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ORIGINAL ARTICLE

Estrogen receptor beta growth-inhibitory effects are repressed through activation of MAPK and PI3K signalling in mammary epithelial and breast cancer cells

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Two thirds of breast cancers express estrogen receptors (ER). ER alpha (ERα) mediates breast cancer cell proliferation, and expression of ERα is the standard choice to indicate adjuvant endocrine therapy. ERbeta (ERβ) inhibits growth *in vitro*; its effects *in vivo* have been incompletely investigated and its role in breast cancer and potential as alternative target in endocrine therapy needs further study. In this work, mammary epithelial (EpH4 and HC11) and breast cancer (MC4-L2) cells with endogenous ERα and ERβ expression and T47-D human breast cancer cells with recombinant ERβ (T47-DERβ) were used to explore effects exerted *in vitro* and *in vivo* by the ERβ agonists 2,3-bis (4–hydroxy–phenyl)-propionitrile (DPN) and 7-bromo-2-(4–hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY). *In vivo,* ERβ agonists induced mammary gland hyperplasia and MC4-L2 tumour growth to a similar extent as the ERα agonist 4,4′,4′′-(4-propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol (PPT) or 17β-estradiol (E2) and correlated with higher number of mitotic and lower number of apoptotic features. *In vitro*, in MC4-L2, EpH4 or HC11 cells incubated under basal conditions, ERβ agonists induced apoptosis measured as upregulation of p53 and apoptosis-inducible factor protein levels and increased caspase 3 activity, whereas PPT and E2 stimulated proliferation. However, when extracellular signal-regulated kinase 1 and 2 (ERK ½) were activated by co-incubation with basement membrane extract or epidermal growth factor, induction of apoptosis by ERβ agonists was repressed and DPN induced proliferation in a similar way as E2 or PPT. In a context of active ERK ½, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (AKT) signalling was necessary to allow proliferation stimulated by ER agonists. Inhibition of MEK ½ with UO126 completely restored ERβ growth-inhibitory effects, whereas inhibition of PI3K by LY294002 inhibited ERβ-induced proliferation. These results show that the cellular context modulates ERβ growth-inhibitory effects and should be taken into consideration upon assessment of ERβ as target for endocrine treatment.

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Keywords: estrogen receptor beta; mammary gland; breast cancer; apoptosis; proliferation; ERK ½

INTRODUCTION

Estrogen receptors (ER) alpha (ERα) and beta (ERβ) are ligand-activated transcription factors with high sequence and structural homology, and similar affinity for the endogenous estrogen 17β-estradiol (E2). ERα and ERβ have overlapping DNA-binding sites, and also regions selectively bound by either receptor.[1,2] Therefore, each receptor can activate different gene expression programs and biological responses.

ERα is essential for mammary epithelial cell (MEC) proliferation and ductal development;[3] while at this stage, ERβ loss has no effect. Instead, lactating mammary glands from $ERβ^{-/-}$ mice show high Ki67, reduced integrin α2 and E-cadherin staining.[4] *In vitro*, ERα ligand activation stimulates HC11 MEC proliferation, whereas ERβ activation induces apoptosis.[5] Ligand activation of ERβ increases E-cadherin protein levels[6,7] and attenuates β-catenin signalling;[7] while ERβ knockdown leads to E-cadherin degradation, β-catenin activation and impaired formation of acinar colonies.[6] Thus, ERβ counteracts ERα growth stimulatory signals and maintains MEC homoeostasis.

ERα stimulates breast cancer growth and it is the standard to indicate adjuvant endocrine therapy.[8] As there is a lack of breast cancer cell lines with endogenous ERβ expression, recombinant ERβ is overexpressed, which often inhibits ERα-induced transcription.[9,10] ERβ re-expression inhibits proliferation,[11,12] migration,[11,13] erbB2/erbB3/AKT (RAC-alpha serine/threonine-protein kinase) signalling[14] and sensitizes cells to tamoxifen and its metabolites.[14,15] Therefore, ERβ expression could be therapeutically exploited with selective agonists.[14] However, effects mediated by recombinant ERβ are mostly ligand independent and may not reflect cellular response *in vivo*.

The study of ERβ in human breast cancer has been stalled by the lack of reliable antibodies, limited amount of cell lines expressing ERβ and the number of splice variants expressed in different cell types and organisms.[16] Epidemiological studies have shown that most ER-positive breast cancers express ERβ, although to a lower extent as compared with normal mammary epithelium.[17,18] In breast tumours, ERβ expression is associated with ERα and progesterone receptor co-expression,[19] and also

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found in ½ of ERα-negative cancers that respond to endocrine therapy.[18] However, ERβ use as prognostic marker is still under debate; although one study found ERβ to be an independent predictor of disease-free survival,[20] another one found ERβ expression uninformative.[21] Further, in ERα-negative cancers, ERβ expression is associated with the proliferation marker Ki67,[22,23] which does not correlate with the proposed tumour-suppressor role of ERβ.

Receptor tyrosine kinases and their downstream effectors target ERs[24] and enhance ER ligand-dependent and -independent transactivation, modulate ER affinity for ligands, DNA and transcriptional co-activators.[25] As ERβ may be targeted by receptor tyrosine kinase signalling, the effects exerted by ERβ agonists should be addressed in more complex systems, ideally *in vivo*. In a mammary cancer model, with endogenous ERα and ERβ expression, in which estrogens induce inhibition of tumour growth, the ERβ-selective agonist 2,3-bis (4–hydroxy–phenyl)-propionitrile (DPN) exerted a similar inhibitory effect as the ERα agonist 4,4′,4′′-(4-propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol (PPT) or E2. This indicates that *in vivo*, ERα and ERβ may trigger similar responses. Interestingly, after a few days, DPN-treated tumours began to grow,[26] suggesting that ERβ-mediated effects may vary depending on the tumour growth stage.

The aim of this study was to analyse effects mediated by endogenous ERβ in mammary epithelial (MECs) and breast cancer cells *in vitro* and *in vivo*. Administration of ERβ agonists *in vivo* stimulated mammary tumour growth and ductal hyperplasia, whereas *in vitro,* ERβ agonists induced apoptosis. p-ERK levels were higher in breast cancer cells grown *in vivo* compared with *in vitro*, and activation of MAPK signalling by basement membrane (BM) or epidermal growth factor (EGF) *in vitro* blocked growth inhibition induced by ERβ agonists. Interestingly, in a context of active MAPK, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling was necessary to allow proliferation induced by ERβ. Taken together, these results indicate that the cellular context can modulate ERβ-mediated effects.

RESULTS

Regulation of mammary epithelial and breast cancer cell growth by ERα and ERβ

Effects exerted on proliferation and apoptosis by the ERβ agonists DPN or 7-bromo-2-(4–hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY), E2 or the ERα agonist PPT in MC4-L2 breast cancer cells or MECs were evaluated.

Proliferation, assessed by bromodeoxyuridine (BrdU) incorporation, increased by 25–50% following incubation with E2 or PPT (Figure 1a) and correlated with ERα expression levels (Supplementary Figures 1A and B). On the contrary, DPN or WAY did not significantly affect BrdU incorporation in any of the cell lines tested (Figure 1a and Supplementary Figure 3A, respectively). Co-treatment with 4OH-tamoxifen (4OH-T) completely prevented E2 and PPT stimulation of proliferation (Supplementary Figure 3B), which indicates that proliferation signals occur through the nuclear receptor pathway. Whether ERα alone or ERα/ERβ heterodimers are involved remains to be established.

Apoptosis was evaluated as caspase 3 (Casp 3) activity (Figure 1b). ERβ activation with DPN (ERβ–DPN) increased Casp 3 activity in all cell lines; although E2 exerted no significant effect on MC4-L2 and HC11 cells but also induced Casp 3 activity in EpH4 cells. Activation of Casp 3 by DPN correlated with upregulation of p53 protein (Figure 1c), which was also confirmed in cells treated with WAY (Supplementary Figure 3C). In addition, E2 and DPN upregulated apoptosis-inducible factor (Aif) in EpH4 and HC11 cells (Figure 1d).

ERβ-induced apoptosis associated to p53 upregulation has been shown in HC11 cells.[27] HC11 and MC4-L2 cells have mutated p53.[28,29] However, transcriptionally inactive p53 mutants can induce apoptosis[30] and p53 also induces apoptosis in the absence of transcription,[31] which may be the case in these cells.

In HC11 cells, PPT increased and DPN reduced cell number, but no net effect was observed with E2.[5] A similar picture was observed in EpH4 cells, while in MC4-L2 cells, E2 also increased cell number (Figure 1e). These effects were reverted by co-treatment with 4OH-T (Supplementary Figure 3D). Therefore, E2, which can activate ERα and ERβ, preferably induced ERα-mediated proliferation in a larger proportion of MC4-L2 breast cancer cells (with higher ERα expression levels) as compared with MECs, in which E2, similarly to ERβ agonists (DPN or WAY) also induced apoptosis through p53 and Casp 3 (EpH4) or Aif (EpH4 and HC11), which resulted in no differences in cell number. It is possible that, as shown previously for HC11 cells,[5] in EpH4 cells, the dual effect exerted by E2 depends on the varying ERα/ERβ ratios throughout the cells.

Treatment of mouse mammary tumours and mammary glands with ERα or ERβ agonists stimulates growth

MC4-L2 tumour growth is stimulated by E2[28] and inhibited by letrozole and raloxifene.[32] To study ERβ–DPN effect on tumour growth, mice bearing MC4-L2 tumours were treated with DPN for 17 days. Tumour growth was significantly stimulated by DPN in a similar way as observed in E2- or PPT-treated groups (Figure 2a), and correlated with upregulation of cyclin D1 protein (Figure 2b) and higher number of mitotic features than the control group (Figure 2c and d, arrows), indicating that DPN induced cell proliferation. In addition, smaller number of apoptotic bodies were observed in DPN-treated group (Figure 2c and d, arrowheads). As DPN exerted similar effects as the ERα agonist PPT, selectivity of DPN treatment *in vivo* was confirmed by quantification of uterine lactoferrin and complement C3 mRNA, which were only upregulated by E2 and PPT[33] (that is, ligand-activated ERα; Supplementary Figure 4A). Therefore, *in vivo,* a dose of ERβ agonist, which does not activate ERα, induced cell proliferation and inhibited apoptosis.

Effects exerted by ERβ agonists DPN and WAY were evaluated in the fourth contralateral mammary gland following 5- or 17-day treatment. Already after 5 days, DPN and to a lower extent WAY or E2 induced ductal growth and lateral branching (Figure 3, left panel) as well as proliferation measured as Ki67 staining (Supplementary Figure 4B). Actively proliferating terminal end buds (arrowheads) were found and areas of ductal hyperplasia were observed (Figure 3, right panel). These results indicate that, as shown in MC4-L2 tumours, activation of ERβ *in vivo* induced proliferation of the mammary epithelium.

Notably, results obtained with ERβ agonists *in vivo* were in clear contrast to those observed *in vitro*.

BM reverts ERβ growth-inhibitory effects

The extracellular matrix influences signalling by ovarian hormones[34] and could modulate effects exerted by ERβ agonists *in vitro* and *in vivo*. To analyse the contribution of the BM to ERβ signalling, MC4-L2 cells were grown with 5% BM (Matrigel). Under these conditions, MAPK signalling was stimulated (that is, upregulation of extracellular signal-regulated kinase 1 and 2 (ERK ½) phosphorylated in thr202/tyr204; p-ERK ½) and downregulated by co-incubation with BM and the MEK ½ inhibitor UO126 (Figure 4a, top panel). Higher p-ERK ½ levels were detected in MC4-L2 tumours compared with MC4-L2 cells grown *in vitro* (Figure 4a, bottom panel), which supports the idea that BM stimulates MAPK signalling.

Basal proliferation was increased by one third in cells grown with BM (not shown); ERβ agonists (DPN or WAY) further exerted a slight but significant increase (Figure 4b and Supplementary Figure 5A, respectively). E2 and ERα-PPT stimulated proliferation in

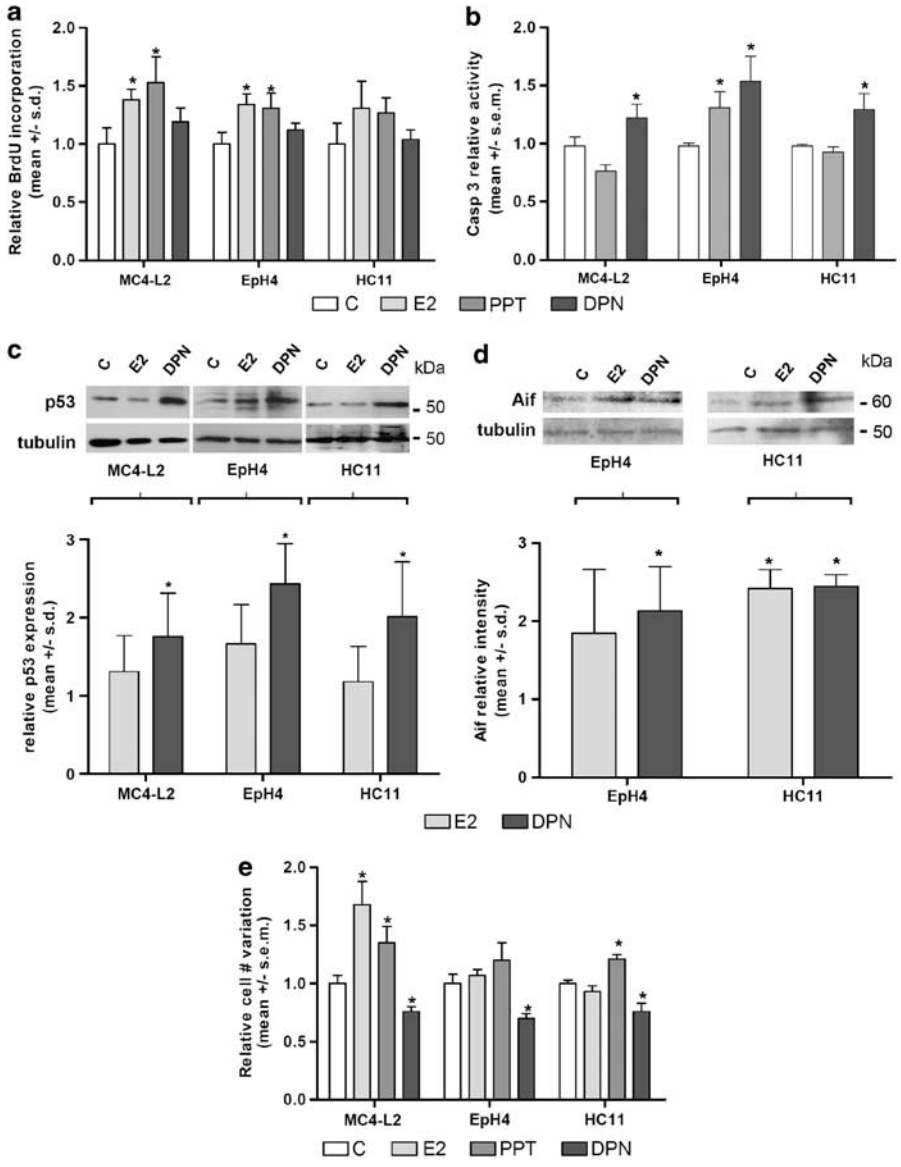


Figure 1. Activation of ERβ with DPN induces apoptosis of mammary epithelial and breast cancer cells. MC4L2 cells were treated with 1 nM E2, 10 nM PPT or 10 nM DPN, whereas EpH4 and HC11 cells were treated with 10 nM E2, 10 nM PPT or 10 nM DPN. (**a**) BrdU incorporation was quantified with an ELISA kit following 3-day incubation with ER agonists. Relative incorporation was calculated by setting untreated control values to 1. A representative experiment of three is shown. (**b**) Casp 3 activity was measured after 24 h treatment using a colorimetric substrate. Relative Casp 3 activity was calculated by normalization of values to the untreated control. Mean ± s.e.m from at least three independent experiments is shown. (**c**, **d**) p53 (**c**) and Aif (**d**) protein levels following 24 h treatment were evaluated by immunoblot. Intensity of the bands was normalized to the value corresponding to the untreated control. (**e**) Cell number was counted after 3-day incubation with each ER agonist and relative cell number variation was calculated by normalizing to untreated control values. In all panels, $^*P<0.05$ vs control.

a similar proportion as in cells without BM (compare index in Figures 1a and 4a). Proliferation induced by any ER agonist used was completely reverted by co-incubation with 4OH-T or UO126 (Figure 4b and Supplementary Figure 5A).

Levels of p53 protein, which in cells grown without BM (− BM) were upregulated by ERβ agonists (Figure 1b and Supplementary Figure 3C), were reduced when cells were grown with BM (+ BM; Figure 4c). Co-incubation of MC4-L2 cells + BM reduced DPN effects on Casp 3 activity to control levels, whereas UO126 completely restored Casp 3 activity as well as upregulation of p53 induced by ERβ–DPN (Figure 4d). In cells + BM, E2 exerted a slight inhibition of Casp 3 activity and no effect on p53 protein levels; yet, E2 co-incubation with UO126 (complete downregulation of ERK ½ levels) increased Casp 3 activity and also upregulated p53 protein (Figure 4d). Therefore, in the absence of active ERK ½, E2 may preferentially activate ERβ and induce apoptosis. Alternatively, active ERα (that is, phosphorylated in AF-1 and AF-2) may be necessary to inhibit ERβ–E2-induced apoptosis by formation of ERα/ERβ heterodimers and transactivation of proliferation genes.

The decrease in MC4-L2 cell number induced by ERβ–DPN in cells –BM (Figure 1e) was not observed in cells cultured + BM; however, co-culture with UO126 completely restored ERβ inhibition of cell number (Figure 4e). These results show that activation of MAPK signalling by BM fully blocks growth-inhibitory effects exerted by ERβ agonists.

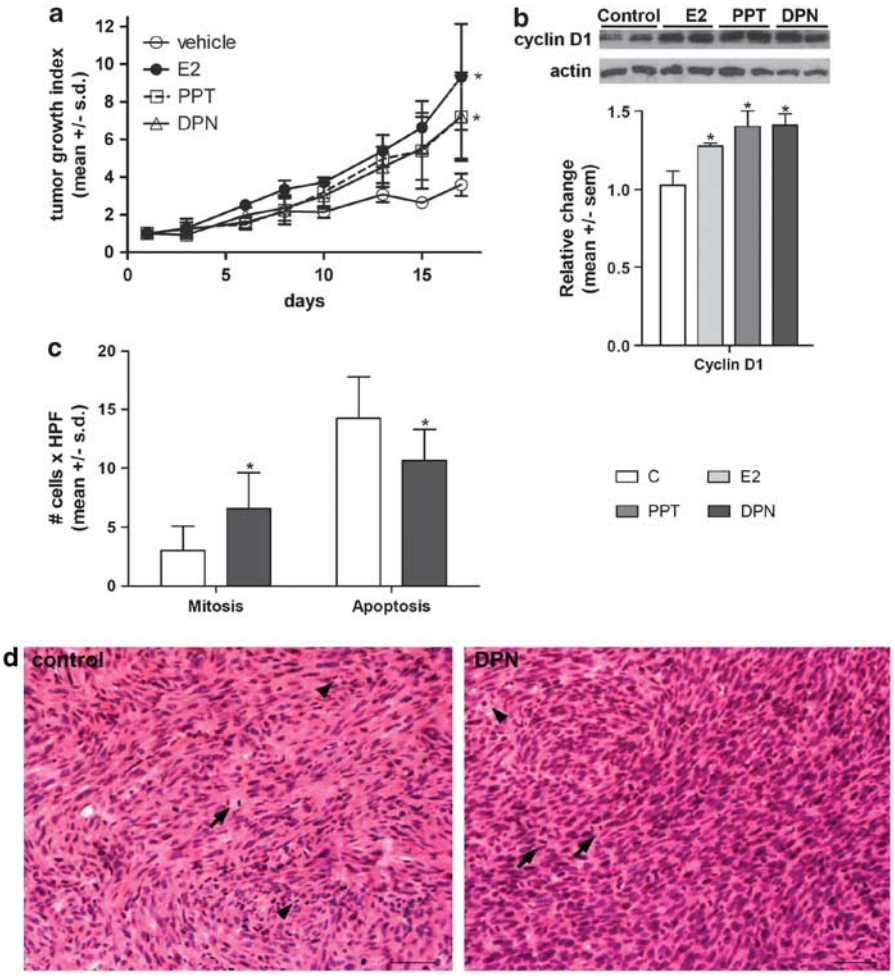


Figure 2. The ERβ agonist DPN increases MC4-L2 tumour growth. (**a**) Effect of ER agonists on tumour growth: animals bearing a 50-mm^2 tumour in the right inguinal mammary fat pad ($n = 4$) were treated with daily s.c. injections of 0.5 μg E2, 100 μg PPT or 50 μg DPN per animal. Tumour growth index was calculated by normalizing each measurement to the tumour size at the beginning of treatment (day 1). (**b**) Cyclin D1 protein expression evaluated in total protein extracts from MC4-L2 tumours after 17-day treatment. The graph bar shows cyclin D1 densitometry analysis. (**c**) Mitosis and apoptosis evaluated in MC4-L2 tumours following 17-day treatment. Tumour sections were stained with H&E and mitotic and apoptotic features were counted in eight high power fields per tumour. (**d**) Representative images of MC4-L2 tumours after 17-day treatment. Arrows indicate mitosis and arrowheads apoptosis. In all panels, $^*P < 0.05$ vs control.

ERK ½ activation by EGF weakens growth-inhibitory effects mediated by ERβ

Mammary glands from mice treated with ERβ agonists showed signs of hyperplasia. It was next investigated if stimulation of MAPK signalling with a mammogenic factor such as EGF, which has been shown to enhance ERα signalling,[35,36] could block growth inhibition induced by ERβ agonists in MECs. EGF (10 ng/ml) upregulated p-ERK ½ in HC11 and EpH4 cells (Figure 5a). In cells grown + EGF, E2, PPT or DPN stimulated proliferation, which was prevented by co-incubation with 4OH-T or UO126 (Figure 5b). Similar results were observed with the ERβ agonist WAY (Supplementary Figure 6A).

Casp 3 activity was reduced to untreated control levels by co-incubation of ERβ agonists with EGF or BM (Figure 5c and Supplementary Figure 6B, respectively), whereas UO126 completely restored Casp 3 activity (Figure 5c and Supplementary Figure 6C). In MECs grown + EGF, p53 and Aif protein levels were either not changed or downregulated by ERβ agonists (Figure 5d). In addition, in cells grown + EGF, cell number increased with E2, PPT or DPN treatment (Figure 5e). Co-incubation of cells with EGF and 4OH-T partially prevented cell number increase induced by ERβ–DPN and co-incubation with UO126 restored ERβ growth inhibition (Figure 5f).

These results indicate that, as observed in MECs, activation of ERK ½ signalling stimulates proliferation and inhibits apoptosis induced by ERβ agonists.

In T47-DERβ cells, ERβ expression activates transcription programs associated with inhibition of cell cycle and apoptosis.[12,37] Therefore, T47-DERβ cells were used to further study if active ERK ½ could block growth inhibition induced by recombinant ERβ in human breast cancer cells.

When ERβ expression remained turned off (− ERβ), cell number was not affected by DPN (Figure 6a), which is consistent with the fact that T47-D cells have no detectable ERβ protein. Following ERβ induction (+ ERβ), cell number was reduced to 50% in a ligand-independent manner. However, in + ERβ cells, incubation + EGF partially increased cell number, and co-incubation with DPN exerted an even greater increase (Figure 6a). ERβ effects in + EGF medium were reverted to –EGF levels by co-incubation with UO126.

Casp 3 activity was increased in T47-DERβ cells expressing ERβ (not shown) and was further increased by the addition of DPN

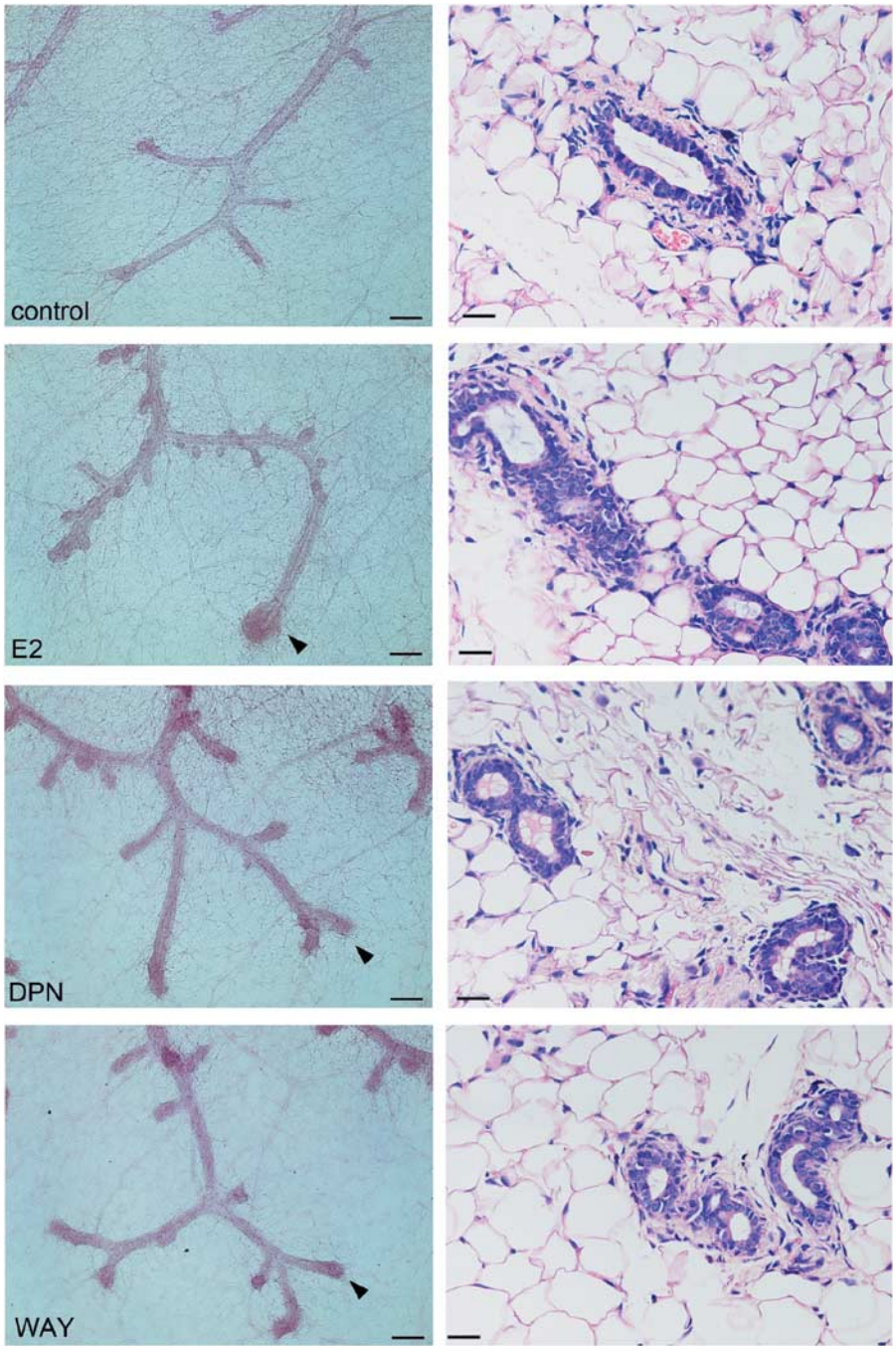


Figure 3. ERβ agonists DPN and WAY induce mammary gland ductal growth. Mammary glands from mice treated daily with 0.5 μg E2, 100 μg PPT, 50 μg DPN or 30 μg WAY, per animal were analysed by whole mount (left panels, 17-day treatment is shown) and for H&E staining (right panels, 5-day treatment is shown). Arrowheads indicate active terminal end buds. Scale bars: whole mount, 0.1 mm; H&E, 25 μm.

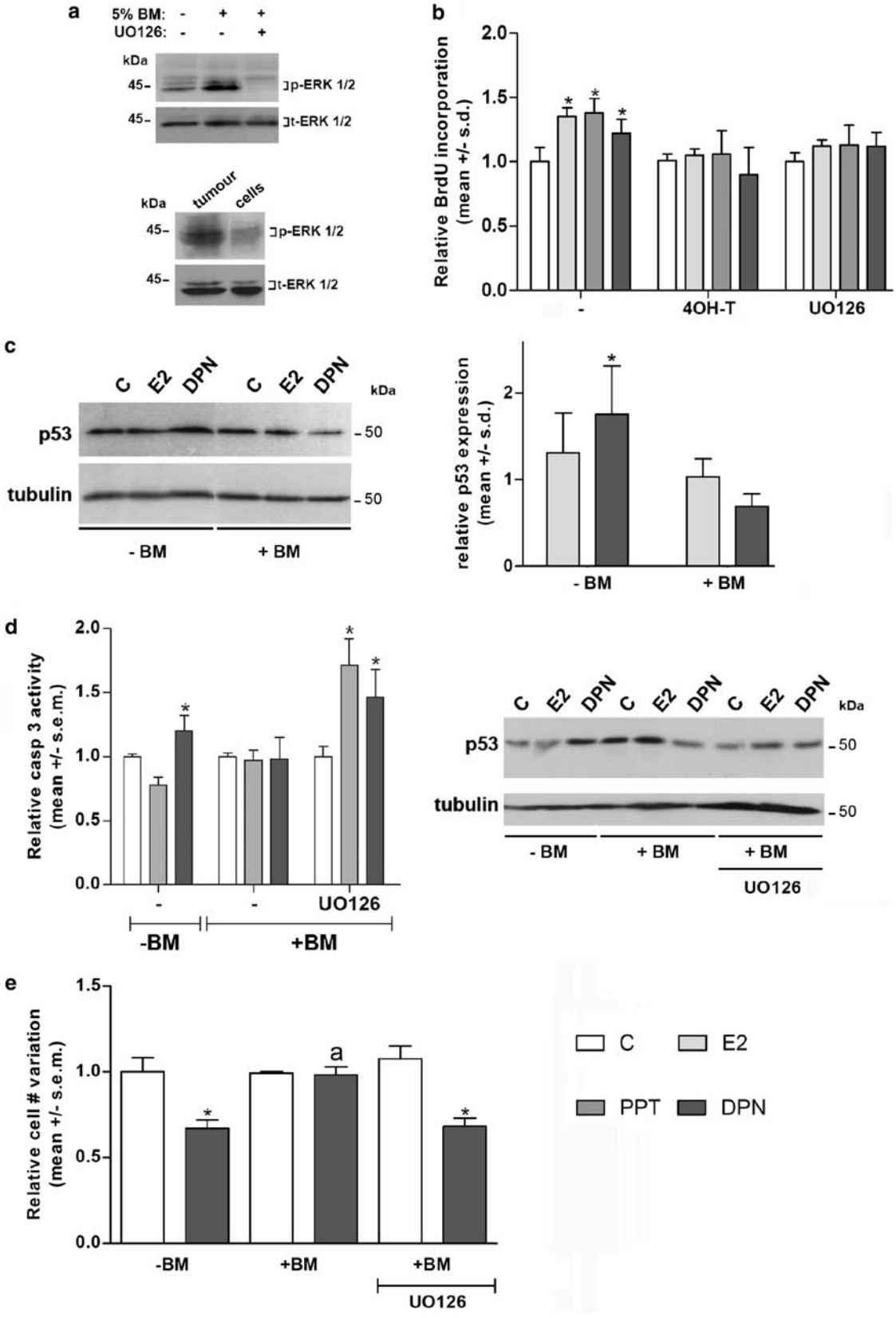
(Figure 6b). EGF addition had no significant effect on Casp 3 activity, but in combination with DPN, decreased Casp 3 activity (Figure 6b), which indicates that as observed in cells with endogenous ERβ expression, caspase-dependent apoptosis induced by ERβ agonists is inhibited by ERK ½ activation. ERβ expression in T47-D cells − EGF, increased Aif and decreased PCNA protein levels, independently of DPN addition (Figure 6c), whereas co-incubation of ERβ-expressing cells + EGF partially reduced Aif and increased PCNA. In addition, Aif levels were further downregulated by DPN, suggesting that in cells + EGF, ligand-dependent stimulation of growth may be due to inhibition of apoptosis pathways by ERβ–DPN.

Thus, ERK ½ signalling modulates ligand-independent effects mediated by recombinant ERβ at the proliferation level and also blocks ligand-dependent and -independent apoptosis induced by ERβ.

Inhibition of PI3K/AKT signalling prevents stimulation of proliferation by ERβ

HC11 MECs require addition of insulin for optimal growth.[38,39] As our initial interest was to explore the effect of ERβ agonists in other cell lines, treatment of cells was always carried out in insulin-containing medium that can activate PI3K and MAPK signalling.

As MAPK/ERK ½ activation inhibited effects exerted by ERβ agonists, it was next investigated if insulin, through activation of PI3K/AKT signalling, could also modulate ERβ activity. In MC4-L2 and EpH4 cells, insulin addition activated PI3K/AKT signalling, but did not affect p-ERK ½ levels. Co-incubation with BM further increased p-AKT levels; however, in MECs, co-incubation with EGF



and insulin did not (Figures 7a and 8a). Upregulation of p-ERK ½ in + BM or + EGF cells was verified. The PI3K inhibitor LY294002 blocked p-AKT upregulation by insulin, and in addition, partially downregulated p-ERK ½ levels in cells grown + BM or + EGF, indicative of cross talk between PI3K and MAPK pathway (Figures 7a and 8a). No effect of Ly294002 was observed on p-ERK ½ levels in cells –BM (Figure 7a) or –EGF (not shown).

Proliferation was not affected by insulin treatment in MC4-L2 breast cancer cells (Figure 7b). However, in EpH4 cells, insulin addition was necessary for E2-induced proliferation and to

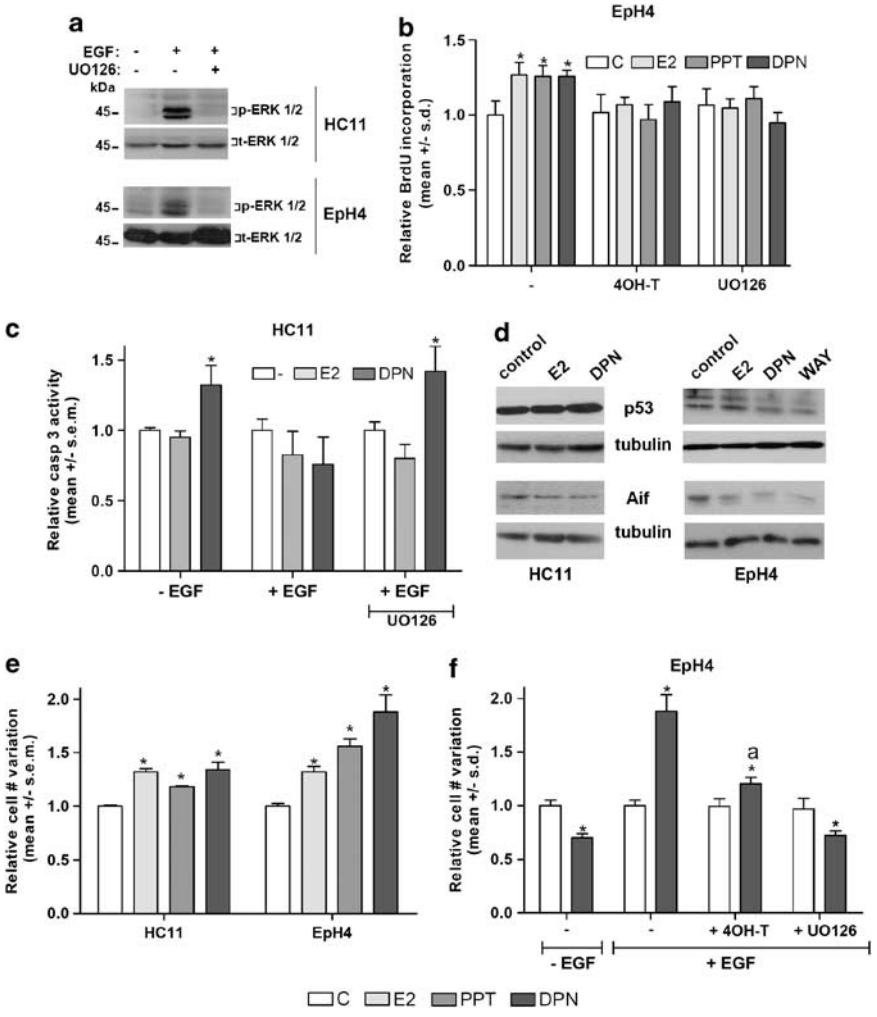


Figure 5. ERK ½ Map kinase activation by EGF changes cellular responses induced by ligand-activated ERβ. HC11 and EpH4 cells were grown with 10 ng/ml EGF and treated with 10 nM E2, 10 nM PPT or 10 nM DPN with or without 100 nM OH-T or 1 μM UO126. (**a**) Immunoblot showing activation of ERK ½ following 24 h incubation with EGF. Representative experiments of three independent cultures. (**b**) BrdU incorporation was quantified in EpH4 cells following 3-day treatment using an ELISA kit. Relative BrdU incorporation was calculated by normalization of values to the agonist untreated control. Representative experiments of three independent cultures. (**c**) Casp 3 activity was measured in HC11 cells following 24 h treatment using a colorimetric substrate. Relative Casp 3 activity was calculated by normalization to the agonist untreated values. Mean ± s.e.m. from three independent experiments is shown. (**d**) p53 and Aif protein levels were analysed by immunoblot in whole-cell extracts prepared after 24 h treatment with ER agonists. Representative experiments of three independent cultures. (**e** and **f**) Number of cells following 3-day incubation was counted and relative cell number variation was calculated by setting untreated control values to 1. Mean ± s.e.m. from four independent experiments is shown. In all cases, $^*P<0.05$ vs control; a, $P<0.05$ vs DPN + BM.

Figure 4. ERK ½ MAPK activation by BM blocks apoptosis induced by ligand activated ERβ. MC4-L2 cells were grown in 5% BM with the addition of 1 nM E2, 10 nM PPT or 10 nM DPN with or without 100 nM OH-T or 1 μM UO126. (**a**) Immunoblot shows p-ERK ½ levels following 24 h incubation (top panel) or in MC4-L2 tumours compared with MC4-L2 cells grown on plastic. Experiments are representative of three independent cultures. (**b**) BrdU incorporation was quantified following 3-day treatment using an ELISA kit. Representative experiments of three independent cultures. Relative BrdU incorporation was calculated by normalization of data to values corresponding to agonist untreated cells. (**c**) p53 protein levels analysed by immunoblot in whole-cell extracts prepared after 24 h treatment with ER agonists. Representative experiments of five independent cultures. The graph shows p53 band intensity values relative to the values from agonist untreated group. (**d**) Casp 3 activity was measured following 24 h treatment using a colorimetric (left panel) substrate. Relative Casp 3 activity was calculated by normalization of data to values corresponding to agonist untreated cells. Mean ± s.e.m. from three independent experiments is shown. Immunoblot showing p53 protein levels following 24 h incubation (right panel). Representative experiments of three independent cultures. (**e**) The number of cells was counted after 3-day treatment and relative cell number variation was calculated by setting untreated control values to 1. In all panels, $^*P<0.05$ vs control; a, $P<0.05$ vs DPN-BM.

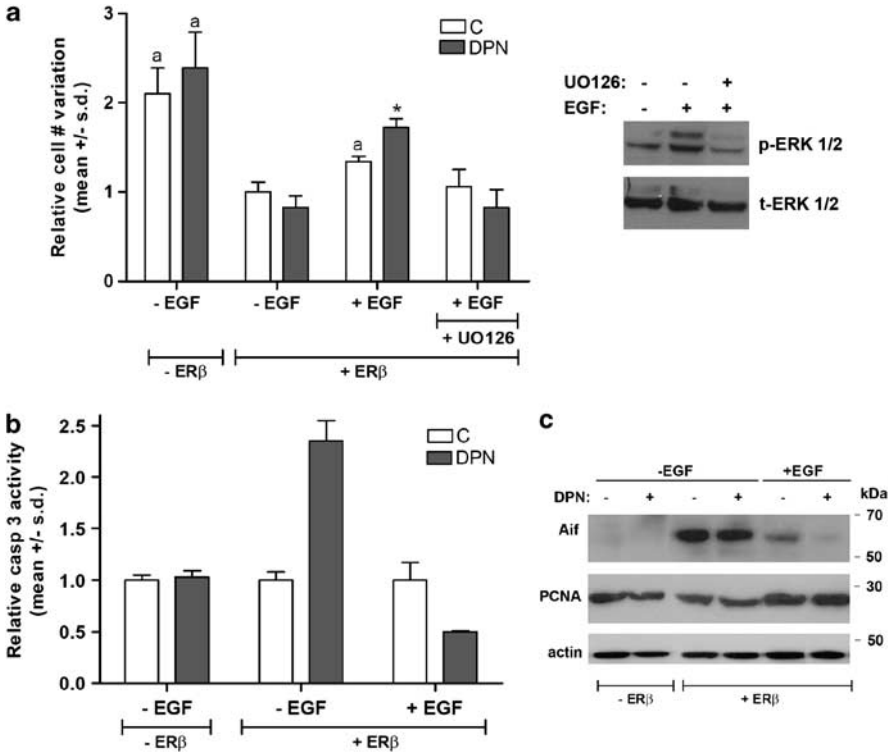


Figure 6. ERK ½ activation by EGF blocks ERβ growth-inhibitory effects in T47-DERβ cells. Cells were cultured with or without ERβ expression for 24 h, as indicated in Materials and methods. Thereafter, cells were treated with 10 nM DPN with or without 10 ng/ml EGF or 1 μM UO126 for the indicated times. (**a**) Left panel: cell number was counted following 3-day incubation and relative cell number variation was calculated by setting untreated + ERβ control values to 1. Representative experiments of two independent cultures. In each experiment, treatments consisted of six replicates ($n = 6$). Right panel: confirmation of inhibition of MAPK signalling following 24 h incubation with MEK ½ inhibitor UO126. (**b**) Casp 3 activity was quantified after 24 h treatment with DPN. Relative Casp 3 activity was calculated by normalization to DPN-untreated values. A representative experiment form three independent cultures is shown. (**c**) Aif and PCNA protein levels in whole-cell lysates prepared following 24 h treatment. Representative experiments of three independent cultures. In all cases, *$P < 0.05$ vs control; a, $P < 0.05$ vs + ERβ–EGF.

prevent ERβ–DPN inhibition of proliferation (Figure 8b). In both cell lines, co-incubation with LY294002 reverted the effects of insulin and completely prevented proliferation induced by E2. In cells + BM or + EGF, LY294002 also inhibited proliferation stimulated by E2 or ERβ–DPN. These results suggest that although MECs are more dependent on insulin signalling, both MECs and breast cancer cells require active ERK ½ and AKT to allow stimulation of proliferation by ERs.

Insulin/PI3K signalling did not affect Casp 3 activity induced by ERβ–DPN (Figures 7c and 8c); however, it was necessary to inhibit E2 induction of Casp 3 in MC4-L2 cells (Figure 7c), which correlated with p53 upregulation under these incubation conditions (Supplementary Figure 5B). In cells grown + BM, inhibition of PI3K/AKT did not restore induction of Casp 3 or p53 upregulation by ERβ–DPN and only slightly increased Casp 3 activity induced by E2 (Figure 7c and Supplementary Figure 5B); possibly through downregulation of p-ERK ½ levels and not of pAKT. In EpH4 cells, inhibition of PI3K/AKT pathway showed a similar effect on induction of Casp 3 by ERβ–DPN (Figure 8c). Consequently, co-treatment with LY294002 + BM or + EGF only prevented ERβ–DPN increase in cell number effects, through inhibition of proliferation but did not revert effects to achieve growth inhibition (Figure 7d and Figure 8d, respectively).

BM induces phosphorylation of endogenous ERβ

MAPK and PI3K signalling enhance ERβ phosphorylation[40] thereby inducing ligand-dependent and -independent ER transcriptional activation.[24] ER transcriptional regulation may be accomplished through direct phosphorylation or through phosphorylation of co-activators associated to the transcriptional machinery. To test if BM induced ER phosphorylation, endogenous ERβ was immunoprecipitated with 14C8 antibody. BM induced ERβ serine phosphorylation (p-Ser), while inhibition of MAPK pathway but not of PI3K/AKT pathway reduced p-Ser levels (Supplementary Figure 7). These results provide evidence that MAPK pathway targets endogenous ERβ for phosphorylation.

DISCUSSION

There is little correlation between the tumour-suppressor role attributed to ERβ and epidemiologic observations. *In vitro* studies have used mostly recombinant ERβ, an experimental setting in which growth-inhibitory effects are generally, although not always, observed.[12–14] These studies have introduced the idea that stimulation of ERβ signalling may protect from breast cancer (that is, high phytoestrogen consumption such as genistein)[41] or could be an alternative choice of therapy for endocrine-resistant tumours.[14,42]

In this work, it is proposed that ERβ-mediated effects are modulated by MAPK/ERK ½ and PI3K/AKT activation. It has long been known that MAPK and PI3K signalling cross talk with ER and, as such, affect ligand-dependent and -independent transcriptional activation of ERα or recombinant ERβ.[24] The novelty of the study presented herein relies in the use of cell lines with endogenous ERα and ERβ expression, the comparison of effects to recombinant

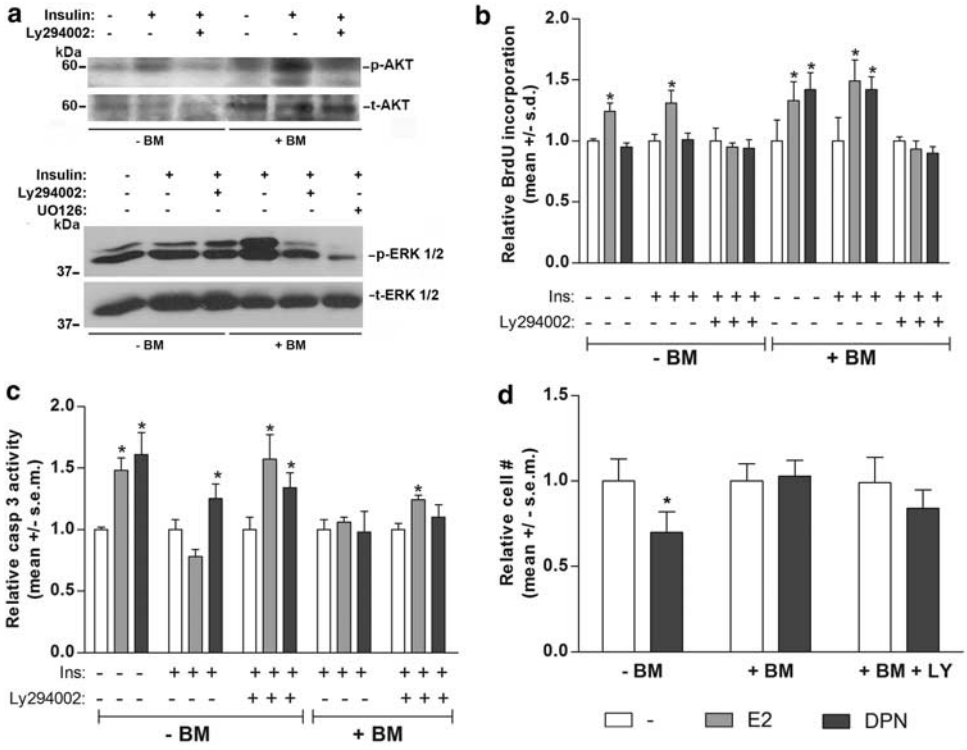


Figure 7. Inactivation of PI3K blocks ER-induced proliferation in MC4-L2 breast cancer cells. Cells were treated with 1 nM E2, 10 nM DPN in the presence or not of 5% BM extract with or without addition of 5 μg/ml insulin and/or 3 μM LY294002. (**a**) p-AKT, total AKT, p-ER ½ and total ERK ½ levels were analysed by immunoblot. Representative experiments of two independent cultures. (**b**) BrdU incorporation was quantified following 3-day treatment using an ELISA kit. Relative BrdU incorporation was calculated by normalization of values to the agonist-untreated control. Representative experiments of two independent cultures. (**c**) Casp 3 activity was measured following 24 h treatment using a colorimetric substrate. Relative Casp 3 activity was calculated by normalization of data to values corresponding to agonist-untreated cells. Mean ± s.e.m. from three independent experiments is shown. (**d**) The number of cells was counted after 3-day treatment and relative cell number variation was calculated by setting untreated control values to 1. Mean ± s.e.m. from three experiments is shown. In all panels, $^*P < 0.05$ vs control.

ERβ and the establishment of the concept that the cellular context determines the response to ERβ agonists.

Main findings from this work can be summarized as follows: ERβ ligand activation increased caspase-dependent (p53 and Casp 3 activity) and -independent (Aif) apoptosis, whereas ERα ligand activation with E2 or PPT required ERK ½ and PI3K activity to stimulate proliferation. Activation of MAPK/ERK ½ by extracellular factors did not enhance ERα-mediated proliferation but reduced ERβ effects on p53, Aif and Casp 3 activity to control levels, as well as allowed ERβ-induced proliferation; such growth stimulatory effects were completely blocked by inhibition of ERK ½ activation with UO126. Additionally, ERK ½ inactivation allowed E2 to preferentially stimulate apoptosis. On the other hand, in a context of active ERK ½, PI3K/AKT signalling was necessary to allow ER-induced proliferation. Taken together, these findings provide a mechanism that should be explored in human breast cancers, particularly aggressive phenotypes such as HER2 and triple-negative or ERα-negative tumours, which express ERβ in a substantial proportion and also present high mitotic index.[43]

An association between MAPK activation, ligand-independent upregulation of ERβ p-Ser levels and inhibition of apoptosis was found. Future studies are needed to establish if ERβ is a direct target of MAPK signalling, whether ligand-independent p-Ser increase affects ERβ affinity for agonists and if it favours formation of homodimers or ERβ/ERα heterodimers. Phosphorylation influences ERα transcriptional activity, affinity for ligands and DNA.[25] Therefore, the complexity of E2 signalling relies on ERα/ERβ ratios[5] and signalling pathways, which phosphorylate ERs and/or their transcriptional co-factors. As such, analysis of the effects exerted by ERβ agonists must consider the fact that complex settings with differential ER posttranslational modifications will most certainly influence ERβ activity.

In the mouse, an ERβ variant that contains an in-frame 18 amino-acid insertion in the ligand-binding domain (ERβ2) has been described. ERβ2 does not bind ligand but may function as transcriptional repressor of full-length ERβ.[44] Mouse cell lines used in this study express low levels of ERβ2 compared with full-length ERβ; therefore, it is likely that most effects mediated by ERβ agonists are not affected by ERβ2. However, the possibility that MAPK or PI3K signalling also target these isoform and in some way modulate ERβ2 interaction with ligand-binding ER forms remains open.

Few studies have analysed the effect of ERβ agonists in the mammary gland or mammary tumours. *In vivo* administration of genistein increased the number of mammary terminal end buds and ductal branching in a similar way as diethylbestrol.[45] As genistein is a weak selective ERβ agonist, it can be argued that in this experimental setting it can also activate ERα. However, in our work, the ERβ-selective agonists DPN and WAY, in concentrations that did not activate ERα-mediated transcription, also increased the number of terminal end buds, lateral branching and Ki67 staining. ERβ$^{-/-}$ glands develop normally;[46] yet, epithelial ERα is essential for ductal proliferation and subsequent morphogenetic steps like side branching and alveologenesis.[3] Therefore, it is likely that ductal proliferation observed in DPN- or WAY-treated mice is mediated by ERα/ERβ heterodimers. However, full differentiation of the mammary epithelium during lactation is compromised by loss of ERβ,[4] an effect which at some stage relies on ERβ inhibition

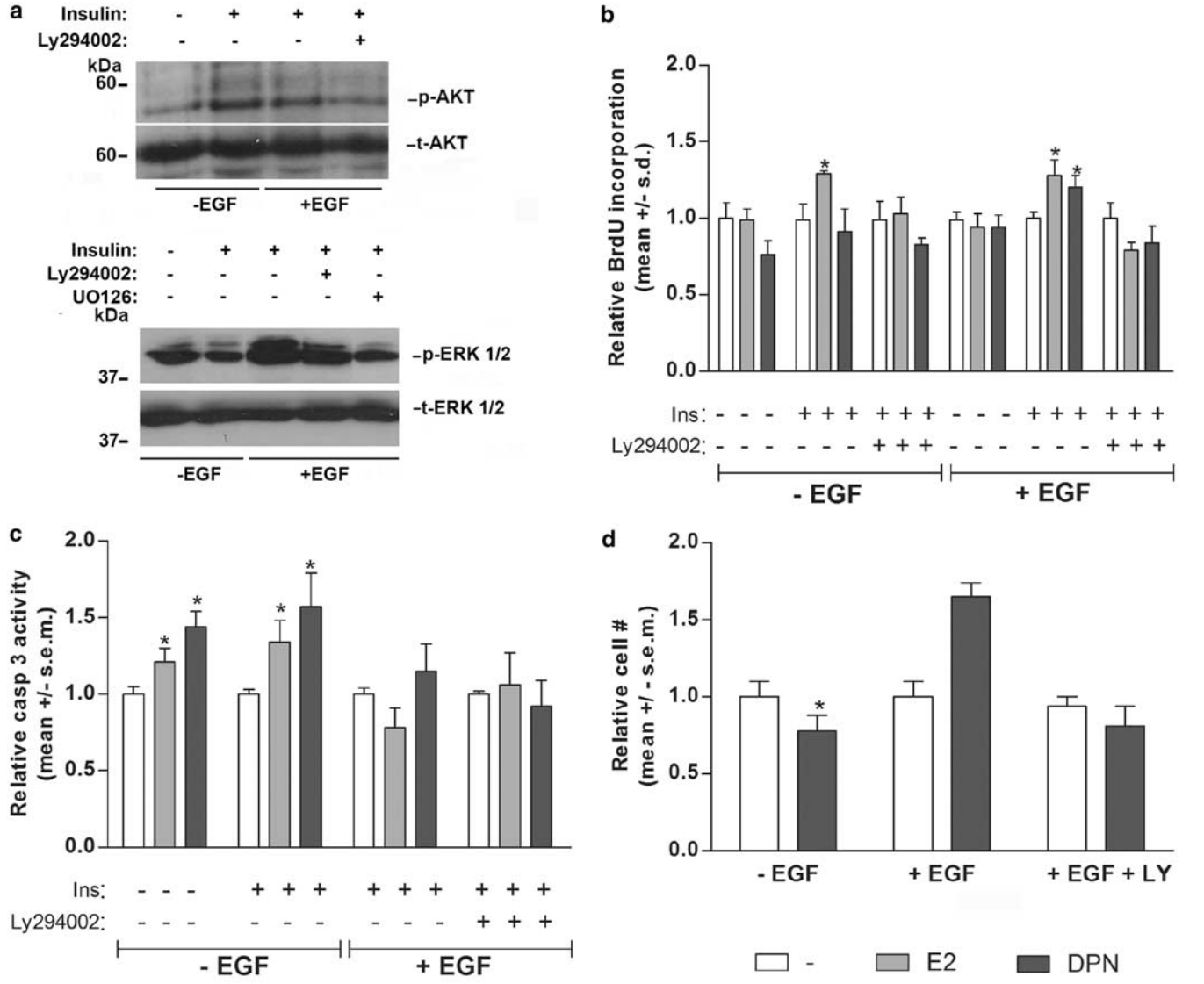


Figure 8. Inactivation of PI3K prevents ER-mediated proliferation in EpH4 mammary epithelial cells. Cells were treated with 1 nM E2, 10 nM DPN in the presence or not of 10 ng/ml EGF with or without addition of 5 μg/ml insulin and/or 3 μM LY294002 (LY). (**a**) p-AKT, total AKT, p-ERK ½ and total ERK ½ levels were analysed by immunoblot. Representative experiments of two independent cultures. (**b**) BrdU incorporation was quantified following 3-day treatment using an ELISA kit. Relative BrdU incorporation was calculated by normalization of values to the agonist-untreated control. Representative experiments of two independent cultures. (**c**) Casp 3 activity was measured following 24 h treatment using a colorimetric substrate. Relative Casp 3 activity was calculated by normalization of data to values corresponding to agonist untreated cells. Mean ± s.e.m. from three independent experiments is shown. (**d**) The number of cells was counted after 3-day treatment and relative cell number variation was calculated by setting untreated control values to 1. Mean ± s.e.m. from three experiments is shown. In all panels, $^*P < 0.05$ vs control.

of E-cadherin internalization and degradation induced by ERα.[6] ERβ overexpression in T-47D cells reduces migration through induction of integrin α1/β1; migration is not restored upon stimulation with EGF.[13] These results suggest selective regulation of ERβ activity by MAPK and PI3K pathway.

The ERβ agonist DPN also induced proliferation and reduced apoptosis of MC4-L2 mouse mammary tumours, which suggests that to understand the role of ERβ in breast cancer, physiological regulation of ERα/ERβ ratios, ER phosphorylation status and MAPK/PI3K activation levels should be taken into consideration. In this context, ERβ phosphorylation on serine 105 was recently identified as a good prognostic factor in response to tamoxifen.[47]

In summary, the cellular context modulates ERβ-induced apoptosis and proliferation. From the clinical point of view, a benefit from ERβ growth-inhibitory effects may still be possible by combining the use of ERβ agonists and receptor tyrosine kinase inhibitors or function-blocking antibodies. Yet, results presented in this work suggest that ERβ phosphorylation status and kinase signalling should be taken into account when analysing ERβ potential as prognostic factor and therapeutic target.

MATERIALS AND METHODS

Hormones and reagents

E2, 4-OH-tamoxifen (4OH-T), insulin, EGF and all chemicals used were from Sigma (St Louis, MO, USA). 4,4′,4″-(4-Propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol (PPT), 2,3-bis (4–hydroxy–phenyl)-propionitrile (DPN), 7-bromo-2-(4–hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY), UO126 and LY294002 were from Tocris (Bristol, UK). Cell culture reagents were from PAA (Pasching, Austria). Commercial BM was from BD Biosciences (Bedford, MA, USA).

Trizol, Superscript III and SYBR-Green Mastermix were from Invitrogen (Paisley, UK). RNA purification columns and on column DNase I were from Qiagen (Hilden, Germany). Immunoblot reagents were from BioRad (Hercules, CA, USA). Primary antibodies: anti-ERα (sc-542), anti-cyclin D1 (sc-753), anti-phospho p42/p42 thr202/tyr204 (sc-16982-R); anti-p53 (sc-99), anti-Aif (sc-13116) and anti–β-actin (sc-130656) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti- pan ERK (#610123), anti-phospho AKT ser473 (#550747) and anti-total AKT (#610860) from BD Biosciences; anti-ERK ½ (#9107) and anti-phospho AKT ser473 (#4060) from Cell Signaling (Beverly, MA, USA); anti-phospho serine (#05–1000) from Millipore (Billerica, MA, USA). Anti-ERβ antibodies were from Abcam (Cambridge, UK). Specificity of mouse anti-ERβ 14C8 antibody has been previously shown with siRNAs to ERβ.[6] Specificity of anti-chicken 14021 antibody has been previously shown using recombinant ERβ.[13] Rabbit anti-ERβ2 and chicken anti-ERβ (503) antibody were a kind gift from Dr Margaret Warner. Anti-ERβ2 antibody was generated by immunizing with a peptide corresponding to the 18 amino-acid insertion found in ERβ2, whereas 503 antibody was generated by immunizing with full-length ERβ1 protein.

Cell culture

HC11 and EpH4 are mouse MECs.[48,49] MC4-L2 cells were obtained from a highly metastatic mouse mammary adenocarcinoma.[28] All cell lines express ERα and ERβ mRNA and protein (Supplementary Information, Supplementary Figures 1A and B, respectively). MC4-L2 tumours express ERα, PR, erbB2[28] and ERβ (Supplementary Figure 1C); MC4-L2 cells do not respond to EGF (unpublished). T47-DERβ cells were obtained by stable expression of ERβ in human T47-D breast cancer cells (ERα +, ERβ −) using a tet-off system.[12,13] In this system, doxycyclin withdrawal induces ERβ expression (Supplementary Figure 1D). In the rodent, an 18 amino-acid insertion in the ligand-binding domain of ERβ originates a splice variant (ERβ2). As ERβ2 can also be detected by the antibodies used herein, expression of ERβ2 was also analysed. In our hands, this antibody works for immunoprecipitation or immunofluorescence. ERβ2 was detected in all cell lines in very low amounts (HC11 not shown, Supplementary Figure 1E). In HC11 cells, ERβ2 was detected in 10% cells as compared with ERβ.

HC11 cells were grown in RPMI 1640 with 5 μg insulin, 10 ng/ml EGF, 5 mg/l gentamicin and 10% FBS. EpH4 and MC4-L2 cells were grown in DMEM/F12 with 5 mg/l gentamicin and 10% FBS. T47-DERβ cells were grown in RPMI 1640 with 10% FBS, 1% penicillin–streptomycin and 10 ng/ml doxycyclin. Cells were preincubated in serum-free RPMI 1640 (2 g/l glucose) or DMEM (4.5 g/l glucose) phenol red-free medium with 5 μg insulin; additionally, ERβ expression was induced in T47-DERβ cells. Following 24 h, cells were treated with 1–10 nM E2, PPT DPN or WAY or same volume of solvent (EtOH) in preincubation medium plus 1% steroid charcoal striped FBS with or without 10 ng/ml EGF or 5% BM. Preincubation in serum-free medium was used to synchronize cells in S phase of cell cycle prior initiation of treatments; yet, similar results were obtained in unsynchronized cells (Supplementary Figure 2).

Cell counting

Twenty thousand cells were seeded in 24-well plates and grown as described above. Following 3-day ligand treatment, cells were detached and duplicates of each well counted in a Neubauer chamber. Cell number variation was calculated by normalizing all values to the agonist untreated control. Results are presented as mean ± s.e.m. of at least three independent cultures, each carried out in sextuplicates.

Proliferation assay

Two thousand cells were seeded in 96-well plates, grown as described above. Following 48 h ligand treatment, medium was refreshed and 10 mM BrdU were added. Twenty-four hour later, cells were fixed and BrdU incorporation quantified with the Colorimetric Cell proliferation ELISA kit (Roche, Mannheim, Germany). Experiments were carried out in quadruplicates and repeated with three independent cultures. Relative variation was calculated by normalizing all values to the agonist untreated control. Results are presented as mean ± s.d. of a representative experiment of at least three.

Caspase 3 assay

Apoptosis was evaluated with the colorimetric Caspase 3 assay kit (CASP-3-C, Sigma). Cells were grown as described above, in 10 cm dishes until 60% confluence. Following 24 h treatment, cells were lysed (2.5 mM HEPES, pH 7.4, 2.5 mM CHAPS and 2.5 mM dithiothreitol) and centrifuged at 14 000 *g*, 10 min, 4 °C. Casp 3 activity was measured in duplicates with Ac-DEVD-pNA substrate. Absorbance units from a parallel reaction in the presence of Casp 3 inhibitor (Ac-DEVD-CHO) were subtracted. Results were normalized to protein concentration in each lysate. Relative Casp 3 activity was calculated by normalizing all values to the agonist untreated control. Unless otherwise stated, results are presented as mean ± s.e.m. of at least three independent experiments.

Animal experiments

Mice were fed *ad libitum* and kept under a 12-h light–dark cycle. Two-month-old virgin BALB/c mice were injected in the left inguinal side with 1×10^6 MC4-L2 cells in PBS. Tumour size was measured every 2 days with a calliper (width × length). Treatment started when tumours reached 0.5 cm^2 (~26 days after injection). Treatment (0.5 μg E2, 150 μg PPT and 50 μg DPN per animal) was administered in daily s.c. injections. Working solutions where prepared in PBS from stocks prepared in EtOH–DMSO (50:50) as shown previously.[33] Four animals were included in each group and the experiment was repeated twice. Following 17-day treatment (when the maximum tumour size allowed by the ethical committee was reached), animals were killed, and tumours, uterus and the contralateral mammary gland were excised. In addition, healthy animals were also treated with same concentrations of E2, PPT or DPN as tumours (daily, s.c.) or WAY (30 mg/kg). Tumours and uteri were either frozen in liquid nitrogen or fixed in 10% formalin for immunohistological studies. Mammary glands, from tumour bearing or from healthy animals were processed for whole mount or fixed in 10% formalin. All animal experimentation was conducted in accordance with standards of humane animal care as outlined by the Stockholm South Ethical Committee of the Swedish National Board for Laboratory Animals. Ethical permit no. S194–07.

Whole mount and histological analysis

The fourth mammary gland was excised, spread on a glass slide and immediately fixed in Carnoýs fixative for 2 h. Samples were gradually rehydrated through a series of decreasing % EtOH and stained in carmine alum over night. Mammary glands were dehydrated, cleared in xylene and mounted with DPX. Quantification of mitosis and apoptosis was carried out by counting typical features on eight high power fields per tumour sample stained with hematoxylin and eosin.

RNA extraction and real-time PCR

RNA was extracted with TRIzol, purified using spin columns. cDNA was synthesized with Superscript III kit and random hexamers using 1 μg DNase-I-treated and purified RNA. Real-time PCR was performed with SYBR-Green Mastermix in an ABI PRISM 7500 (Applied Biosystems, Carlsbad, CA, USA). Conditions were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 40 s. All runs were performed in triplicate and specific amplification was checked with melting curve analysis. Complement C3, Gapdh and lactoferrin primer sequences were extracted from Frasor *et al.*;[33] 18S from Helguero *et al.*;[6] ERα and ERβ sequences will be provided upon request. The standard curve method was used to determine RNA levels.

Whole-cell extracts and immunoblot

Cells were lysed in 1% NP-40, 50 nM Tris-HCl (pH 7.5), 140 nM NaCl, 2 nM EDTA with protease and phosphatase inhibitors, placed 20 min on ice and centrifuged at 14 000 *g*, 20 min, 4 °C. In all, 50 μg total proteins were resolved by 7.5 or 10% SDS–PAGE and transferred onto a PVDF membrane. Luminescence was detected with ECL plus (Amersham, Buckinghamshire, UK). Experiments were repeated at least three times in each cell line. Band intensity was quantified with Quantity One software (BioRad) and normalized to loading control (tubulin) intensity. Normalized values were then divided by the untreated control value. Data is presented in graph bars showing relative intensity variation (to the control = 1).

Immunoprecipitation

Cells were lysed in 50 mM Tris-HCl (pH 8), 5 mM EDTA, 150 mM NaCl, 1% triton X-100, 10% glycerol with protease and phosphatase inhibitors. One microgram total protein was incubated with 2 μg anti-ERβ 14C8 antibody[50] prebound to magnetic beads overnight at 4 °C. Then, bead-antibody complexes were washed thoroughly with 0.1% tween-20/PBS and resuspended in Laemmlís buffer for subsequent immunoblot detection.

Statistical analysis

Statistical significance was analysed using Graph Pad Software. Differences between treated and untreated groups were evaluated with one-way ANOVA and Dunnet's or Tukey's post test. Significant differences in immunoblot band intensity were analysed using two tailed *t*-test.

ABBREVIATIONS

Aif, apoptosis-inducible factor; Akt, RAC-alpha serine/threonine-protein kinase; BM, basement membrane; BrdU, bromodeoxyuridine; Casp 3, caspase 3; DPN, 2,3-bis (4–hydroxy–phenyl)-propionitrile; E2, 17β-estradiol; erbB-2, receptor tyrosine-protein kinase erbB-2; erbB-3, receptor tyrosine-protein kinase erbB-3; EGF, epidermal growth factor; ERK ½, extracellular signal-regulated kinase 1 and 2; Mek 1, ERK activator kinase 1; 4OH-T, 4-hydroxytamoxifen; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PPT, 4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol; p-Ser, phosphorylated serine

CONFLICT OF INTEREST

Jan-Åke Gustafsson is consultant of KaroBio AB and BioNovo. All the other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)