Prolactin and oestrogen synergistically regulate gene expression and proliferation of breast cancer cells

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Abstract

The pituitary hormone prolactin (PRL) plays an important role in mammary gland development. It was also suggested to contribute to breast cancer progression. *In vivo* data strongly supported a crucial role of PRL in promoting tumour growth; however, PRL demonstrated only a weak, if any, pro-proliferative effect on cancer cells *in vitro*. Several recent studies indicated that PRL action *in vivo* may be influenced by the hormonal milieu, e.g. other growth factors such as 17β-oestradiol (E_2). Here, we explored the potential interplay between PRL and E_2 in regulation of gene expression and cell growth. PRL alone induced either a weak or no proliferative response of T47D and BT-483 cells respectively, while it drastically enhanced cell proliferation in E_2-stimulated cultures. Affymetrix microarray analysis revealed 12 genes to be regulated by E_2, while 57 genes were regulated by PRL in T47D cells. Most of the PRL-regulated genes (42/57) were not previously described as PRL target genes, e.g. *WT1* and *IER3*. One hundred and five genes were found to be regulated upon PRL/E_2 co-treatment: highest up-regulation was found for *EGR3*, *RUNX2*, *EGR1*, *MAFF*, *GLIPR1*, *IER3*, *SOCS3*, *WT1* and *AREG*. PRL and E_2 synergised to regulate *EGR3*, while multiple genes were regulated additively. These data show a novel interplay between PRL and E_2 to modulate gene regulation in breast cancer cells.

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Introduction

Prolactin (PRL) and 17β-oestradiol (E_2) are among the key regulators of mammary gland development and differentiation (Hennighausen & Robinson 2005). In breast cancer, a mitogenic effect of E_2 is well known, whereas the role of PRL is still unclear. PRL is a peptide hormone primarily secreted by the pituitary gland, but extrapituitary synthesis by e.g. breast cancer cells has also been reported (Clevenger *et al*. 1995, Ginsburg & Vonderhaar 1995, Bhatavdekar *et al*. 2000). PRL acts through the PRL receptor (PRLR) belonging to the class I cytokine receptor family. PRLR is a non-tyrosine kinase receptor which dimerises upon ligand binding and transduces intracellular signals mainly via the JAK/STAT, ERK1/2 and AKT pathways (Clevenger *et al*. 2003). A role of PRL in breast cancer is increasingly accepted. Epidemiological data indicate that the level of circulating PRL correlates with an increased risk of breast cancer and the occurrence of metastasis (Tworoger & Hankinson 2008). Although PRLR has been reported to be over-expressed in the majority of breast cancers, we recently demonstrated that some of the antibodies used in these studies were not specific for PRLR. Moreover, the available antibodies with proved PRLR specificity have a relatively low sensitivity thus leaving the question open: to what extent is the functional PRLR present in breast tumours (Galsgaard *et al*. 2009). *In vivo* data support a key role of PRL in cancer progression. In mice, transgenic

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over-expression of PRL induced formation of mammary tumours (Wennbo *et al*. 1997), many of which were oestrogen receptor (ER)-positive (Rose-Hellekant *et al*. 2003). PRLR knockout studies have shown a decrease of the mammary tumour growth rate (Oakes *et al*. 2007). Based on the *in vivo* data, PRL was suggested to be a growth factor for cancer cells. However, *in vitro* PRL promoted only a weak (Chen *et al*. 1999) or no (Chen *et al*. 2010) proliferative response of breast cancer cells. It is thus possible that the effect of PRL *in vivo* is tuned by other factors present in the hormonal milieu around the tumour, e.g. growth factors such as E_2.

E_2 is a steroid hormone produced primarily by the ovaries. In postmenopausal women, E_2 is produced in extragonadal tissues (e.g. the breast) by conversion of adrenal steroid hormones. The majority of breast cancer cells are responsive to, or dependent on, E_2 supply, and increased E_2 synthesis is found in breast tumour tissue (Suzuki *et al*. 2008). E_2 acts through ER that belongs to the nuclear receptor superfamily of transcription factors. E_2 binding triggers receptor homodimerisation, phosphorylation and binding to specific oestrogen response elements (ERE) located in the promoter regions of target genes. Transcriptional activity is induced after recruitment of nuclear coactivators (e.g. steroid receptor coactivators and p300). Ligand-bound ER can also regulate transcription independently of ERE binding, either by using integral proteins bridging the ERE bound ER and other transcriptional complexes or as a co-activator directly on existing AP1/ or Sp1/coactivator complexes (DeNardo *et al*. 2005). Growth factor signalling pathways enhance ER phosphorylation and transcriptional activity (Thomas *et al*. 2008), which may occur also in the absence of E_2 (Bunone *et al*. 1996). Mitogenic effects of E_2 on breast tumour growth have been well described and adjuvant endocrine therapy targeting either the ER or the E_2 synthesis is routinely used in the clinic. The pure anti-oestrogen ICI 182 780 (Fulvestrant/Faslodex) is used in the therapy of advanced breast cancer (Howell *et al*. 2004). It causes complete abrogation of the transcriptional activity of the ER, followed by a rapid degradation of the ER protein (Marsaud *et al*. 2003).

Two recent studies have addressed growth-promoting effects of PRL and E_2 and have examined signalling pathways involved (Gonzalez *et al*. 2009, Chen *et al*. 2010). PRL was shown to stimulate Ser118 phosphorylation of ER, the modification which was suggested to potentiate transcriptional activity of the unliganded ER or to stabilise ER allowing maintenance of a response to E_2. In the present study, we focused on the individual and combined effects of the two hormones on gene expression using PRLR/ER-positive breast cancer cell lines as an *in vitro* model.

Materials and methods

Hormones and inhibitors

Human recombinant PRL was expressed in *Escherichia coli* and purified as described previously (Svensson *et al*. 2008); E_2 was purchased from Sigma–Aldrich. ICI 182 780 was obtained from Tocris Bioscience (Bristol, UK).

Cell cultures

The human breast cancer cell lines T47D and BT-483 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). T47D cells were routinely cultured in DMEM with phenol red (Invitrogen), supplemented with 10% FCS (Invitrogen), 2 mM glutamax (Invitrogen), 10 μg/ml human insulin (Novo Nordisk A/S, Bagsvaerd, Denmark), 100 U penicillin and 100 μg/ml streptomycin (Invitrogen) (referred in the manuscript as 10% FCS medium). BT-483 cells were routinely cultured in RPMI 1640 with phenol red (Invitrogen), supplemented with 20% FCS, 10 μg/ml human insulin (Novo Nordisk A/S), 100 U penicillin and 100 μg/ml streptomycin (referred as 20% FCS medium). Cells were propagated in a 5% CO_2 humidified atmosphere at 37 °C. To study the effects of PRL or E_2, cells were cultured in phenol red-free DMEM (Invitrogen) supplemented with 10% charcoal/dextran-stripped FBS (Hyclone, Logan, UT, USA), 2 mM glutamax, 1 mM sodium pyruvate, 100 U penicillin and 100 μg/ml streptomycin (referred in the manuscript as 10% CSS medium). In all the experiments, vehicle (ethanol) was added to PRL-treated and control cultures.

Cell proliferation

Cells (1.2×10^4 cells) were seeded in 96-well multidishes (Nunc, Soeborg, Denmark) in 10% CSS medium. Next day, the medium was replaced, and the cells were cultured for 3 (T47D) or 6 (BT-483) days in the absence or presence of hormones and/or inhibitors. Six replicates per treatment were analysed. To measure cell proliferation, the cells were incubated with 0.5 μCi ^{3}H-thymidine (GE Healthcare, Fairfield, CT, USA, specific activity 59.0 Ci/mmol) for 4½ h. Cells were detached by trypsin, harvested using a 96-well cell harvester, and subsequently washed four times with

PBS and three times with H_2O followed by fixation in 96% ethanol. ^{3}H-thymidine incorporation was measured by liquid scintillation counting using a TopCount NXT (Perkin Elmer, Waltham, MA, USA). At least three independent experiments were performed. Data analysis was done using GraphPad Prism 4.0 software (Graphpad Software, La Jolla, CA, USA). Results were considered significant when $P<0.05$.

Total RNA extraction

T47D cells (1.0×10^7) were plated in 92 mm dishes (Nunc) in 10% CSS medium. Next day, the medium was replaced, and the cells were cultured in the absence or presence of 20 nM of PRL and/or 1 nM E_2 for 6 h. Cells were washed twice with ice-cold PBS and lysed in 5 ml TRIzol (Invitrogen). In the experiments involving ICI 182 780, T47D cells were pre-treated with 100 nM ICI 182 780 for 30 min prior to adding PRL and/or E_2. BT-483 cells (4.0×10^4) were plated in quadruplicates in 96-well plates (Nunc) containing 10% CSS medium. Next day, the medium was replaced, and the cells were treated with 20 nM or PRL and/or 1 nM E_2 for 24 h. Cells were washed once in cold PBS and lysed in 100 µl TRIzol. Chloroform was added to TRIzol cell homogenate in the ratio 1:5 and whirl mixed for 15 s. Homogenates were incubated 2–3 min at room temperature and centrifuged 15 min at 10 000 ***g*** (4 °C). The water phase was further processed to purify RNA using an RNeasy MinElute Cleanup Kit (Qiagen). RNA integrity was confirmed (RIN-scores were all reported to be 10.0) on an Agilent 2100 Bioanalyser using total RNA nano chips (Agilent Technologies, Santa Clara, CA, USA).

GeneChip analyses

Five micrograms of total RNA from each sample of three independent experimental groups ($n=3$) were labelled using Affymetrix One-Cycle Target Labelling and Control Reagents following the manufacturer's instructions. Labelled targets were hybridised to Human Genome U133 Plus 2.0 Arrays (full transcriptome) in a Hybridisation Oven 640 (Affymetrix, Santa Clara, CA, USA), then washed and stained in a Fluidics Station 450 (Affymetrix). GeneChips were scanned using a GeneChip Scanner 3000. Normalisation and statistical analyses were carried out using R (http://www.R-projects.org) and Bioconductor packages (http://www.bioconductor.org). Specifically, the affylmGUI was applied for normalising the data (RMA-normalisation) and for calculating the false discovery rates (Benjamini & Hochberg 1995) for differentially regulated genes between the compared contrasts. Clustering of log2 fold ratios was done hierarchically by complete linkage and Euclidean distance. The Heatplus and geneplotter packages (Bioconductor) were used for generation of the clustered heatmap.

Quantitative real-time PCR

cDNA was prepared using 1 µg of total RNA from each sample, random primers and Superscript II Reverse Transcription reagents (Invitrogen) according to the manufacturer's instructions. Five microlitres of 10-fold diluted cDNA (corresponding to 0.04 µg converted RNA per sample) were used as a template to analyse the expression of selected genes by quantitative real-time PCR (qPCR). For BT-483 cells, 150 ng total RNA from each sample were used. qPCR was performed using TaqMan PCR core reagents (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 7900HT Sequence Detection System. Relative quantification of gene expression levels was performed using the relative standard curve method as outlined in User Bulletin #2 (ABI Prism 7900HT sequencing detection system; Applied Biosystems). Briefly, a standard curve for each gene was made using the same 2–1000-fold serial dilution of cDNA. Each curve was then used to calculate relative amounts of target mRNA in the samples. Primers and FAM-labelled probes for test and control genes were ordered as Assays-on-Demand from Applied Biosystems. Assay order numbers for these genes were: *WT1*, Hs01103754_m1; *EGR1*, Hs00152928_m1; *EGR3*, Hs00231780_m1; *IER3*, Hs00174674_m1; *CITED2*, Hs01897804_s1; *RUNX2*, Hs01047976_m1 and *TBP*, Hs99999910_m1. Data were analysed using the ABI Prism SDS 2.2 software (Applied Biosystems). Expression levels for each mRNA were normalised to the *TBP* mRNA level, which by Affymetrix GeneChip analysis was shown to be invariant in treated and control samples.

Western blotting

T47D cells were seeded in 60 mm plates (Nunc) in 10% CSS medium. Next day, the medium was renewed, and 20 nM PRL and/or 1 nM E_2 were added for 20 min or 24 h. For intracellular signalling studies, cells were rinsed in ice-cold PBS and snap frozen in liquid nitrogen prior to lysis in RIPA buffer. Western blot analysis was carried out as described previously (Galsgaard *et al.* 2009). Primary antibodies against ERα, phospho-STAT5^{Y694}, phosho-STAT3^{Y705}, phospho-AKT^{S473}, phospho-ERα^{S118}, STAT5, STAT3, AKT and ERK

were purchased from Cell Signaling Technologies (Danvers, MA, USA); pERK1/2 (anti-active MAPK) from Promega; ERα (clone SP1) from Lab Vision Corporation (Thermo Fisher Scientific, Fremont, CA, USA), PRLR (clone 1A2B1) from Zymed (Invitrogen) and β-actin from AbCam (Cambridge, UK). HRP-conjugated goat anti-mouse and swine anti-rabbit were purchased from Dako (Glostrup, Denmark). Three independent experiments were performed.

Results

PRLR and ER expression in human breast cancer cell lines

In order to select a cell line to study the effects of PRL and E_2 in breast cancer, we examined the PRLR and ER protein expression in 16 different human breast cancer cell lines. A high expression level of both PRLR and ER was detected in the ductal carcinoma cell lines T47D and BT-483 (Fig. 1). Thus, these two cell lines were selected as models for further analysis.

PRL and E_2 synergistically stimulate breast cancer cell proliferation

Dose–response growth experiments for T47D cells were performed to elucidate the potency of E_2 and PRL in stimulation of cell proliferation. Cells were exposed to the hormones for 3 days in a phenol red-free medium containing 10% of steroid-stripped serum, and the proliferation rate was measured using ^{3}H-thymidine incorporation. PRL and E_2 stimulated the T47D cell proliferation in a dose-dependent manner (Fig. 2A and B). A plateau was reached at concentrations ≥ 1 and ≥ 20 nM for E_2 and PRL respectively.

Both PRL and E_2 are implicated in breast cancer development and can be present simultaneously in the tumour microenvironment. Therefore, we investigated the possible interplay between the growth factors on breast cancer cell proliferation. Cells were stimulated either with PRL alone or in combination with E_2. PRL alone mediated a weak (twofold) stimulatory effect on T47D breast cancer cell growth. Strikingly, PRL/E_2 co-treatment resulted in a profound increase of the cell proliferation rate (Fig. 2C).

Gonzalez *et al*. (2009) have recently reported that PRL may induce transcriptional activity of ER in an E_2-independent manner. To investigate the contribution of ER to the PRL- and E_2-stimulated cell proliferation, we used the pure ER antagonist ICI 182 780 (Faslodex, Fulvestrant). ICI 182 780 binds to ER with high affinity and abrogates its transcriptional activity, followed by a rapid degradation of the receptor (Marsaud *et al*. 2003) and hence inhibition of breast cancer cell proliferation (Rasmussen *et al*. 2007). As expected, ICI 182 780 completely abolished E_2-stimulated proliferation (Fig. 2D), but did not influence PRL-induced cell proliferation. The profound synergistic effect of PRL/E_2 co-treatment was abolished by ICI 182 780, but only to the level obtained with PRL alone. No agonistic or toxic effects were observed with ICI 182 780 alone. Thus, our results indicate that PRL-induced cell proliferation is independent of ER.

To investigate whether the synergetic effect of PRL and E_2 was cell line specific, we examined the effect of the hormones on another PRLR+/ER+ cell line, BT-483. Since the BT-483 cell line grows extremely slowly, the proliferation was analysed after 6 days of culture in the presence or absence of PRL and E_2. PRL significantly enhanced the E_2-stimulated proliferation, while no effect was observed by PRL alone (Fig. 2E).

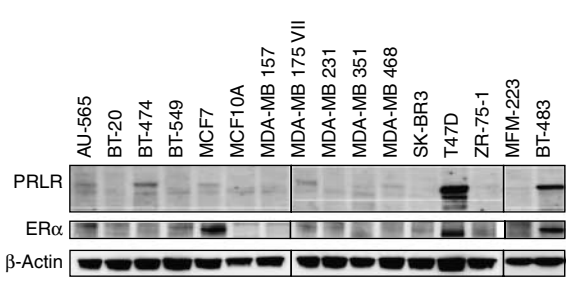

Figure 1 Western blot analysis of the PRLR and ER protein expression in the indicated human breast cancer cell lines. Expression of PRLR was assessed previously in this panel of cell lines (described in Galsgaard *et al*. (2009)). Expression of ER was done by re-probing of the same membrane with an anti-ERα-specific antibody. β-Actin staining was used as loading control.

Regulation of gene transcription by PRL and E_2

To elucidate the molecular mechanism of the synergistic growth-promoting effect of PRL and E_2, a genomic approach was used to compare gene expression profiles. Full transcriptome profiles were examined using Affymetrix GeneChips. T47D cells were treated for 6 h with PRL and/or E_2 in 10% CSS medium. Three independent experiments were performed. With a false discovery rate (FDR) of 5%, E_2 regulated the expression of only 12 genes (at least twofold), including *MYC* that is a well-defined E_2 target gene. Fifty-seven different genes were regulated by PRL at least twofold and with a FDR of 5%, including the suppressors of cytokine signalling (*CISH*, *SOCS2* and *SOCS3)*, which are well known downstream targets of the JAK/STAT pathway.

However, most of the PRL-regulated genes (42/57) were not previously described in connection with PRL, e.g. cancer-associated genes as GLI pathogenesis-related 1 (*GLIPR1*), tumour protein p63 (*TP63*/*TP73L*), Wilms' tumour 1 (*WT1*), immediate early response 3 (*IER3*), dual specificity phosphatases 4 and 6 (*DUSP4*, *DUSP6*), guanylate binding protein 1 (*GBP1*), egl nine homologue 3 (*EGLN3*) and the tumour necrosis factor receptor superfamily member 11A precursor (*TNFRSF11A*, alias *RANK*). Thus, PRL profoundly regulated gene transcription compared with E_2 under the conditions used in this study. Interestingly, 105 genes were regulated in response to PRL/E_2 co-treatment, indicating that PRL and E_2 cooperatively regulate gene expression. Early growth response 3 (*EGR3*), runt-related transcription factor 2 (*RUNX2*) and early growth response 1 (*EGR1*) were the most markedly up-regulated genes in response to PRL/E_2 co-treatment. Table 1 shows a list of up- and down-regulated genes ($\geq$2-fold, FDR of 5%). A complete list of all regulated genes (FDR of 5%) is provided in Supplementary Table 1–3, see section on supplementary data given at the end of this article.

A clustered heatmap of genes regulated $\geq$2-fold (with a FDR of 5%) by PRL/E_2 co-treatment was created for visualisation and comparison of PRL and E_2 single treatments to PRL/E_2 co-treatment (Fig. 3). As indicated in the dendrogram, the individual samples clustered well together according to the treatments. The PRL/E_2 co-treatment cluster was more similar to the PRL cluster than to the E_2 cluster. The heatmap also visualises that the increased number of genes regulated in response to co-treatment was mainly due to additive effects of PRL and E_2.

A cluster of 11 different genes (13 probe sets) was specifically regulated by E_2. Interestingly, a clear additive or even synergistic effect of PRL/E_2 co-treatment was observed on *EGR3* mRNA expression (Fig. 3). Likewise, transcripts mainly regulated by PRL were focused in a cluster of 19 different genes (28 probe sets). No significant additive effect of PRL/E_2 co-treatment was found on any of the 19 genes compared with PRL single treatment.

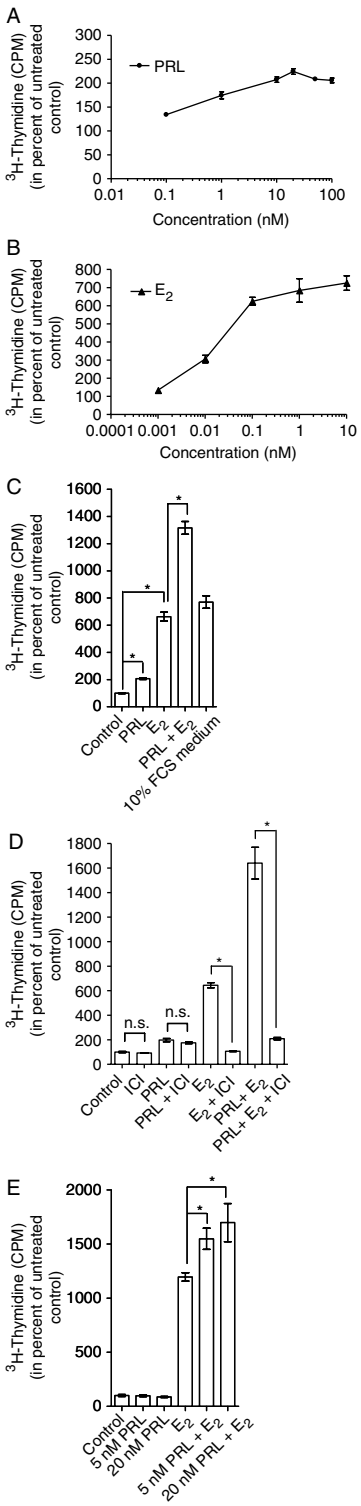

Figure 2 PRL- and E_2-stimulated cell proliferation. (A–D) T47D cells were treated for 3 days with indicated hormones and/or inhibitors in 10% CSS medium. (A and B) Dose–response growth experiments with PRL and E_2 were performed in parallel cultures. (C) Cells were treated with 20 nM PRL and/or indicated concentrations of E_2. The last column shows growth in a medium containing 10% FCS. (D) Cells were treated with 20 nM PRL and/or 1 nM E_2 and/or 100 nM ICI. (E) BT-483 cells were treated for 6 days in 10% CSS medium with indicated concentrations of PRL$\pm$1 nM E_2. Proliferation was estimated using ^{3}H-thymidine incorporation. All data are expressed in percentage of an untreated 10% CSS control culture. Mean values $\pm$S.E.M. (n=6) from a representative of at least three independent experiments are shown. Prolactin (PRL), 17β-oestradiol (E_2) and ICI 182 780 (ICI). *Denotes that $P<0.05$; not significant (NS).

Table 1 Microarray analysis of prolactin (PRL)- and 17β-oestradiol (E_2)-induced gene regulation. Cells were treated with 20 nM PRL and/or 1 nM E_2 for 6 h in 10% CSS medium. Total cellular RNA was isolated, and genome-wide mRNA levels were determined using Affymetrix GeneChip microarray analysis. Three independent experiments were performed. The table shows a ranked list of the up- and down-regulated genes. Genes with a false discovery rate (FDR) of 5% and at least twofold regulated are included. For genes represented by more than one probe set, only the highest score is shown. Gene names are given according to the HUGO Gene Nomenclature Committee (http://www.genenames.org). For more detailed information see Supplementary Tables 1–3, see section on supplementary data given at the end of this article

E_2	PRL	PRL/E_2
>3x up-regulated: *RASGRP1* (4.8x), *MPPED2* (3.3x), *ADRA2A* (3.2x), *CXCL12* (3.1x), *EGR3* (3.1x)	>3x up-regulated: *GLIPR1* (4.3x), *GBP3* (4.0x), *TP73L* (3.2x), *SOCS2* (3.1x)	>3x up-regulated: *EGR3* (5.1x), *RUNX2* (4.0x), *EGR1* (4.0x), *MAFF* (3.8x), *GLIPR1* (3.8x), *IER3* (3.5x), *SOCS3* (3.4x), *WT1* (3.3x), *AREG* (3.3x), *CLIC6* (3.2x), *RASGRP1* (3.2x), *CISH* (3.2x), *OBFC2A* (3.2x), *HEY2* (3.1x), *DUSP4* (3.1x), *CA13* (3.0x)
2–3x up-regulated: *STC1*, *MYC*, *ZNF703*, *KCTD6*, *RBM24*, *NPY1R*, *KCNK5*	2–3x up-regulated: *CA13*, *EGR1*, *CRH*, *RUNX2*, *WT1*, *IER3*, *OBFC2A*, *PTHLH*, *BHLHB8*, *CISH*, *SOCS3*, *DUSP6*, *TNIK*, *DUSP4*, *SPRED1*, *GBP1*, *EGLN3*, *MAFF*, *LMCD1*, *RAB7B*, *TMEM106A*, *TNFRSF11A*, *SNORA9*, *NOG*, *PHLDA2*, *TNS4*, *MAG1*, *SLC16A9*, *TMEM46*, *MAP3K5*, *ALDH1A3*, *CLIC6*, *ABCG2*, *TMC5*, *AREG*, *BACH2*, *ARSG*	2–3x up-regulated: *DOK7*, *DUSP6*, *CRH*, *SNORA9*, *SPRED1*, *BHLHB8*, *MYC*, *PDZK1*, *ADRA2A*, *NOG*, *HS3ST3B1*, *THBS1*, *SOCS2*, *RUNX1*, *RRS1*, *RAB7B*, *SLC7A11*, *ACOX2*, *AMD1*, *B4GALT1*, *MYB*, *ARSG*, *CXCL12*, *HSPC111*, *LMCD1*, *PFKFB3*, *KITLG*, *ZNF703*, *CMTM7*, *MPPED2*, *STC2*, *TNIK*, *KIAA0133*, *TP73L*, *BCL6*, *SLC16A9*, *TNS4*, *PPRC1*, *PHLDA2*, *PEO1*, *GBP3*, *PDCD2L*, *IFRD1*, *PMAIP1*, *KLF10*, *ARTN*, *RBM24*, *CCND1*, *MARS2*, *CHSY1*, *ZBTB24*, *CCDC86*, *LYAR*, *BYSL*, *CDK5R1*, *TFAP2C*, *HK2*, *PTHLT*, *KCNQ4*
	2–3x down-regulated: *ANGPT1*, *FBXO32*, *PFAAP5*, *RALGPS2*, *BCL6*, *SH3RF2*, *CLDN1*, *AKAP9*, *SLC2A13*, *ODZ2*, *PCMTD1*, *BCAR4*, *SHANK2*, *ANK3*, *LRRC3A*	2–3x down-regulated: *ANK3*, *ANGPT1*, *AKAP9*, *ATXN1*, *FLJ41603*, *SH3RF2*, *CDH10*, *TP53INP1*, *ODZ2*, *PCMTD1*, *PFAAP5*, *INADL*, *DLG2*, *CG012*, *CLDN1*, *DST*, *RAB18*, *CITED2*, *CHES1*, *PRNP*, *CYP39A1*, *SLC2A13*, *CYBRD1*, *CYP4Z2P*, *ALF*, *RIN2*, *HIST1H2BG*, *BLNK*
	>3x down-regulated: *KLHL24* (3.7x)	>3x down-regulated: *KLHL24* (4.3x), *FBXO32* (3.4x)

Quantitative PCR analysis of selected PRL- and E_2-regulated genes

To confirm the results of the microarray analysis, we examined the mRNA expression of a panel of selected genes by qPCR analysis. *EGR3*, *EGR1*, *WT1*, *IER3*, CBP/p300-interacting transactivator 2 (*CITED2*) and *RUNX2* were chosen as examples of genes regulated by either E_2 (*EGR3*) or PRL (*EGR1* and *WT1*) or additively (*IER3*, *CITED2* and *RUNX2*). In general, a very high consistence was found between the microarray results and qPCR data (Fig. 4A).

In line with the microarray data (Table 1), *EGR3* appeared to be the most up-regulated gene in response to PRL/E_2 co-treatment, and was also shown to be synergistically up-regulated (~14-fold) by qPCR compared with E_2 alone (approximately eightfold). A low PRL-induced *EGR3* expression was as well observed by qPCR analysis (approximately twofold, $P<0.05$). As expected, the *EGR1* and *WT1* mRNA expression was up-regulated by PRL. E_2 potentiated the PRL-induced *EGR1* expression, while no effect was found by E_2 alone. *IER3*, *CITED2* and *RUNX2* were all confirmed to be additively regulated by PRL and E_2.

To elucidate whether ER contributed to the PRL-induced gene expression, cells were pre-incubated with the anti-oestrogen ICI 182 780 prior to the treatment with PRL and E_2. ICI 182 780 completely abolished the E_2- and/or PRL-induced *EGR3* gene transcription as well as the E_2-induced *IER3* expression (Fig. 4B). In contrast, ICI 182 780 did not influence PRL-stimulated *EGR1* or *IER3* transcription. Thus, PRL regulated the *EGR1* and *IER3* expression independently of ER

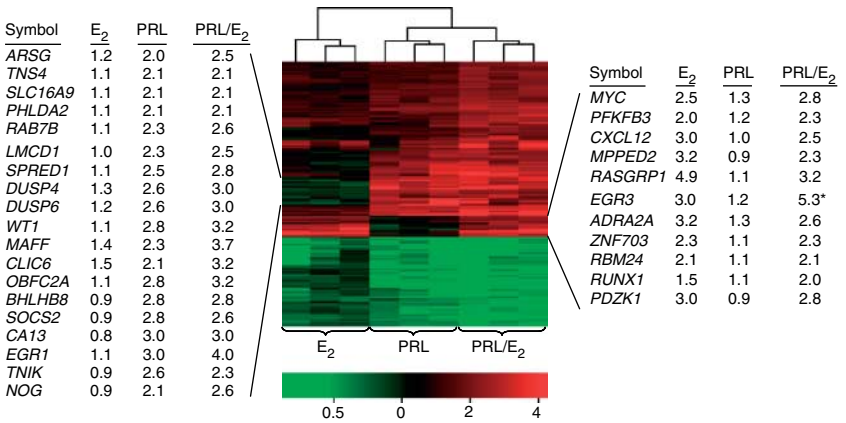

Figure 3 PRL and E_2 cooperate to regulate gene transcription. Clustered heatmap of genes regulated ≥2-fold in response to PRL/E_2 co-treatment (FDR=5%, *$P<0.05$). The corresponding heatmaps of the genes regulated by single treatments are included. A cluster of genes mainly regulated by PRL is shown to the left, whereas a cluster of genes mainly regulated by E_2 is shown to the right. For genes represented by more than one probe set, only the highest score is shown.

function, while the PRL effect on *EGR3* required a functional ER.

Since a synergistic effect of PRL and E_2 was also observed on BT-483 cell proliferation (Fig. 2E), we analysed expression of *EGR3*, the gene which was most regulated by PRL/E_2 in T47D cells. As found for T47D cells, PRL stimulated a weak but significant up-regulation of *EGR3*, while E_2 markedly induced the gene expression after 6 h of treatment (Fig. 4C). Since BT-483 cells grow very slow and thus might exhibit a delayed growth response to PRL/E_2 compared with T47D cells, we also analysed the *EGR3* gene expression level after 24 h. PRL significantly potentiated E_2-induced *EGR3* expression, while no effect was observed by PRL alone.

PRL rapidly induced multiple intracellular signalling events, including ER phosphorylation, and cooperated with E_2 to enhance sustained ER and ERK1/2 activity

To elucidate the signalling events underlying the gene regulation, we assessed the most prominent PRL- and E_2-induced signalling pathways. Using western blot analysis, we observed PRL-induced phosphorylation of STAT5, STAT3, ERK1/2 and AKT after a short-term (20 min) treatment of T47D cells. Short-term E_2 treatment did not stimulate any of the above-mentioned signalling pathways and did not influence PRL-induced phosphorylation (Fig. 5). Both PRL and E_2 induced phosphorylation of ER (Ser118). Surprisingly, the PRL-induced ER phosphorylation was profound, whereas the effect of E_2 was only marginal. Reduced activity of all the pathways was evident in response to long-term (24 h) PRL treatment compared with short-term PRL exposure. Notably, extended E_2 exposure also induced a weak phosphorylation of ERK1/2 and ER (Ser118), which was enhanced upon PRL/E_2 co-treatment. Phosphorylation of AKT, STAT3 or STAT5 was not affected by the PRL/E_2 co-treatment.

Discussion

A large number of studies have proved the importance of PRL and E_2 for mammary gland development and function. Both the growth factors were also implicated in breast cancer pathogenesis. Since PRL and E_2 may be present simultaneously in the tumour milieu, the hormones may potentially influence each others action. It was previously reported that PRL and E_2 co-operatively regulate T47D cell growth (Chen *et al*. 1999, 2010). In the present study, we explored the underlying mechanism of PRL and E_2 on breast cancer cell proliferation and gene expression. We selected T47D and BT-483 cells as model systems, since high expression levels of both PRLR and ER were detected in these cell lines. PRL alone promoted a weak but significant twofold stimulation of T47D breast cancer cell proliferation, while no effect was observed in BT-483 cells. A drastic synergistic effect on T47D cell proliferation was observed upon co-treatment with PRL and E_2. The synergistic growth-stimulating effect of PRL and E_2 was also evident for BT-483 cells. Cooperation between PRL and E_2 in stimulating cancer cell proliferation is in agreement with the effect of PRL and E_2 in mammary gland development: PRL and E_2 cooperate to stimulate breast epithelium proliferation

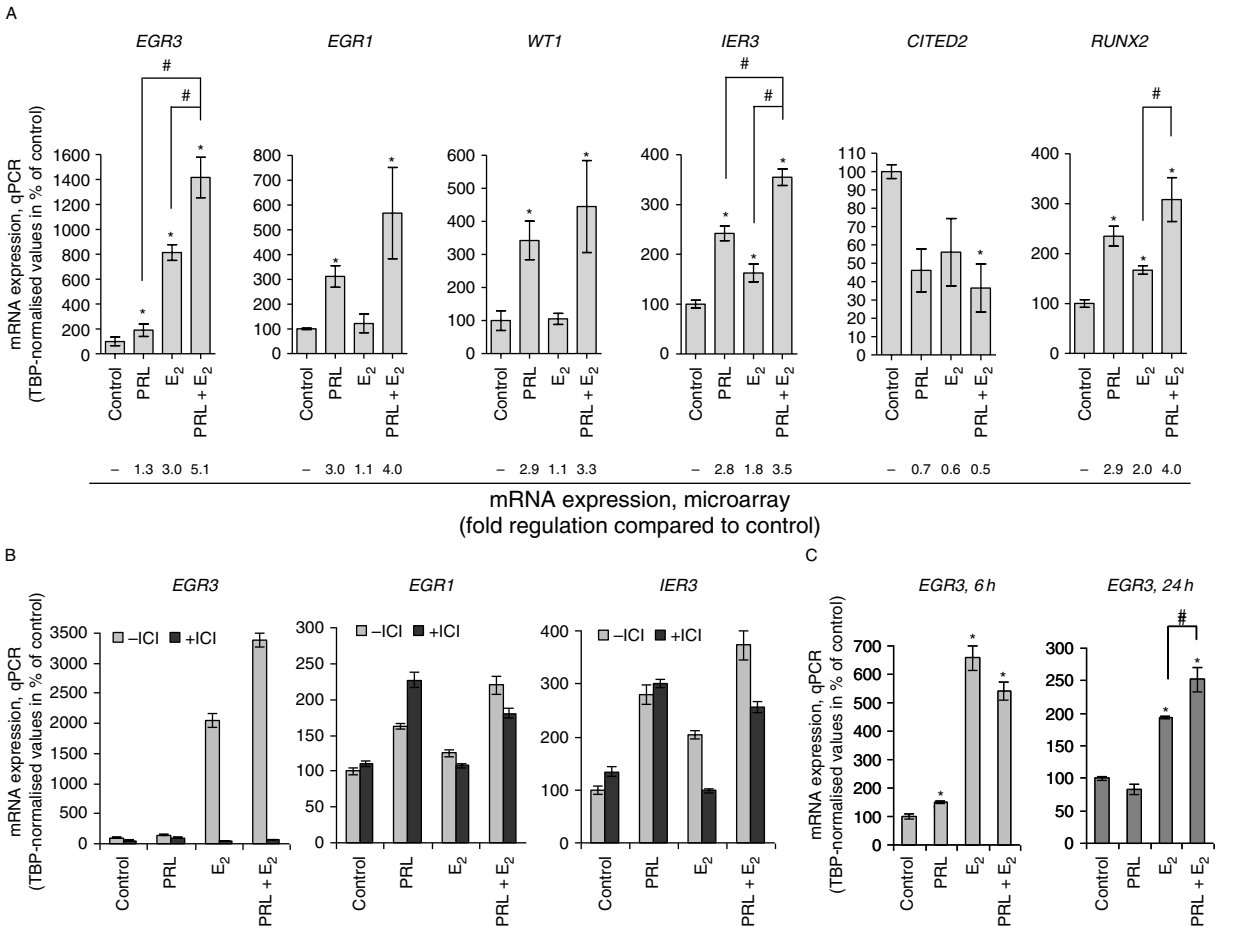

Figure 4 qPCR analysis of PRL- and E_2-regulated genes. (A) Expression of six genes identified by microarray analysis as targets of PRL, E_2 or E_2/PRL was assessed by qPCR. Results represent mean ± S.E.M. of three independent experiments. (B) The contribution of ER was further investigated for the regulation *EGR3*, *EGR1* and *IER3*. T47D cells were pre-treated with 100 nM ICI 182 780 (ICI) for 30 min prior to 6 h of 20 nM PRL and/or 1 nM E_2 treatment. Results represent mean ± S.E.M. of three technical replicates. (C) *EGR3* gene regulation in BT-483 cells. BT-483 cells were treated with 20 nM PRL and/or 1 nM E_2 for 6 and 24 h. Results represent mean ± S.E.M. of four biological replicates. The expression level of each of the genes was normalised to the level of the invariantly expressed TBP transcript. *TBP*-normalised mRNA values are shown in percentage of the untreated control. Statistically significant ($P<0.05$) differences between single treatment versus control (*) and single treatment versus co-treatment (#) are denoted. For comparison, the corresponding mRNA expression levels obtained by Affymetrix microarray analysis are shown below the qPCR graphs for each gene.

during pregnancy, whereas PRL induces differentiation and milk protein synthesis after delivery when the oestrogen level has dropped (Hennighausen & Robinson 2005).

In order to investigate the gene expression profile underlying the observed proliferative response, a microarray analysis has been performed using T47D cells. We found *EGR3* to be the most up-regulated gene upon PRL/E_2 co-treatment. A role of EGR3 in cell proliferation has been demonstrated by Liu *et al.* (2008). The synergistic induction of the *EGR3* mRNA expression in both T47D and BT-483 cells upon PRL/E_2 co-treatment correlates with the observed synergistically induced cell proliferation.

E_2 single treatment also stimulated the expression of *EGR3*, which is in agreement with both *in vitro* and *in vivo* data published by Creighton *et al.* (2006). A minor induction of *EGR3* transcription (1.5–2-fold by qPCR) was as well observed upon a PRL single treatment in T47D and BT-483 cells. We found that the anti-oestrogen ICI 182 780 abolished both E_2- and PRL-induced *EGR3* expression, indicating that the expression was due to ER transcriptional activity.

PRL induced a phosphorylation of ER (Ser118), which is in agreement with recent findings (published during preparation of this manuscript) (Gonzalez *et al.* 2009, Chen *et al.* 2010). Phosphorylation of ER (Ser118) has been demonstrated to correlate with

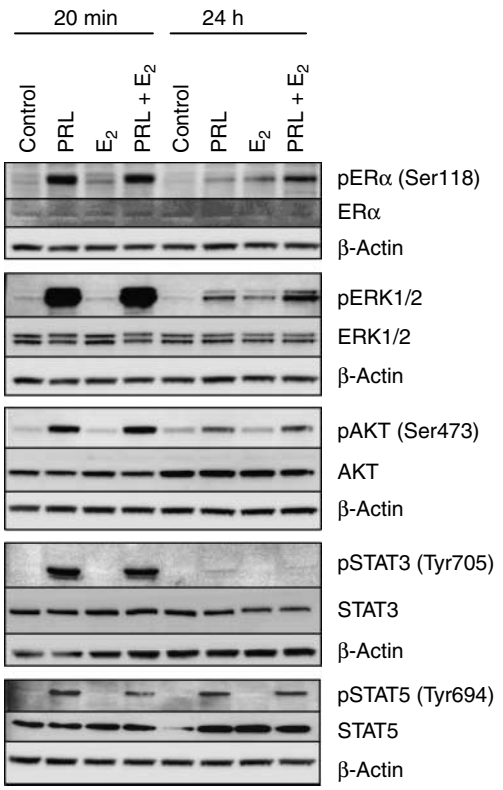

Figure 5 PRL- and E_2-induced cell signalling. T47D cells were seeded in 10% CSS medium. Next day, cells were treated with 20 nM PRL and/or 1 nM E_2 for either 20 min or 24 h as indicated. Cellular proteins were analysed by western blotting with the indicated antibodies against phosphorylation state or total protein forms. β-Actin was used as protein loading control. A representative of three independent experiments is shown.

increased transcriptional activity of ER, also independently of E_2 (Gonzalez *et al*. 2009). Surprisingly, we found that E_2 induced only a minor ER (Ser118) phosphorylation, while a profound response was detected upon PRL treatment. We speculate that the synergistic PRL/E_2-stimulated *EGR3* expression is due to the PRL-induced phosphorylation of the liganded ER providing increased transcriptional activity of the receptor.

We also showed that PRL, but not E_2, strongly induced the gene expression of *EGR1* and *WT1* in T47D cells. A further up-regulation of *EGR1* was found upon PRL/E_2 co-treatment. Like *EGR3*, both *EGR1* and *WT1* encode proteins which belong to the EGR family of zinc finger transcription factors (EGR1–4 and WT1; Madden & Rauscher 1993). EGR1 and WT1 bind to similar GC-rich promoter sequences (Hamilton *et al*. 1998). EGR1 is primarily an enhancer of transcription, while WT1 is a repressor (Lee & Haber 2001). EGR1 seems to play a pro-oncogenic role in prostate cancer (Baron *et al*. 2006), and EGR1$^{-/-}$ mice have a delayed prostate tumourigenesis (Abdulkadir *et al*. 2001). Ectopic expression of EGR1 has been reported to increase tumour growth in xenografted athymic mice (Scharnhorst *et al*. 2000). Knockdown of EGR1 resulted in a reduced proliferation rate of MCF7 human breast cancer cells *in vitro* and decreased growth of MDA-MB-231 tumour xenografts in immunocompromised mice (Mitchell *et al*. 2004). These data suggest that EGR1 might play a central role in PRL and PRL/E_2-stimulated T47D cell proliferation, which we observed in this study. Transcription of *EGR1* was previously shown to be controlled by ERK1/2 signalling in breast cancer cells (Chen *et al*. 2004). We speculate that up-regulation of the *EGR1* expression upon PRL/E_2 co-treatment is due to the observed cooperation between the hormones in enhancing ERK1/2 activity. The anti-oestrogen ICI 182 780 did not influence PRL-induced *EGR1* expression or cell proliferation, indicating that PRL-stimulated T47D cell growth is independent of ER activity.

WT1 was initially discovered as a tumour suppressor gene in the paediatric kidney malignancy, Wilms' tumour. Later it was found that WT1 also exhibits characteristics of an oncogene (Rivera & Haber 2005). The expression level of both WT1 and EGR1 positively correlates with clinical stage and prognosis in Wilms' tumour disease (Ghanem *et al*. 2000). Several isoforms of WT1 with distinct effects have been reported. One of the isoforms was shown to cause oncogenic transformation of breast cancer cells, whereas another caused breast cancer cell cycle arrest (Burwell *et al*. 2007). Down-regulation of all WT1 isoforms using siRNA in T47D breast cancer cells resulted in reduced levels of cyclin D1, phosphorylated Rb and S-phase content, implying a role of WT1 in breast cancer cell growth (Caldon *et al*. 2008). Here we show that PRL induced the expression of *WT1*, which can as well contribute to the cell proliferation. To our knowledge, no previous study described *WT1* as a PRL target gene.

PRL rapidly induced ERK1/2, AKT, STAT3 and STAT5 signalling. Consistent with the finding that E_2 alone upon short-term treatment did not stimulate phosphorylation of either STATs, ERK1/2 or AKT and only marginally of ER, we found only 12 genes to be regulated by E_2 after 6 h of treatment. The relatively low number of E_2-regulated genes compared with previous publications (Frasor *et al*. 2003, Creighton *et al*. 2006) may at least in part be due to the shorter period of E_2 deprivation prior to treatment in the present study (1 vs 3 days (Creighton *et al*. 2006) or 4 days (Frasor *et al*. 2003)). T47D cells in this study were routinely cultured in the presence of insulin as

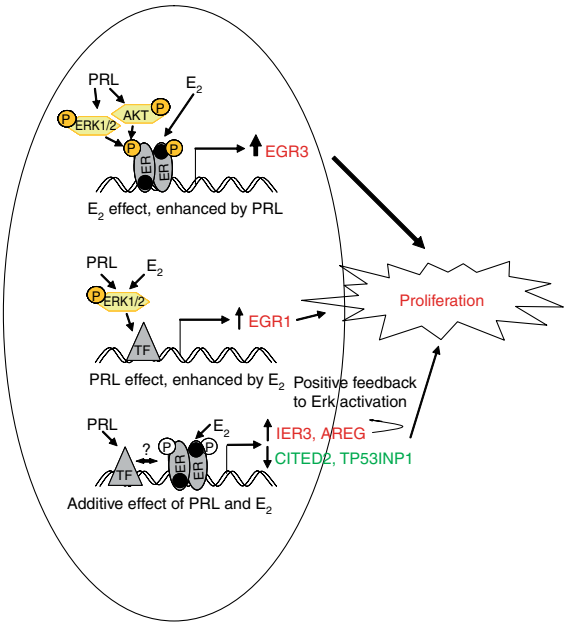

Figure 6 PRL and E_2 cooperate to regulate gene expression and cell proliferation; Model. Three mechanisms are suggested for cooperative gene regulation by PRL and E_2: (A) PRL may potentiate E_2-induced gene transcription by phosphorylation of ER leading to increased transcription of ER target genes, as observed for *EGR3*. (B) E_2 may enhance PRL-induced gene transcription by further activating the ERK1/2 pathway, which causes increased transcriptional activity of PRL-stimulated transcription factors, as found for *EGR1*. (C) PRL and E_2 may cooperate to regulate the same target genes via either direct or indirect interactions at the promoter regions. Additive regulation was observed for several genes, including *IER3*, *AREG*, *CITED2* and *TP53INP1*. The IER3 protein and amphiregulin (encoded by AREG) may cause a further activation of ERK1/2, e.g. upon long-term PRL/E_2 co-treatment. All described genes were previously shown to be implicated in cell proliferation.

recommended by ATCC. Prior to stimulation, the medium was exchanged and treatments were conducted in the absence of insulin. Potentially, this may also influence the observed effects which differ from the previously published data.

Phosphorylation of ER (Ser118) was suggested to be, at least in part, mediated by ERK1/2 and/or AKT signalling pathways upon epidermal growth factor (EGF; Kato *et al*. 1995) or PRL (Chen *et al*. 2010) stimulation. We report here that PRL and E_2 cooperate to enhance a sustained phosphorylation of ER (Ser118) as well as of ERK1/2, but not of AKT. Therefore, the observed sustained ER phosphorylation may be mediated by ERK1/2. Sustained ERK1/2 activation was shown to be required for stabilisation and hence activity of the c-FOS protein (Murphy *et al*. 2002), cyclin D1 gene expression and cell cycling of fibroblasts (Weber *et al*. 1997, Yamamoto *et al*. 2006). In line, we find cyclin D1 (CCND1) significantly up-regulated (2.1-fold) in the microarray analysis by PRL/E_2 co-treatment.

Multiple genes were additively regulated by PRL/E_2 co-treatment. Among these, *AREG* and *IER3* were up-regulated, while the tumour protein p53-inducible nuclear protein 1 (*TP53INP1*) and *CITED2* were down-regulated. We speculate that the observed effect of PRL/E_2 co-treatment on the gene transcription may be due to either improved activity of specific transcriptions factors, as reported for PRL/E_2-induced activation of activating protein 1 (AP-1; Gutzman *et al*. 2005, Safe & Kim 2008), or a physical association between PRL/E_2-induced transcription factors (as shown for STAT5/ER (Bjornstrom *et al*. 2001, Wang & Cheng 2004), Sp-1/ER and AP-1/ER (Safe & Kim 2008)).

Amphiregulin (encoded by *AREG*; McBryan *et al*. 2008, Willmarth & Ethier 2008) and the IER3 protein (Yang *et al*. 2006) have been demonstrated to play a role in breast cancer progression. E_2 is reported to regulate both *AREG* (McBryan *et al*. 2008) and *IER3* (Yang *et al*. 2006) expression. Decreased *AREG* expression was observed in PRLR−/− mice (Ormandy *et al*. 2003), while no data connect PRL and IER3. AREG promoter activity was previously demonstrated to be regulated by RAF/MEK activity and Sp1 (Shao *et al*. 2004). The *IER3* gene expression was found to be induced by ERK1/2- and/or AKT-regulated transcription factors Sp1, p53 or NFκB (Im *et al*. 2002, Hoshiya *et al*. 2003). Since we found both ERK1/2 and AKT to be activated by PRL, it is possible that ERK1/2 and/or AKT play a role also in PRL-induced *AREG* and *IER3* expression. Interestingly, the IER3 protein is shown to interact directly with ERK1/2 and thereby increase ERK1/2 activity (Garcia *et al*. 2002), while amphiregulin can be secreted and binds to EGF receptor followed by ERK1/2 phosphorylation (Wang *et al*. 2008). Thus, up-regulation of *IER3* and *AREG* may contribute to the sustained ERK1/2 activation observed in this study.

We show here that PRL/E_2 co-treatment down-regulated *TP53INP1* expression. Down-regulation of the TP53INP1 protein was found in pancreatic cancer patients. Over-expression of *TP53INP1* in a pancreatic cancer cell line drastically reduced its capacity to form tumours in mice (Gironella *et al*. 2007). *In vitro*, *TP53INP1* induced cell cycle arrest and cell death (Tomasini *et al*. 2005). Thus, down-regulation of *TP53INP1* by PRL/E_2 might represent yet another mechanism promoting cancer cell growth.

Another gene, *CITED2*, whose expression was down-regulated upon PRL/E_2 co-treatment, was also implicated in cancer. It has been demonstrated that

colon cancer cell invasion was increased upon *CITED2* down-regulation (Bai & Merchant 2007). In addition, CITED2 was reported to inactivate the heterodimeric transcription factor hypoxia-inducible factor-1 (HIF1α/β, HIF1; Shin *et al*. 2008). HIF1 has been implicated in both angiogenesis and tumour metastasis. HIF1α is stabilised under hypoxia conditions, oncogene activation or loss of tumour suppressors, while HIF1β is constitutively expressed (Denko 2008). Interestingly, PRL/E_2 co-treatment increased the expression level of HIF1β (*ARTN*). Ectopic expression of HIF1β was recently reported to promote proliferation of breast cancer cells *in vitro* and growth of tumour xenografts in mice (Kang *et al*. 2009). Thus, enhanced HIF1 expression and activity might also contribute to the observed proliferative response upon PRL/E_2 co-treatment.

We also demonstrated enhanced expression of *RUNX2* upon PRL, E_2 and PRL/E_2 co-treatment. PRL was previously found to regulate *RUNX2* expression in human pre-osteoblast cells (Seriwatanachai *et al*. 2009), and a direct interaction between ER and RUNX2 was reported to influence RUNX2-mediated transcription (Khalid *et al*. 2008). RUNX2 is involved in tumour invasion and especially bone metastasis (Pratap *et al*. 2006). Also EGR1 and EGR3 have been proposed to be involved in breast cancer cell migration, invasion and angiogenesis (Mitchell *et al*. 2004, Suzuki *et al*. 2007, Liu *et al*. 2008). Although our study has primarily focused on the role of PRL and E_2 interactions in breast cancer cell proliferation, the hormones may also be implicated in other aspects of cancer progression. A proposed model for the hormonal crosstalk is shown in Fig. 6.

In conclusion, we have demonstrated a drastic synergistic effect of PRL and E_2 on gene expression and breast cancer cell proliferation. A positive correlation between the level of circulating PRL and the incident of ER-positive breast tumours has been reported (Tworoger & Hankinson 2008). This correlation might have functional implications. The novel interplay between PRL and E_2 presented here indicates that PRL can potentiate the progression of the ER+ tumours, and hence provides a rational for a combination therapy targeting both PRL and E_2 signalling in the ER+/PRLR+ breast cancer patients.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0326.

Declaration of interest

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

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