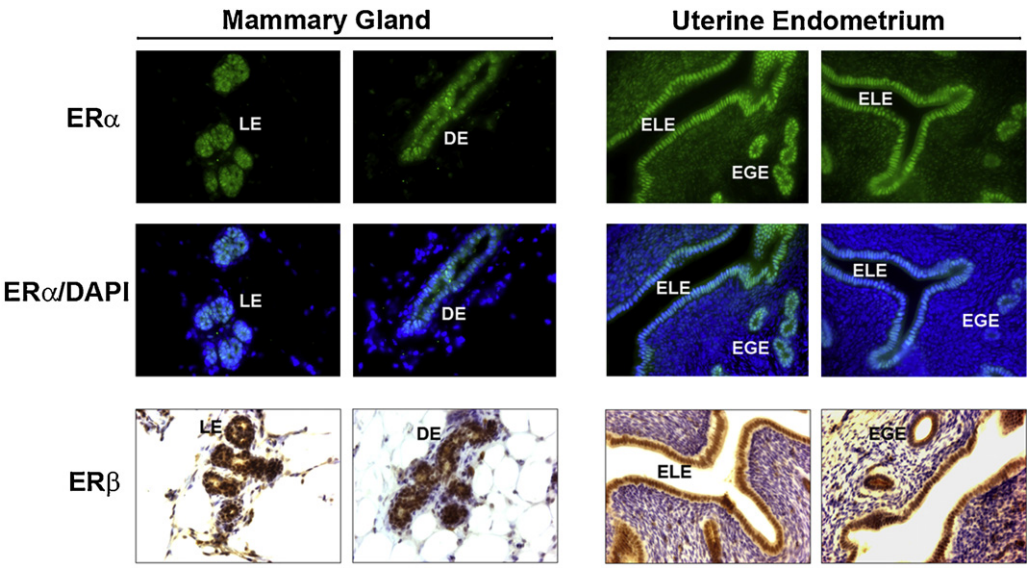

Fig. 1. Immunostaining of ERα and ERβ in the mammary gland and uterine endometrium of ovariectomized rats. Five to six week old rats were ovariectomized (OVX), and mammary gland and uterus tissues were harvested two weeks after OVX. The expression pattern of ERα was determined by IF staining (green fluorescence, top panels). Cell nuclei were counterstained with DAPI (blue fluorescence). Images of ERα staining and DAPI staining were overlaid to show the localization of ERα-staining cells (middle panels). The expression pattern of ERβ was determined by IHC staining with DAB as substrate to give brown color (bottom panels). In the mammary gland, ERα staining was detected in more than 50% of luminal epithelial cells; ERβ was expressed in almost all epithelial cells and some stromal cells. In the endometrium, ERα was detected in almost all epithelial cells and stromal cells; ERβ expression was detected mainly in the luminal and glandular epithelial cells. DE, ducts; ELE, endometrial luminal epithelial cells; EGE, endometrial glandular epithelial cells; LE, lobules. Magnification, 400×. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3.3. Inhibition of PPT induced amphiregulin expression by DPN in the mammary gland

In the mammary gland, ERα mediates cell proliferation by paracrine regulation and the epidermal growth factor (EGF) family member amphiregulin plays critical role in the paracrine action of ERα [11,31,68]. Consistent with its estrogenic activity to promote cell proliferation as shown above, PPT significantly increased amphiregulin expression (Fig. 4). DPN alone did not affect much of amphiregulin expression; when co-administered with PPT, PPT induced amphiregulin increase was significantly reduced by DPN in the PPT/DPN group (Fig. 4). These data suggest that the inhibitory effect of DPN on PPT-induced mammary cell proliferation may be mediated by suppression of amphiregulin expression.

3.4. DPN does not inhibit the estrogenic activity of PPT in the uterus

Uterine wet weight (UWW) and morphology were used to determine the uterotrophic effect of PPT and DPN (Fig. 5A, and data not shown). In the OVX control group, the uterus was very thin in diameter. Consistent with other studies [36,37,60–62,65,69], PPT treatment increased uterine diameter and uterine wet weight, whereas uterus morphology and UWW in the DPN group were very similar to that of the control group (Fig. 5A). Co-administration of DPN with PPT did not inhibit or enhance PPT's effect on UWW (Fig. 5A).

Immunostaining of BrdU-labeled cells was used to determine endometrial cell proliferation rate in different groups of rats (Fig. 5B and C). In comparison to the control group, PPT treatment group showed significantly higher percentage of BrdU-labeled endometrial luminal and glandular cells (Fig. 5B and C). DPN treatment did not affect the percentage of BrdU-labeled endometrial luminal and glandular cells (Fig. 5B and C). When PPT and DPN were co-administered in the PPT/DPN group, PPT-increased cell proliferation was not inhibited by DPN (Fig. 5B and C). These data are consistent with that the estrogenic activity in the uterus is mediated by ERα and that ERβ-selective agonist DPN had little effect on the estrogenic activity of ERα-selective agonist PPT in the uterus [36,37,60–62,65,69].

3.5. The estrogenic activity of PPT in the uterus is opposed by progesterone and the opposing function of progesterone in the uterus is not affected by DPN

The adverse effect of estrogenic activity on uterine endometrium can be opposed by progestins [3,4]. Consistent with that the estrogen action in the uterus is mediated by ERα, we found that the estrogenic activity of ERα-agonist PPT can be opposed by progesterone (Fig. 5). The UWW and the percentage of BrdU-labeled endometrial epithelial cells in the PPT/P4 group were significantly lower than that of the PPT group (Fig. 5). UWW and the percentage of BrdU-labeled endometrial epithelial cells in the PPT/P4/DPN group were similar to that of the PPT/P4 group (Fig. 5). These data indicate that progesterone has opposing effect on PPT in the uterus tissues and DPN does not affect the opposing function of progesterone.

3.6. The inhibitory function of DPN in mammary cell proliferation remains effective when used together with PPT and progesterone

Progestins when used alone have little effect on mammary cell proliferation; however, progestins and estrogen have synergistic effect on mammary cell proliferation and progestins also have synergistic effect with PPT in promoting mammary gland end bud development [31,33,56,70]. Consistently, we found that the percentage of BrdU labeled mammary proliferating cells was significantly higher in the PPT/P4 group than that of the PPT group (Figs. 2 and 6). To determine whether the inhibitory effect of DPN on mammary cell proliferation is affected by progesterone, DPN was co-administered with PPT/P4. The percentage of BrdU labeled mammary cells in the PPT/P4/DPN group was significantly lower

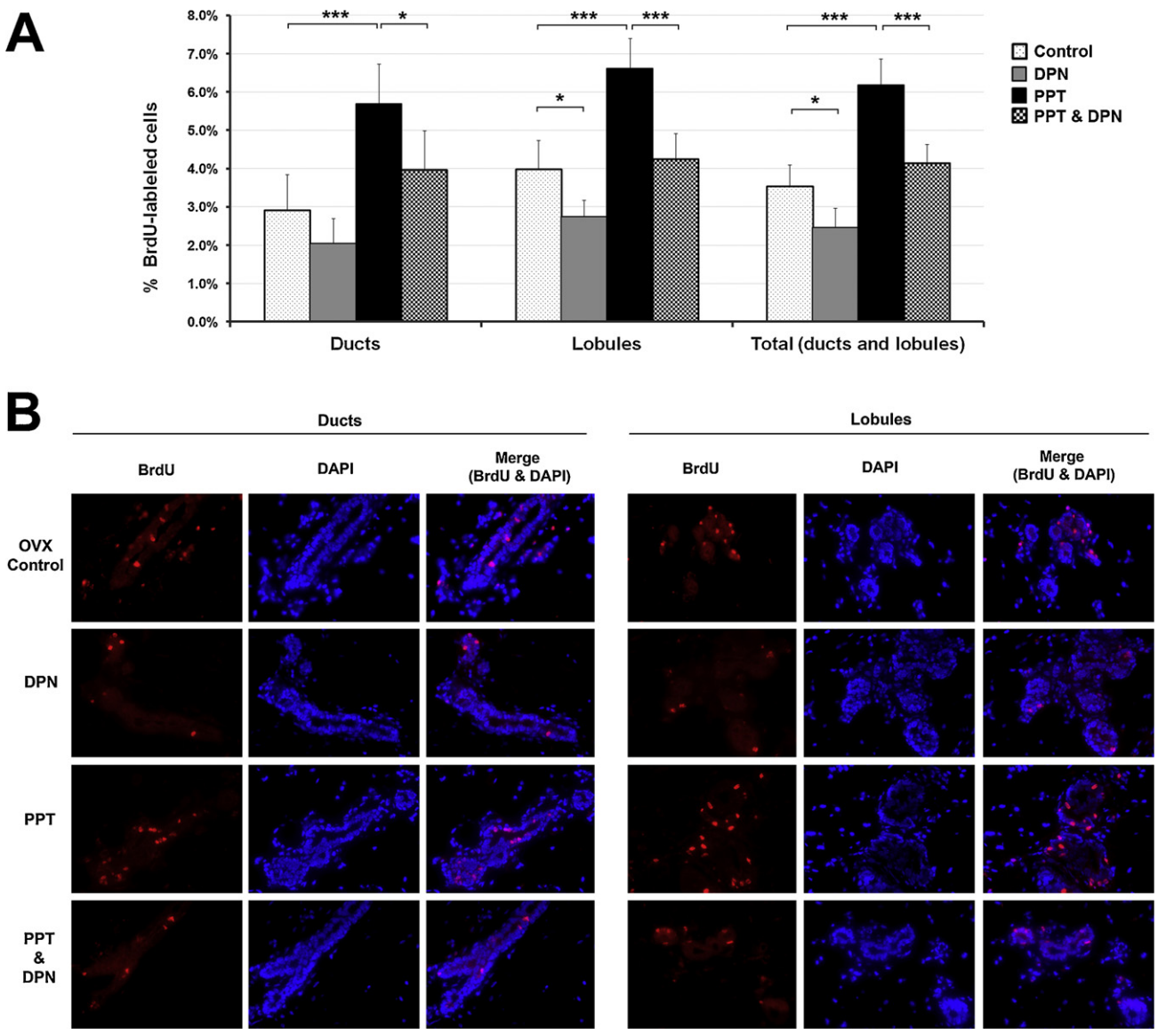

Fig. 2. DPN counteracts the proliferative effect of PPT in the mammary gland of OVX rats. OVX rats were rested for two weeks before treatment for 3 days with DMSO (control), DPN, PPT, PPT and DPN (PPT/DPN), respectively. BrdU was injected concurrently with each drug administration to label proliferating cells. (A) Percentage of BrdU-labeled proliferation cells in the mammary gland of different groups of rats. Mammary epithelial cells in ductal and lobular structures were counted separately, and the total is the combination of ductal and lobular cells. DPN slightly decreased mammary cell proliferation. PPT significantly increased mammary epithelial cell proliferation. When co-administered with PPT, DPN significantly decreased mammary cell proliferation caused by PPT. Data are shown as means ± SD. The p values are as follows: 0.45 for DPN vs. control in the ducts, 0.03 for DPN vs. control in the lobules and totals; <0.001 for PPT vs. control in the ducts, lobules, and total; 0.023 for PPT&DPN vs. PPT in the ducts, and <0.001 for PPT&DPN vs. PPT in the lobules and total ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (B) Representative microimages showing BrdU-labeled proliferation cells in the mammary ductal and lobular structures of different groups of rats. BrdU-labeled cells were detected by immunofluorescent staining with BrdU stained with red fluorescence; nuclei were counterstained with DAPI to give blue fluorescence. Images of BrdU staining and DAPI staining were merged to show the location of BrdU-labeled cells. Magnification, 400×. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

than that of the PPT/P4 group, indicating that the inhibitory function of DPN was still effective when co-administrated with PPT/P4 (Fig. 6).

4. Discussion

Mammary gland and uterine endometrium are classic estrogen regulated organs/tissues and the estrogen action is mediated mainly by ERα [7–11,30,36,37]. Excessive estrogenic activity, such as estrogen-based postmenopausal hormone replacement therapy, exposure to environmental endocrine disruptive agents with estrogenic activity, early menarche or late menopause, is associated with increased risk for breast cancer and/or endometrial cancer [71–73]. Accumulating evidence indicates that ERβ may function as a tumor suppressor and therefore can be exploited for cancer prevention and therapy [9,47,48]. However, *in vivo* animal model studies on the role of ERβ and its interaction with ERα in mammary cell proliferation and tumorigenesis are very limited and the expected tumor suppressor function was not observed in the studies using ERβ-selective agonists BAG and ERB-041 [30,56,57]. It is unknown whether ERB-041 or other ERβ-selective agonists have additive/synergistic or antagonistic effect with ERα-selective agonist PPT in mediating mammary cell proliferation. In this study, we used ER-selective agonists PPT and DPN to activate ERα and ERβ separately in an OVX rat model. We demonstrated for the first time that the estrogenic activity of PPT in the mammary gland, including cell proliferation and amphiregulin expression, can be opposed by

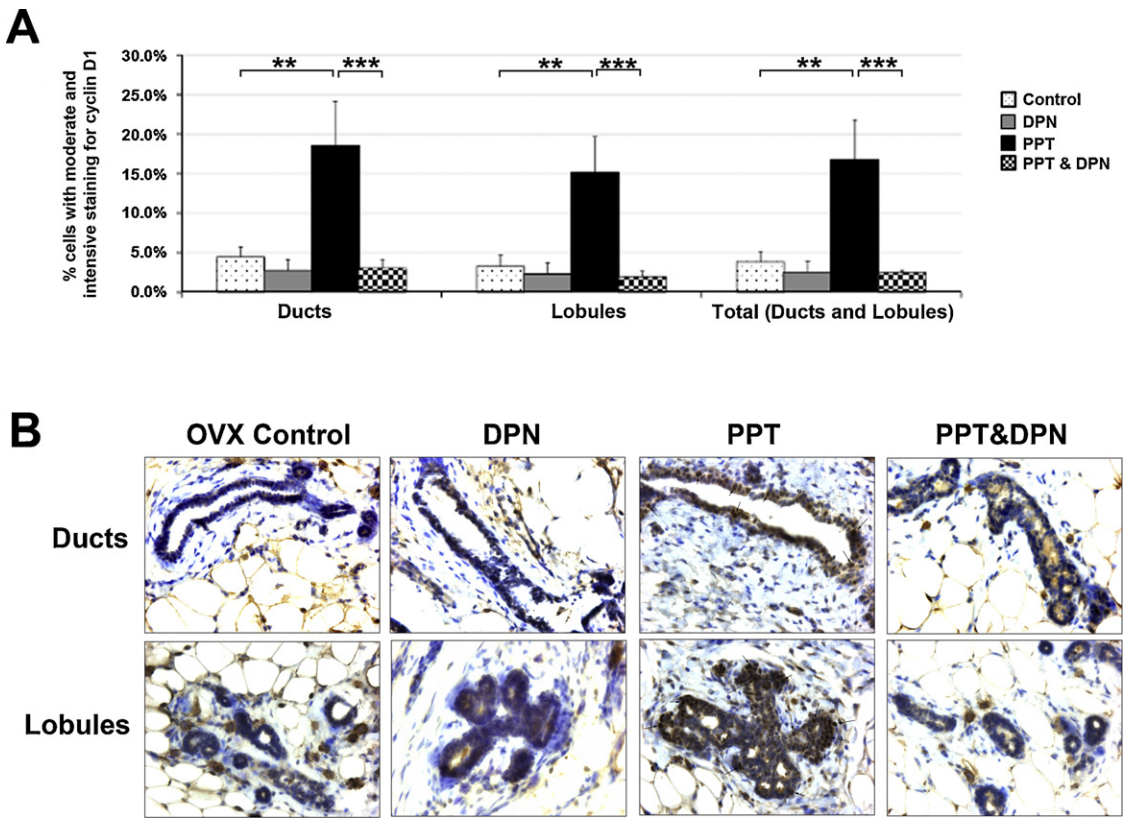

Fig. 3. DPN inhibits PPT induced cyclin D1 expression in the mammary gland. (A) Percentage of cells with moderate and intensive staining for cyclin D1 in the mammary gland of different groups of rats. Mammary epithelial cells in ductal and lobular structures were counted separately, and the total is the combination of ductal and lobular cells. Cyclin D1 was detected by immunohistochemical staining using DAB as the substrate (brown color) and hematoxylin for counterstaining (blue color). Cells were counted as unstained, weakly stained, moderately stained, and intensively stained for cyclin D1. Cells with moderate or intensive staining were calculated as cyclin D1-staining cells and the percentage of those cells were shown in the graph as means ± SD. PPT significantly increased the percentage of cyclin D1 staining cells. When co-administered with PPT, DPN significantly decreased the percentage of cyclin D1 staining cells caused by PPT. The *p* values are as follows: 1.00 for DPN vs. control in the ducts, lobules, and totals; 0.002 for PPT vs. control in the ducts, lobules, and totals; 0.001 for PPT&DPN vs. PPT in the ducts, lobules, and totals (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). (B) Representative cyclin D1 IHC staining images of ductal and lobular structures in the mammary gland of the four groups of rats. Cells with intensive cyclin D1 staining (arrows) were found in the mammary gland treated with PPT and barely observed in the mammary gland of the other three groups (control, DPN, PPT/DPN). Magnification, 400×. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

an ERβ-selective agonist in an animal model. We further showed that the estrogenic activity of PPT in the uterus can be opposed by progesterone, and that DPN and progesterone do not interfere with each either for their respective opposing functions in the mammary gland and uterus. These findings indicate that the adverse effect of ERα-mediated estrogenic activity in different organs/tissues can be opposed by different drugs via different mechanisms, which may be explored for the development of better hormone replacement therapy regimen with less risk for breast and endometrial cancer.

The differential effect of DPN on the estrogenic activity of PPT in the mammary gland and uterus supports that the effect of DPN activated ERβ on target cells is cell type dependent. In addition, the effect of ERβ on cellular activities of target cells is ligand type dependent and the results from ERβ-selective agonists including DPN may not represent the physiological role of ERβ bound to estrogen [9,47,53,54]. The effect of DPN on mammary cell proliferation in this study is quite different from that of the ERβ-selective agonist BAG in the study by Cheng et al. using an OVX mouse model [30]. It is unlikely that the difference is caused by different rodent

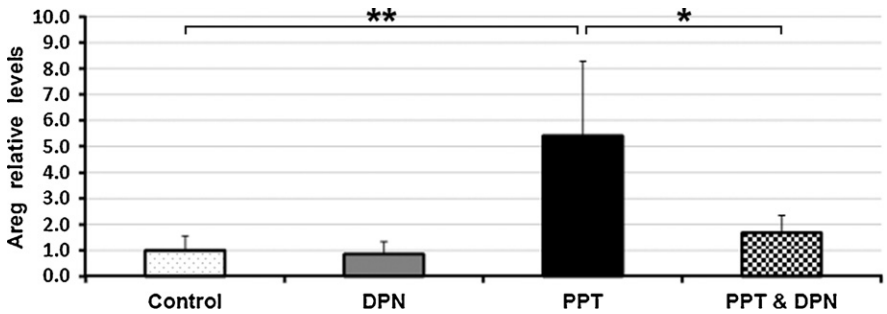

Fig. 4. DPN inhibits PPT induced amphiregulin expression in the mammary gland. RNA levels of amphiregulin were determined by quantitative real-time RT-PCR; β-actin was used as internal control. PPT but not DPN significantly increased amphiregulin expression. Amphiregulin expression in the PPT/DPN group was significantly lower than that in the PPT group. Data are shown as means ± SD. The *p* values are as follows: 0.003 for PPT vs. control; 0.013 for PPT&DPN vs. PPT (*$p < 0.05$; **$p < 0.01$).

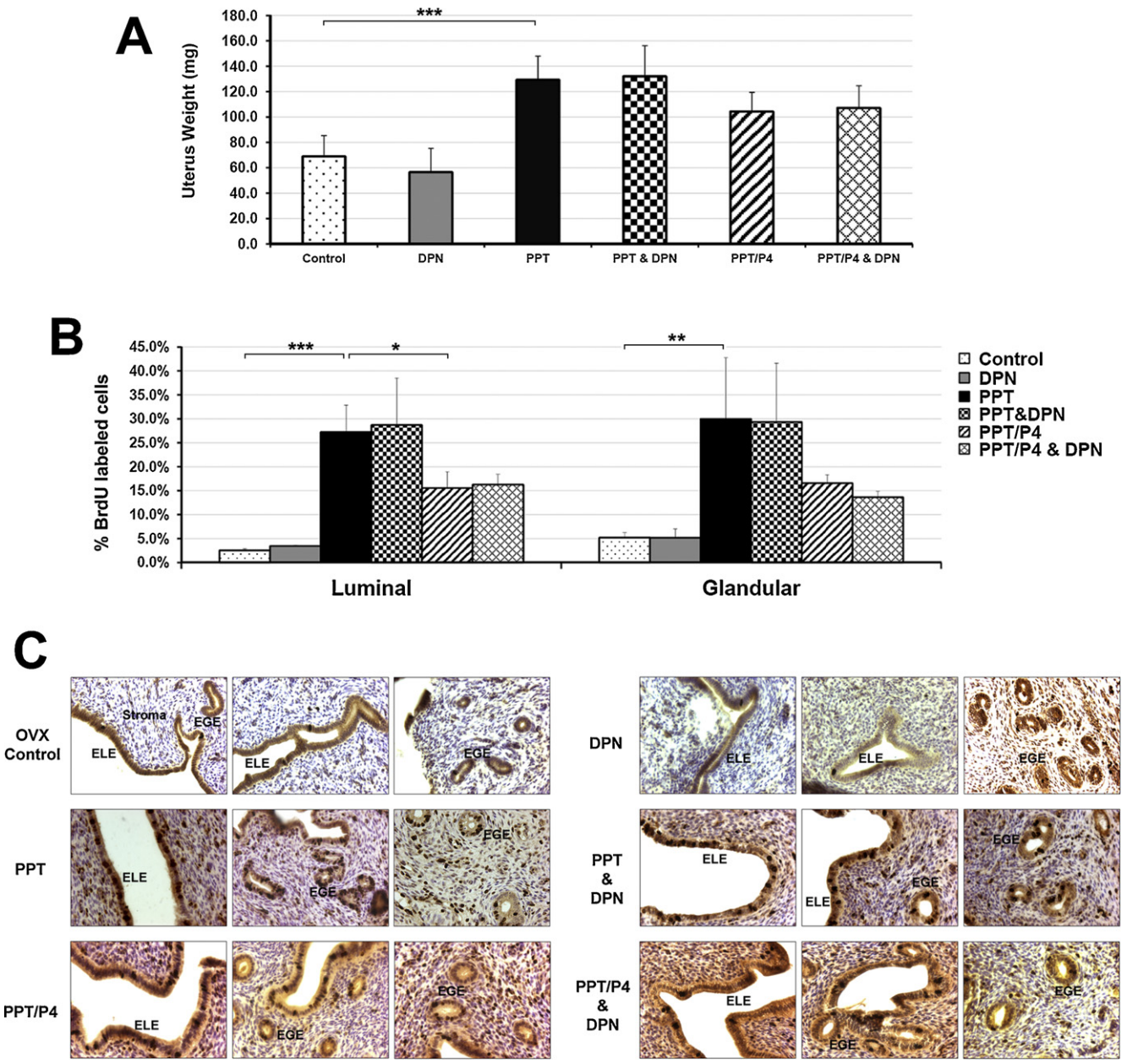

Fig. 5. The estrogenic activity of PPT in the uterus is opposed by progesterone but not by DPN. (A) Uterine wet weight (UWW) in different groups of rats. PPT but not DPN had uterotrophic activity. The uterotrophic activity of PPT was opposed by progesterone but not by DPN. Data are shown as means ± SD. $^{***}p < 0.001$ for the PPT group vs. the control group. (B) Percentage of BrdU-labeled endometrial luminal and glandular epithelial cells. PPT but not DPN significantly increased endometrial luminal and glandular epithelial cell proliferation. The effect of PPT on endometrial epithelial cell proliferation was opposed by progesterone but not by DPN. Data are shown as means ± SD. The p values are as follows: <0.001 for PPT vs. control in luminal epithelial cells, 0.003 for PPT vs. control in glandular epithelial cells; 0.014 for PPT/P4 vs. PPT in luminal epithelial cells, and 0.078 for PPT/P4 vs. PPT in glandular epithelial cells ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (C) Representative microimages showing BrdU-labeled proliferation cells in endometrial luminal and glandular cells. BrdU-labeled cells were detected by immunohistochemical staining using DAB as substrate (brown color) and hematoxylin for counterstaining. ELE, endometrial luminal epithelial cells; EGE, endometrial glandular epithelial cells. Magnification, 400×. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

species, but it will be valuable to evaluate ERβ-selective agonists in more than one species. It is more likely that different ERβ-selective agonists including DPN and BAG may exert different effects; in that case, it will be valuable to determine whether other ERβ-selected agonists besides DPN have anti-proliferative function in the mammary gland.

The uterotrophic activity of PPT and several ERβ-selective agonists has been well studied in different rodent models. In all these models, PPT has been shown with estrogenic activity to increase uterine wet weight or to stimulate endometrial cell proliferation [36,62,65]. For the ERβ-selective agonists evaluated for uterotrophic activity, 8β-VE2 is the only one known with uterotrophic activity [9,30]. In the OVX rodent models, DPN does not have uterotrophic activity [61,69]. Data on whether the uterotrophic activity of PPT can be suppressed by DPN is inconsistent in different animal models. In the OVX model, our data are consistent with that DPN does not suppress the uterotrophic activity of PPT [60]. In ArKO (aromatase knock-out) and ovary intact immature mouse models, DPN does not have uterotrophic activity but may have some inhibitory activity and reduces the estrogenic activity of PPT [62,65]. From these different models, it is clear that DPN does not have uterotrophic activity and may instead

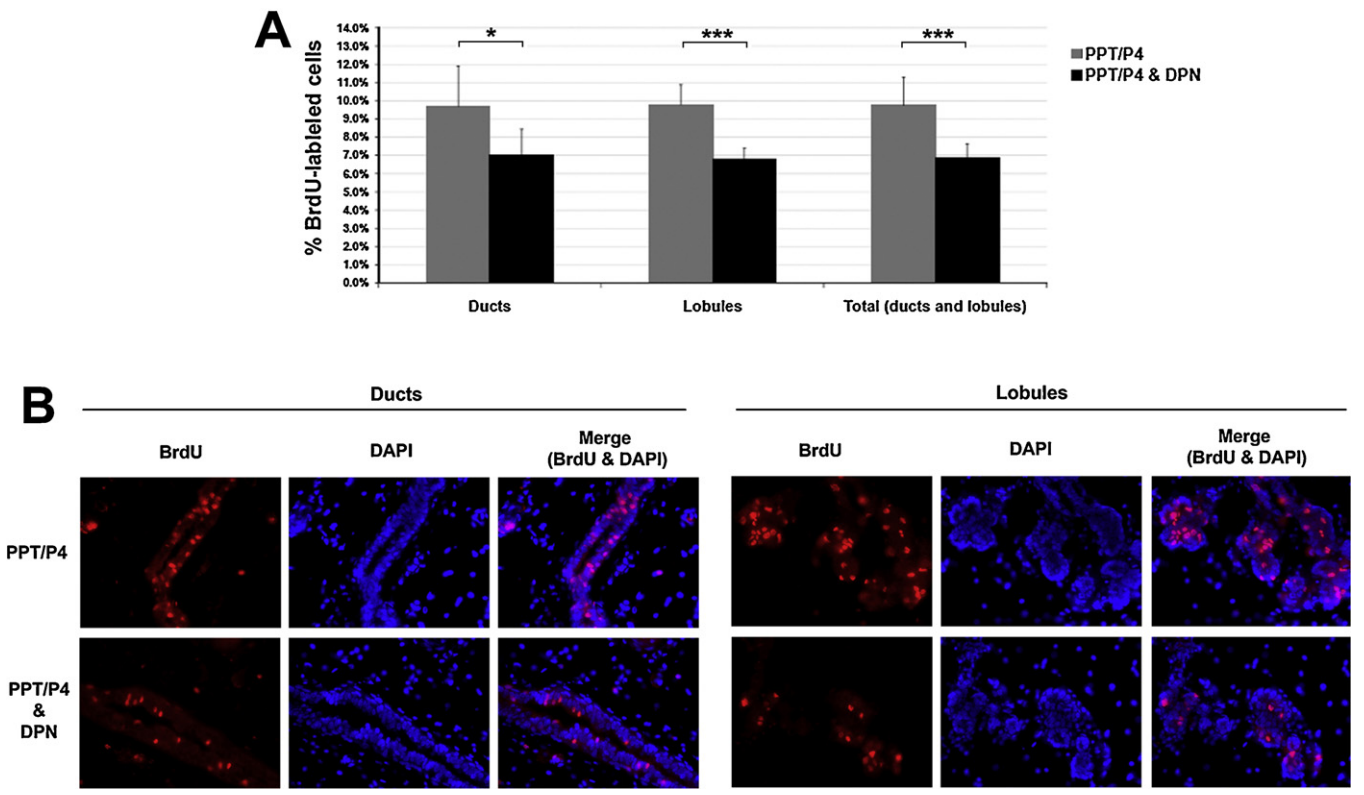

Fig. 6. DPN inhibits mammary cell proliferation induced by PPT and progesterone. (A) Percentage of BrdU-labeled mammary epithelial cells. See Fig. 2 to compare with other groups of rats. PPT and progesterone (P4) were synergistic in promoting mammary epithelial cell proliferation. When co-administered with PPT/P4, DPN significantly decreased mammary cell proliferation caused by PPT/P4. Data are shown as means ± SD. The p values are as follows: <0.001 for PPT/P4 vs. PPT in the ducts, lobules, and totals; 0.02 for PPT/P4 & DPN vs. PPT/P4 in the ducts, <0.001 for PPT/P4 & DPN vs. PPT/P4 in the lobules and totals ($^{*}p < 0.05$; $^{***}p < 0.001$). (B) Representative microimages showing BrdU-labeled proliferation cells in the mammary ductal and lobular structures. BrdU-labeled cells were detected by immunofluorescent staining with BrdU stained with red fluorescence; nuclei were counterstained with DAPI to give blue fluorescence. Images of BrdU staining and DAPI staining were merged to show the location of BrdU-labeled cells. Magnification, 400×. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

inhibit the ERα-mediated uterotrophic activity to a certain extent. The reason why DPN has a more pronounced suppressive effect on PPT activity in the mammary gland vs. the uterus is not clear. It could be related to the relative expression levels of ERβ or the relative ratios of ERα/ERβ in these two types of tissues. It is worth noting that the relative expression patterns of ERα and ERβ are different in these two types of tissues, which might also account for the differential effect of DPN in these two types of tissues. As shown in Fig. 1, ERβ expression is more widespread than ERα in the mammary gland; in the uterine endometrium, ERα expression is more widespread than ERβ. The mammary gland and uterine endometrium respond differently to Tamoxifen, indicating that the cellular contexts, such as coactivators or corespressors, might be different in these two types of tissues that may also be related to the differential effect of DPN [74].

In the PPT/DPN group of rats in this study, the estrogenic activity of PPT in the mammary gland is almost completely abrogated by DPN, as determined by mammary cell proliferation rate and expression of cyclin D1 and amphiregulin. The mammary cell proliferation rate in the PPT/P4/DPN group is significantly lower than that in the PPT/P4 group, indicating that ERα-mediated activity that functions synergistic with progesterone is significantly inhibited by DPN. However, the mammary cell proliferation rate in the PPT/P4/DPN group is significantly higher than that in the control and PPT/DPN groups, and even a little higher than the PPT group. These data indicate that when co-administered with PPT/P4, PPT activated ERα activity is not completely blocked by DPN. Further studies are needed to determine whether the inhibitory function of DPN is dose-dependent and whether the inhibitory function of DPN can be further increased at other doses.

ERα-mediated activity is believed to be responsible for the increased risk for breast cancer and endometrial cancer in women under HRT. As shown in this study using the OVX postmenopausal rat model, ERα-mediated estrogenic activity activated by PPT in the mammary gland and uterus can be differentially opposed by DPN and progesterone, respectively. It will be interesting to determine whether a regimen containing DPN such as PPT/DPN/progestin is a better option than the estrogen-plus-progestin regimen. When considering inclusion of DPN or ERβ-selective agonist in the hormone replacement therapy regimen, at least two types of effects need to be considered. The first concern is whether ERβ-selective agonist has adverse effects in different organs/tissues. The other concern is that whether some of the beneficial effects of estrogenic activity are abolished or some of the adverse effects of estrogenic activity are enhanced. Based on the reported animal model studies involving many organ systems, it seems that PPT and DPN might be good candidates with regards to the beneficial vs. adverse effects. Here we will briefly discuss the effects on the mammary gland, uterine endometrium, hot flashes, and bone metabolism. As aforementioned, DPN can inhibit the adverse effect of PPT in the mammary gland and DPN has no adverse effect on uterine endometrium. With regards to hot flashes, both PPT and DPN have been shown to prevent hot flashes in rodent models [36,66]. For bone metabolism, bone sparing activity is found in PPT but not found in several ERβ-selective agonists including ERB-041; the effect of DPN on bone metabolism is unknown [9,36]. Further

studies will be needed to determine the effect of DPN on bone metabolism and its effect on the bone sparing activity of PPT. In summary, these functional studies of DPN in animal models suggest that DPN merits consideration for the development of better hormone replacement therapy regimen.

5. Conclusions

Estrogen-based postmenopausal hormone replacement therapy increases the risk for breast and uterine endometrial cancers; estrogen action in the mammary gland and endometrium is mediated mainly by ERα [1,2,7–11,30,36,37]. In this study using an ovariectomized postmenopausal rat model and the ERα-selective agonist PPT and ERβ-selective agonist DPN, we provide *in vivo* evidence that ERα-mediated estrogenic activity in the mammary gland can be opposed by ERβ-selective agonist DPN. Suppressing PPT-induced amphiregulin expression by DPN may account for its opposing function in mammary gland cell proliferation. The estrogenic activity of ERα-agonist PPT in the uterus can be opposed by progesterone. These findings indicate that the adverse effect of ERα-mediated estrogenic activity in different organs can be opposed by different mechanisms, which may be explored for the development of better hormone replacement therapy regimen with less risk for breast and endometrial cancer.

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