Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 88 (2004) 69–77
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Endogenous human prolactin and not exogenous human prolactin induces estrogen receptor α and prolactin receptor expression and increases estrogen responsiveness in breast cancer cells☆

Jennifer H. Gutzman[a,b], Kristin K. Miller[a], Linda A. Schuler[a,b,*]

[a] *Department of Comparative Biosciences, 2015 Linden Drive, University of Wisconsin–Madison, Madison, WI 53706, USA*
[b] *Molecular and Environmental Toxicology Graduate Program, 1710 University Avenue, University of Wisconsin–Madison, Madison, WI 53726, USA*

Received 26 May 2003; accepted 27 October 2003

Abstract

Prolactin (PRL) and estrogen act synergistically to increase mammary gland growth, development, and differentiation. Based on their roles in the normal gland, these hormones have been studied to determine their interactions in the development and progression of breast cancer. However, most studies have evaluated only endocrine PRL and did not take into account the recent discovery that PRL is synthesized by human mammary cells, permitting autocrine/paracrine activity. To examine the effects of this endogenous PRL, we engineered MCF7 cells to inducibly overexpress human prolactin (hPRL). Using this Tet-On MCF7hPRL cell line, we studied effects on cell growth, PRLR, ERα, and PgR levels, and estrogen target genes. Induced endogenous hPRL, but not exogenous hPRL, increased ERα levels as well as estrogen responsiveness in these cells, suggesting that effects on breast cancer development and progression by estrogen may be amplified by cross-regulation of ERα levels by endogenous hPRL. The long PRLR isoform was also upregulated by endogenous, but not exogenous PRL. This model will allow investigation of endogenous hPRL in mammary epithelial cells and will enable further dissection of PRL effects on other hormone signaling pathways to determine the role of PRL in breast cancer.

Keywords: Prolactin; Prolactin receptor; Estrogen; Breast cancer

1. Introduction

In the normal mammary gland, the polypeptide hormone prolactin (PRL) and the steroid hormones, estrogen and progesterone, act synergistically to increase mammary gland growth, development, and differentiation (reviewed in [1–4]). Based on their roles in the normal gland, these hormones have been studied to determine their function in the development and progression of breast cancer. While the mitogenic activity of estrogen in mammary tumor cells has been well-established, appreciation of this activity for PRL is relatively recent [5–9]. Epidemiological studies that have evaluated circulating PRL levels in an attempt to show a correlation with breast cancer risk have been controversial. While several studies suggest a positive correlation between circulating PRL levels and breast cancer risk factors (reviewed in [10]), drug therapies to reduce serum PRL levels, such as the use of bromocriptine, have been unsuccessful in treating the disease [11,12]. However, these studies evaluated only endocrine PRL and did not take into account the recent discovery that PRL is synthesized and secreted by human mammary cells, permitting autocrine/paracrine activity [13–15]. Study of this local PRL production is necessary to determine its contribution to breast cancer and potentially improve breast cancer treatment modalities.

Understanding regulation of the PRL receptor (PRLR) and the estrogen receptor (ER) within the mammary gland is key to determine how these hormones interact and exert their mitogenic effects. PRL has been shown to regulate responsiveness to ovarian hormones in several female reproductive tissues including the ovary and uterus, as well as the normal mammary gland [16,17]. In vivo studies of these relationships are complicated by indirect actions of PRL on both the ovary and the stromal compartment of the mammary gland, and have been shown to vary with genotype in the mouse [18]. PRLR$^{-/-}$ and ERα$^{-/-}$ mice have defects in mammary

Abbreviations: hPRL, human prolactin; E2, 17β-estradiol; lPRLR, long prolactin receptor isoform; iPRLR, intermediate prolactin receptor isoform; PgR, progesterone receptor; ERα, estrogen receptor α; NIDDK, Institute of Diabetes and Digestive and Kidney Disease

☆ Preliminary studies of this work were presented at the 82nd Annual Meeting of the Endocrine Society, June 2000, Toronto, Canada.

* Corresponding author. Fax: +1-608-263-3926.
E-mail address: schulerl@svm.vetmed.wisc.edu (L.A. Schuler).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2003.10.008

gland lobuloalveolar development, and ductal growth and differentiation, respectively [19,20]. Although levels of mammary ERα in $PRLR^{-/-}$ mice, and PRLR in $ER\alpha^{-/-}$ mice have not been reported, the pattern of progesterone receptor (PgR) expression during mammary development in $PRLR^{-/-}$ mice was disrupted [2]. In a defined in vitro system, normal murine mammary epithelial cells responded to exogenous PRL with a small (25%) increase in ER and a three-fold increase in PgR [21]. However, despite the importance of ovarian hormone responsiveness in the design of therapeutic targets and prognostic indicators for breast cancer, the role of PRL in control of ER and PgR is less clear in mammary tumor cells. In primary neoplasms, several groups have described a positive correlation between ER and PgR, and PRLR levels [22–24]. However, other groups did not find a relationship [25–29]. In the breast cancer cell line, MCF7, Ormandy et al. did not see any effect of human prolactin (hPRL) treatment on ER mRNA levels or specific binding after 24 h, but did observe a small increase in PgR (<two-fold) [24]. In contrast, in an earlier report, Shafie and Brooks observed that hPRL induced a small increase (<two-fold) in ER in the same cell line, after 48 h [30]. Neither study addressed endogenous hPRL production, nor did they evaluate functional effects of PRL on estrogen responsiveness.

To examine the effects of endogenously synthesized mammary hPRL, we engineered MCF7 cells, an extensively studied hormone responsive breast cancer cell line, to inducibly overexpress hPRL. We used this Tet-On MCF7hPRL cell line to study effects of endogenous hPRL on cell growth, PRLR, ERα, and PgR levels, and estrogen target genes. Induced endogenous hPRL, but not exogenous hPRL added to the media, increased ERα levels as well as estrogen responsiveness in the Tet-On MCF7hPRL cells, suggesting that effects on breast cancer development and progression by estrogen may be amplified by cross-regulation of ERα levels by endogenous hPRL.

2. Materials and methods

2.1. Materials

The following antibodies were used for Western analyses: hPRL (anti-hPRL-IC-5) was obtained from the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) and Dr. Parlow; ERα (NCL-ER-6F11/2) from Novocastra Laboratories Ltd. (United Kingdom); lPRLR (PRLr-ECD 35-9200) and iPRLR (PRLr 34-4800) from Zymed (San Francisco, CA, USA); cyclin D1 (MS-210-P1) from Neomarkers (Fremont, CA, USA); progesterone receptor (NCL-PGR-312) from Novocastra (United Kingdom); and Bcl-2 (sc-492) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Recombinant hPRL (Lot AFP795) was obtained through the National Hormone and Pituitary Program, NIDDK, and Dr. Parlow. The ERE-luciferase construct contains three oxytocin receptor estrogen responsive elements located upstream of a luciferase reporter [31]. All other reagents were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.2. Development of stable Tet-On MCF7hPRL cells

Tet-On MCF7 cells that had been selected for high responsive transcriptional activator (rtTA) activity were a gift from Dr. John Pink, Case Western University. Tet-On MCF7 cells were maintained in RPMI 1640 medium containing Tet System approved 10% fetal bovine serum (Clontech), penicillin and streptomycin, insulin, and geneticin (G418) 400 μg/ml. hPRL cDNA [32] was blunt end cloned into the *Xba*I site of the Tet-On system pTRE vector (Clontech), and the construct confirmed by sequencing. Tet-On MCF7 cells were co-transfected with pTRE-hPRL and pTK-Hyg (Clontech), and stable transfectants were selected using hygromycin B (200 μg/ml). The resulting clones were screened for inducible hPRL expression in response to doxycycline using RT-PCR and these were used for further experiments. Tet-On MCF7hPRL cells were maintained in the same media as for Tet-On MCF7 cells with the addition of hygromycin B. Four to five days prior to experiments with estrogen, cells were changed to steroid free, phenol-red free RPMI 1640 containing 5% Tet System approved FBS (Clontech) that had been stripped three times with dextran coated charcoal (3 × CSS) to remove endogenous steroids.

2.3. RT-PCR and Western analysis

For hPRL mRNA analysis, cells were plated in six well plates at 1×10^5 cells per well, serum starved for 24 h, and treated −/+ doxycycline as indicated. RNA was isolated using the RNeasy kit (Qiagen) and RT-PCR was performed as previously described [7]. For Western analysis, Tet-On MCF7hPRL cells were plated at 1×10^6 cells per 100 mm dish, serum starved for 24 h, and treated −/+ 0.5 μg/ml doxycycline for different times, or cells were treated with 0.5 μg/ml doxycycline for 48 h followed by treatment with 1 nM E2 or 4 nM hPRL as indicated. Cell lysates were harvested and analyzed as previously described [7]. Primary antibody concentrations were as follows: hPRL 1:10,000; ERα 1:1000; lPRLR 1:2000; iPRLR 1:6250 (0.04 μg/ml); cyclin D1 1:500; PgR 1:1000; and Bcl-2 1:1000. Signals were quantitated using Molecular Dynamics, Inc. (Sunnyvale, CA) Densitometer and ImageQuant, version 4.2a software.

2.4. Secreted hPRL detection and quantitation

Conditioned media was collected from Tet-On MCF7 parental cells and Tet-On MCF7hPRL cells that were treated without or with doxycycline as indicated. Nb2 cells obtained from Dr. Lewis Sheffield, were employed to measure bioactive secreted hPRL in the Tet-On MCF7hPRL conditioned

media [33–35]. Nb2 cells were maintained in Fischer's Media, containing 10% horse serum, 10% FBS, and 0.1 mM β-mercaptoethanol. Nb2 cells were blocked in the cell cycle for 24 h prior to the assay in the media above without 10% FBS. Cells were then plated at 10^5 cells per well in a 96 well plate and incubated with the conditioned media or hPRL standards (0–3 ng/ml) for 72 h. After 72 h, total cell number was determined by the MTS assay (Promega, Madison, WI, USA) according to manufacturer's protocol. The amount of secreted hPRL was calculated from the hPRL standard curve. For detection of hPRL protein in Tet-On MCF7hPRL cell conditioned media by Western, 500 μl of conditioned media was harvested. Protein from conditioned media was precipitated with 1.5 ml of ice-cold acetone, incubated at −20 °C for 2 h, and centrifuged at 14,000 × *g* for 10 min. Precipitated protein pellets were allowed to dry slowly, resuspended in 10 μl of protein sample buffer, and analyzed by Western as described above.

2.5. Tet-On MCF7hPRL cell proliferation

Tet-On MCF7hPRL cells were grown in estrogen free media for 4 days then plated at 5×10^5 cells per 60 mm tissue culture dish. Cells were changed to serum free media for 24 h and treated for 48 h with or without vehicle, 0.5 μg/ml doxycycline, and/or 1 nM E2. Cell number was analyzed as previously described [7].

2.6. Reporter gene assay

Tet-On MCF7hPRL cells were grown in estrogen free media for 4 days then cells were plated into 12 well tissue culture plates at 3×10^5 cells per well. Cells were serum starved for 24 h, then transiently transfected using SuperFect (Qiagen) as previously described [36]. After 3 h, the transfection complex was replaced with serum free media −/+ doxycycline and −/+ E2 at different concentrations for 6 h. Cells lysates were harvested and analyzed for luciferase and β-galactosidase activity as described [36].

3. Results

3.1. Development and characterization of a mammary epithelial tumor cell line that inducibly overexpresses hPRL

To study the local production of hPRL in human mammary epithelial tumor cells, we developed a MCF7hPRL inducible cell model using the Tet-On Gene Expression System (Clontech). As diagrammed in Fig. 1, when the Tet-On MCF7hPRL cells are treated with doxycycline, the rtTA recognizes the tet responsive element (TRE) on the responsive plasmid and activates hPRL transcription. To confirm that doxycycline treatment of Tet-On MCF7hPRL cells increased hPRL transcription and hPRL protein expression,

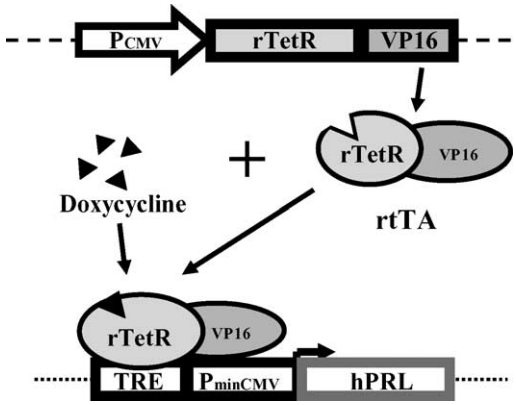

Fig. 1. Schematic representation of induction of hPRL production in the Tet-On MCF7hPRL cell model. MCF7 cells were stably transfected with the reverse Tet responsive transcriptional activator regulator plasmid. The rtTA consists of the reverse Tet repressor (rTetR) fused to the VP16 transactivation domain of herpes simplex virus, under the control of a cytomegaloviral promoter (PCMV). Next, the Tet-On MCF7 cells were stably transfected with the pTRE responsive plasmid expressing human prolactin, under the control of a tet responsive element followed by a minimal cytomegaloviral promoter (PminCMV). In the presence of doxycycline, the rtTA binds to the TRE in the responsive plasmid and activates transcription of hPRL.

cells were treated without and with doxycycline and analyzed by RT-PCR and Western blots. Tet-On MCF7hPRL cells treated with doxycycline showed a marked induction of hPRL mRNA (Fig. 2A) and protein expression (Fig. 2B) compared to the untreated control, indicating that the Tet-On MCF7hPRL cells are functioning properly.

In order to confirm that this induced hPRL could be secreted and was therefore capable of paracrine activity, conditioned Tet-On MCF7hPRL cell media was collected and analyzed for bioactive hPRL using the rat pre–T lymphoma cell line Nb2. Nb2 cells express elevated levels of a high affinity PRLR isoform, and depend on PRL for growth, making them extremely sensitive to PRL and ideal for detection of secreted active PRL [33–35]. To optimize doxycycline treatment conditions, the Tet-On MCF7hPRL cells were subjected to increasing concentrations of doxycycline for variable times. Following treatment, conditioned media was harvested and secreted hPRL was detected by the Nb2 cell bioassay. As shown in Fig. 2C, hPRL secretion was detected in response to low concentrations of doxycycline, reaching a maximum at 0.5 μg/ml. We chose this concentration for further experiments. Although we detected hPRL mRNA, and protein in cell lysates as early as 24 h, hPRL activity was not appreciably increased in the media using this assay until 48 h after doxycycline treatment.

Using this bioassay, we determined the concentration of hPRL secreted by the doxycycline-induced cells by comparing the conditioned media to hPRL standards. Conditioned media from Tet-On MCF7hPRL cells treated with doxycycline for 72 h contained about 10 ng/ml of hPRL (100 ng PRL/1×10^6 cells/72 h) and untreated cells contained about 0.5 ng/ml of hPRL (5 ng hPRL/1×10^6 cells/72 h) (Fig. 3A).

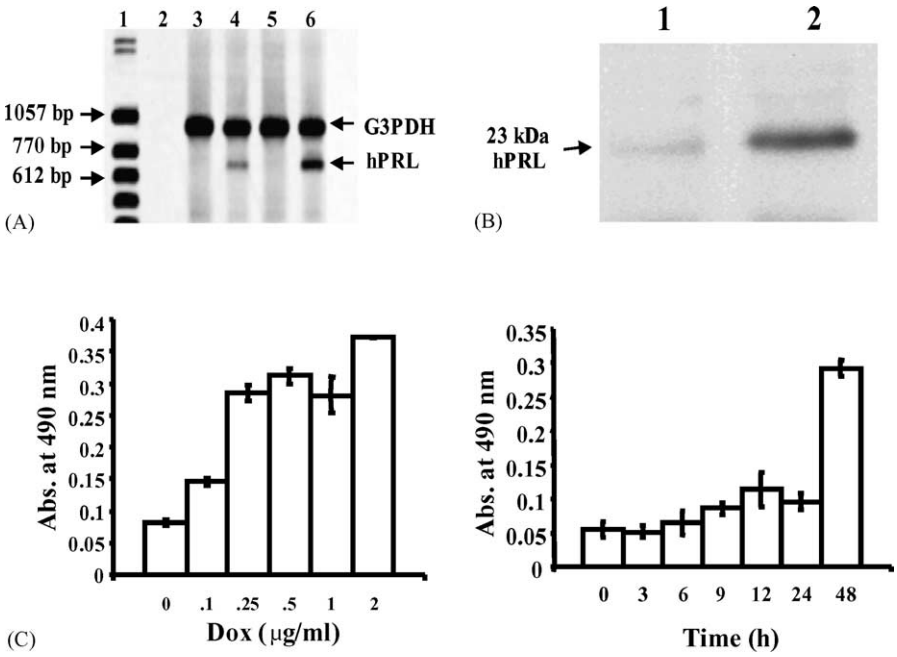

Fig. 2. Doxycycline induces hPRL mRNA, protein expression, and secretion in Tet-On MCF7hPRL cells. (A) RT-PCR analysis of mRNA isolated from cells cultured for 24 and 48 h without or with 0.5 μg/ml doxycycline. Lane 1: lambda DNA ladder; lane 2: negative control without the addition of cDNA to the PCR reaction; lane 3: 24 h without doxycycline; lane 4: 24 h with 0.5 μg/ml doxycycline; lane 5: 48 h without doxycycline; lane 6: 48 h with 0.5 μg/ml doxycycline. G3PDH was used as a positive internal control in all samples. (B) Representative Western analysis of hPRL protein induction by doxycycline. Cells were treated without (lane 1) or with (lane 2) 0.5 μg/ml doxycycline for 24 h. Cell lysates were harvested and 30 μg of protein was analyzed. (C) Dose–response and time-dependence of doxycycline-induced hPRL secretion by Tet-On MCF7hPRL cells. Cells were washed and serum starved for 24 h, then treated with increasing concentrations of doxycycline for 48 h in serum free media (left) or treated with 0.5 μg/ml doxycycline in serum free media for different times (right). Ten microliters of harvested conditioned media was diluted in 40 μl of serum free media; bioactive secreted hPRL was detected using Nb2 cell proliferation (see Section 2). Each value is the mean ± S.D. of three replicates.

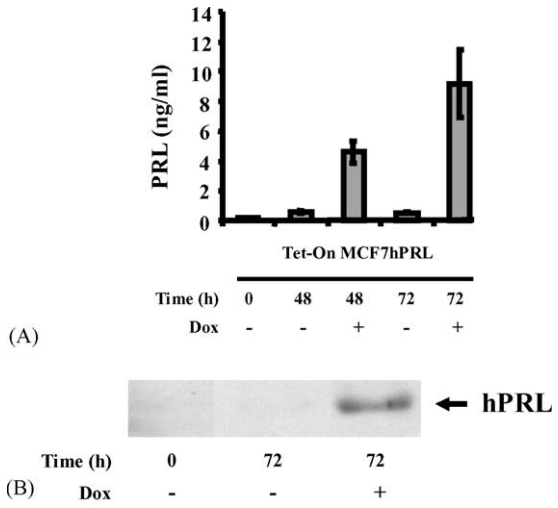

Fig. 3. Quantitation of dox-inducible hPRL secretion by Tet-OnMCF7 hPRL cells. (A) Tet-On MCF7hPRL cells were serum starved for 24 h then treated without or with 0.5 μg/ml doxycycline for 48 or 72 h. The amount of bioactive secreted hPRL was quantitated using Nb2 cell proliferation compared to hPRL standards. Each value is the mean ± S.D. of triplicates. (B) Representative Western analysis of hPRL in the conditioned media of Tet-On MCF7hPRL cells. Cells were serum starved for 24 h then treated without or with 0.5 μg/ml doxycycline in serum free media. Five hundred microliters of conditioned media was harvested at 0 and 72 h and precipitated with acetone prior to analysis.

The latter is similar to the amount of hPRL secreted by cultured breast cancer cells in previous reports [13,14]. In this system, doxycycline treatment induced a 20-fold increase in hPRL secretion compared to basal levels. hPRL secretion by the control Tet-On MCF7 parental cell line, without the pTRE responsive plasmid, was similar to uninduced Tet-On MCF7hPRL cells (data not shown). To confirm the induction of secreted hPRL, protein in conditioned media from doxycycline treated Tet-On MCF7hPRL cells was precipitated with acetone and analyzed by Western blot (Fig. 3B). These studies indicate that the Tet-On MCF7hPRL cells express high levels of hPRL mRNA, hPRL protein, and secrete hPRL into the media when induced with doxycycline.

3.2. Tet-On MCF7hPRL cells proliferate faster in response to doxycycline-induced hPRL and E2

Next we examined the effect of doxycycline-induced hPRL on cell growth in the presence and absence of 17β-estradiol (E2). Tet-On MCF7hPRL cells were grown in 3 × CSS serum for 5 days prior to the experiment to minimize exposure to estrogens. Cells were plated in 3 × CSS and then serum starved for 24 h ($t = 0$), prior to treatment with doxycycline, E2 or both together. Viable cells were then counted 48 h after treatment (Fig. 4). The rate of apoptosis was extremely low (data not shown); therefore,

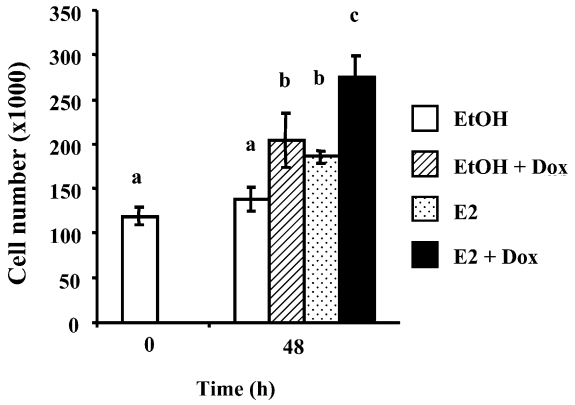

Fig. 4. Growth of Tet-On MCF7hPRL cells in response to doxycycline and E2 treatment. Representative experiment. Cells were serum starved for 24 h then treated without or with vehicle, 0.5 μg/ml doxycycline, and 1 nM E2. Viable cells were counted at 0 and 48 h. Results are expressed as the mean ± S.D. of triplicate plates. Different letters denote significantly different values analyzed by one-way ANOVA, followed by Student–Newman–Keuls multiple comparison test ($P < 0.05$).

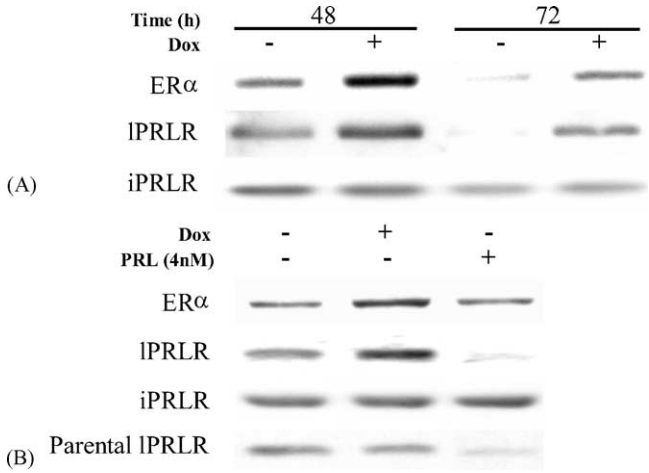

Fig. 5. Dox-induced endogenous hPRL increases ERα and lPRLR, but not iPRLR in Tet-On MCF7hPRL cells. (A) Representative Western analysis of ERα, lPRLR and iPRLR in cell lysates that were serum starved for 24 h then treated without or with 0.5 μg/ml doxycycline for 48 or 72 h. (B) Representative Western analysis of ERα, lPRLR, and iPRLR in Tet-On MCF7hPRL cell lysates, and lPRLR in Tet-On parental cell lysates. Cells were serum starved for 24 h then treated without or with 0.5 μg/ml doxycycline, and vehicle or 4 nM hPRL for 48 h.

these changes represent primarily proliferation. Numbers of doxycycline treated cells increased 1.5-fold over untreated controls ($P < 0.05$), similar to E2 treatment, indicating that increased endogenous hPRL production can stimulate Tet-On MCF7hPRL cell proliferation. Cells treated with both doxycycline and E2 together showed a further increase ($P < 0.05$) over doxycycline or E2 treatment alone. These results are consistent with previous data showing breast cancer cells proliferate in response to these hormones [7,9] and confirm that endogenous PRL has mitogenic effects in this system.

3.3. Doxycycline-induced hPRL increases ERα and lPRLR, but not iPRLR expression in Tet-On MCF7hPRL cells

MCF7 cells express multiple PRLR isoforms generated by alternative splicing, including the long PRLR (lPRLR) and the intermediate PRLR (iPRLR), each with different signaling capabilities. The predominant isoform in these cells, the lPRLR, is the most studied PRLR isoform and is a classic type I cytokine receptor. It mediates signals to many pathways, including the well-studied Jak/Stat pathway (reviewed in [10]). The iPRLR isoform has a truncated cytoplasmic domain and a unique 13 amino-acid sequence after the extended box 2 region. It also activates the Jak/Stat pathway, but it is less effective in mediating signals to Fyn [37]. MCF7 cells also express ERα, although they have undetectable levels of ERβ (Wade Welshons, personal communication).

To investigate the effect of increased endogenous hPRL on expression of these receptors, we treated Tet-On MCF7hPRL cells with or without doxycycline for 48 or 72 h and analyzed the cell lysates for ERα, lPRLR, and iPRLR by Western blot (Fig. 5A). After 48 h of doxycycline treatment, cells showed a three-fold increase in ERα and a two-fold increase in lPRLR expression, indicating that induced hPRL upregulated these receptors. Doxycycline treatment had no effect on iPRLR levels, indicating that this effect was isoform specific. A similar trend was seen at 72 h, although levels of receptors were uniformly lower. Levels of ERα, lPRLR, and iPRLR were not altered in the parental Tet-On MCF7 cells treated with doxycycline (data not shown).

Because PRL has been reported to up- or down-regulate PRLR at different targets depending on dose and experimental system (reviewed in [38–40]), we compared the effects of exogenous hPRL and doxycycline-induced endogenous hPRL. As shown in Fig. 5B, lPRLR levels decreased and ERα expression was unchanged when cells were treated with 4 nM exogenous hPRL for 48 h, in contrast to the upregulation of both receptors following increased endogenous synthesis. The iPRLR isoform was not changed by either treatment. Treatment with lower concentrations (0.04 and 0.4 nM) of exogenous hPRL also had no effect on ERα, lPRLR, or iPRLR levels (data not shown). The parental Tet-On MCF7 cells responded similarly to exogenous hormone. lPRLR was downregulated (Fig. 5B), and ERα levels were not altered by any of the treatments (data not shown).

3.4. Doxycycline-induced hPRL increases Tet-On MCF7hPRL cell responsiveness to E2

The number of estrogen receptors expressed within a cell has been shown to be a limiting factor for E2 responsiveness [41]. Because we observed an increase in ERα in response to increased endogenous hPRL, we examined the ability of the Tet-On MCF7hPRL cells to mediate the classical E2 response, activation of transcription via an

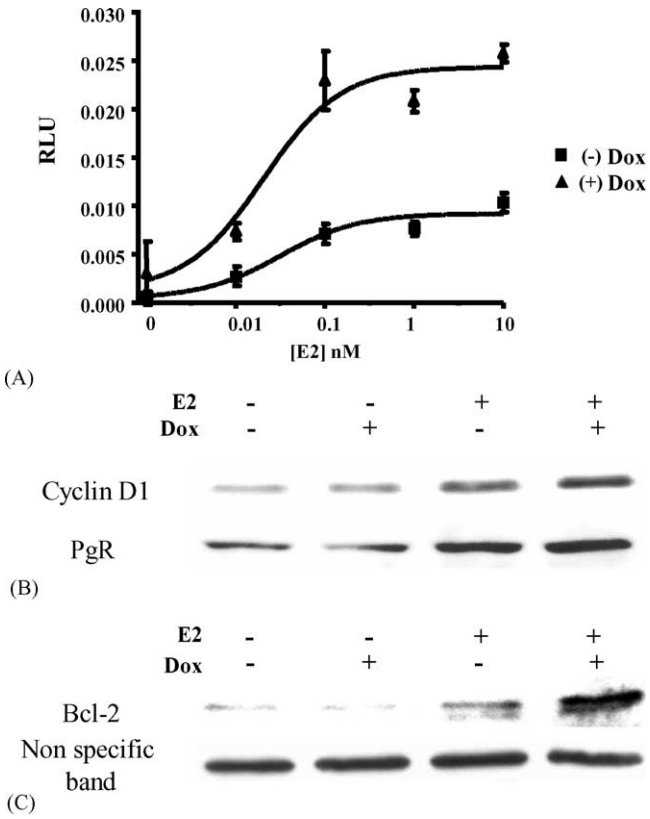

Fig. 6. Dox-induced hPRL production increases E2 sensitivity in Tet-On MCF7hPRL cells. (A) Representative experiment. Tet-On MFC7hPRL cells were plated in 5% 3 × CSS for 24 h then serum starved for 24 h in the presence of 0.5 μg/ml doxycycline. Cells were transiently transfected with an ERE-luciferase reporter and β-galactosidase control construct, then treated with different concentrations of 17β-estradiol (E2) for 6 h. Cells lysates were assayed for luciferase activity, and β-galactosidase activity was used to correct for transfection efficiency. The graph shows relative luciferase units (RLU) as a function of E2 concentration on a logarithmic scale. Data were analyzed using a nonlinear regression equation (Prism 3.0). Points represent the mean ± S.D. of triplicates. (B) and (C) Representative Western analysis of cyclin D1 and PgR (B) or Bcl-2 (C) in Tet-On MCF7hPRL whole cell lysates. Cells were serum starved for 24 h, treated without or with 0.5 μg/ml doxycycline for 48 h and then treated with vehicle control or 1 nM E2 for 6 h (B) or 48 h (C).

ERE enhancer (Fig. 6A). Cells that were pre-treated with doxycycline for 48 h showed a three-fold increase in maximal ERE-luciferase activity compared to the vehicle treated cells, although the ED_{50} was not significantly different. We also examined effects on expression of other known E2 targets including the cell cycle regulator, cyclin D1; the classic E2 responsive protein, progesterone receptor; and the anti-apoptotic protein, Bcl-2 [3,42–46]. Cells were estrogen withdrawn, pre-treated with doxycycline for 48 h, and then treated with or without 1 nM E2 (Fig. 6B and C). Doxycycline treatment alone modestly increased cyclin D1 levels, consistent with our previous data showing that PRL increases cyclin D1 transcription [7,36]. E2 treatment alone increased cyclin D1 levels 3.5-fold, also consistent with previous reports [42,43,47]. Interestingly, treatment with E2 and doxycycline together increased cyclin D1 levels 4.5-fold over untreated control, showing an increased efficacy of E2 treatment following doxycycline. Doxycycline treatment did not alter PgR, but slightly enhanced the effect of E2. When Tet-On MCF7hPRLR cells were treated with both E2 and doxycycline together, they showed almost a 10-fold increase in Bcl-2 levels compared to the lack of change when treated with doxycycline alone and a three-fold increase when treated with E2 alone. Together, these data indicate that doxycycline-induced hPRL not only increases ERα levels, but also in doing so, increases the responsiveness of the Tet-On MCF7hPRL cells to E2.

4. Discussion

The complex interactions between hPRL and other hormones within the mammary gland important for normal development (reviewed in [1–4]) suggest a role for these hormones in the development and progression of breast cancer. The discovery that mammary epithelial cells produce and secrete hPRL [13–15] has redirected breast cancer research in this area. However, studying autocrine/paracrine actions of PRL in the mammary gland has been challenging. Here we developed a hormonally responsive, mammary tumor cell line that inducibly expresses and secretes hPRL as a tool to study endogenous hPRL and how it may regulate receptor levels as well as responsiveness to other hormones in breast cancer.

One mechanism of hormone interaction is cross-regulation of their receptors. Endogenous hPRL increased ERα protein levels substantially, which was associated with a robust increase in E2 responsiveness, measured by ERE-luciferase activity, and activation of E2 target genes, including cyclin D1 and Bcl-2. These targets are important for cell cycle progression and inhibition of cell death, respectively, and both are implicated in breast cancer [48–50]. The inability of induced PRL to similarly augment the PgR response was unexpected, and may be explained by maximal stimulation of this pathway by E2 alone. PRL induces transcription of ERα through the Jak2/Stat5 pathway in the rodent corpus luteum (reviewed in [16]). In light of the well characterized ability of this pathway to mediate many PRL actions (reviewed in [10]), and its recognized importance in mammary development [51,52], the Jak2/Stat5 pathway is likely to be involved in PRL regulation of ERα at this target as well.

The status of ERα expression in breast cancer is critical for prognosis and therapeutic strategies. However, few models for studying regulation of ERα expression are available. Expression of a PRL transgene within murine mammary epithelium resulted in ERα+, as well as ERα− mammary tumors [53]. While these findings in vivo do not demonstrate direct induction of mammary ERα expression by PRL, the lack of this tumor population in response to other mammary oncogenes suggests that this relationship also may be important in tumorigenesis. These models of locally produced

PRL provide tools to dissect this potentially very significant relationship in breast cancer development and progression.

Endogenous hPRL also selectively increased the major PRLR isoform, lPRLR, without affecting iPRLR protein expression. Multiple factors have been shown to govern total PRLR expression at different target tissues [38–40]. Receptor levels may increase due to changes in transcription, translation, or protein stability, or decrease due to receptor degradation and/or processing. While long-term PRL treatment in vivo upregulated mammary PRLR [54,55], and higher circulating PRL during various physiologic states can be associated with elevated mammary PRLR [56–58], these models are complicated by complex indirect effects and feedback loops. In vitro, treatment with exogenous ligand generally results in PRLR downregulation, following internalization and subsequent degradation [59–61], similar to the effect of exogenous PRL in the present studies. A very low concentration (0.04 nM) of PRL slightly increased (50%) PRL binding in another breast cancer cell line, but the effect was lost at 0.4 nM PRL [62]. Interestingly, engineering the ERα− breast cancer cell line, MDA-MB-435, to constitutively overexpress PRL, also resulted in elevated lPRLR [63].

It is likely that transcription plays a major role in the increase in lPRLR seen here. Exogenous PRL increased activity of one of the PRLR promoters via Stat5 in pancreatic β-cells in vitro [64]. Analyses of the PRLR promoter in both rodents and humans have revealed additional elements that have been shown to mediate a response to PRL in other systems, including AP-1 and Sp1 sites, as well as sites for interaction with C/EBPβ [65–69]. However, additional studies are required to determine the effective mechanism for this response in both normal and neoplastic epithelial cells.

The substantial increase in ERα in response to increased endogenous hPRL compared to the modest, or lack of effect, using exogenous hPRL in the parent cell line [24,30], and the marked disparity between the effects of exogenous and increased endogenous hPRL on both ERα and lPRLR levels in the present study, are consistent with important differences in the mechanism of action of endogenously produced and exogenously added hPRL. When compared to normal physiological and commonly used experimental concentrations, the amount of hPRL secreted into the media by the Tet-On MCF7hPRL cells is very low, accumulating to concentrations near the Kd of the PRLR only after 72 h [70]. This phenomenon is similar to that described for autocrine production of hGH by MCF7 cells [71]. One possible explanation for this difference is that the locally synthesized PRL may be post-translationally modified, resulting in a different spectrum of activity compared to the unmodified hPRL from NIDDK. For example, phosphorylated hPRL has been shown to inhibit growth promoting effects of unmodified PRL, while potently stimulating effects associated with differentiation (reviewed in [72]); the latter might include increased ERα expression. The approximate agreement between mitogenic PRL activity measured by the Nb2 assay, and immunoreactive PRL in the conditioned media in our studies, does not support this as a major factor. However, our data do not rule out some modification of a minor component that could account for our findings. Alternatively, endogenous hPRL may have access to different pathways than PRL secreted by the pituitary and presented to the plasma membrane. Such “intracrine” signaling by PRL has been suggested in other cell types [73–75], as well as by other growth factors including epidermal growth factor, platelet derived growth factor, and renin-angiotensin [76–78]. These possibilities are currently under investigation.

We have developed a model to study the effects of endogenous hPRL in mammary tumor cells to better understand the autocrine/paracrine signaling of PRL in these cells. Endogenous hPRL, but not exogenous hPRL, upregulated both lPRLR and ERα protein levels, and substantially increased the responsiveness of these cells to E2. This model will allow investigation of endogenous hPRL in mammary epithelial cells and will enable further dissection of PRL effects on other hormone signaling pathways to determine the role of hPRL in breast cancer. These studies may help to develop new prognostic indicators and treatments for the disease.

Acknowledgements

This work was supported by Army grant DAMD17-01-1-0460, NIH grant RO1 CA 78312, and Molecular and Environmental Toxicology Training grant T32-ES07015. The authors would like to thank Drs. Fern Murdoch, Michael Fritsch, Charles Czuprynski, Jyoti Watters and Jack Gorski (University of Wisconsin–Madison) and Dr. John Pink, (Case Western University), for reagents and helpful discussions; Dr. Ameae Walker (University of California-Riverside) and Drs. Matthew Schroeder and Juu-Chin Lu (University of Wisconsin–Madison) for assistance with experiments, and Dr. Wade Welshons for access to unpublished data.

References

[1] N.D. Horseman, Prolactin and mammary gland development, J. Mammary Gland Biol. Neoplasia 4 (1999) 79–88.

[2] S.L. Grimm, T.N. Seagroves, E.B. Kabotyanski, R.C. Hovey, B.K. Vonderhaar, J.P. Lydon, K. Miyoshi, L. Hennighausen, C.J. Ormandy, A.V. Lee, M.A. Stull, T.L. Wood, J.M. Rosen, Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development, Mol. Endocrinol. 16 (2002) 2675–2691.

[3] G. Shyamala, Y.C. Chou, S.G. Louie, R.C. Guzman, G.H. Smith, S. Nandi, Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, J. Steroid Biochem. Mol. Biol. 80 (2002) 137–148.

[4] R.C. Hovey, J.F. Trott, E. Ginsburg, A. Goldhar, M.M. Sasaki, S.J. Fountain, K. Sundararajan, B.K. Vonderhaar, Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal

branch growth in the mammary gland, Dev. Dyn. 222 (2001) 192–205.
[5] R. Das, B.K. Vonderhaar, Prolactin as a mitogen in mammary cells, J. Mammary Gland Biol. Neoplasia 2 (1997) 29–40.
[6] M. Llovera, C. Pichard, S. Bernichtein, S. Jeay, P. Touraine, P.A. Kelly, V. Goffin, Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation, Oncogene 19 (2000) 4695–4705.
[7] M.D. Schroeder, J. Symowicz, L.A. Schuler, Prolactin modulates cell cycle regulators in mammary tumor epithelial cells, Mol. Endocrinol. 16 (2002) 45–57.
[8] G. Fuh, J.A. Wells, Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines, J. Biol. Chem. 270 (1995) 13133–13137.
[9] W.Y. Chen, P. Ramamoorthy, N. Chen, R. Sticca, T.E. Wagner, A human prolactin antagonist, hPRL-G129R, inhibits breast cancer cell proliferation through induction of apoptosis, Clin. Cancer Res. 5 (1999) 3583–3593.
[10] C.V. Clevenger, P.A. Furth, S.E. Hankinson, L.A. Schuler, Role of prolactin in mammary carcinoma, Endocrinol. Rev. 24 (2003) 1–27.
[11] J.P. Peyrat, P. Vennin, J. Bonneterre, B. Hecquet, B. Vandewalle, P.A. Kelly, J. Djiane, Effect of bromocriptin treatment on prolactin and steroid receptor levels in human breast cancer, Eur. J. Cancer Clin. Oncol. 20 (1984) 1363–1367.
[12] J.C. Heuson, A. Coune, M. Staquet, Clinical trial of 2-Br-alpha-ergocryptine (CB154) in advanced breast cancer, Eur. J. Cancer 8 (1972) 155–156.
[13] C.V. Clevenger, W.-P. Chang, W. Ngo, T.L.M. Pasha, K.T. Montone, J.E. Tomaszewski, Expression of prolactin and prolactin receptor in human breast carcinoma: evidence for an autocrine/paracrine loop, Am. J. Pathol. 146 (1995) 695–705.
[14] E. Ginsburg, B.K. Vonderhaar, Prolactin synthesis and secretion by human breast cancer cells, Cancer Res. 55 (1995) 2591–2595.
[15] M. Zinger, M. McFarland, N. Ben Jonathan, Prolactin expression and secretion by human breast glandular and adipose tissue explants, J. Clin. Endocrinol. Metab. 88 (2003) 689–696.
[16] J. Frasor, G. Gibori, Prolactin regulation of estrogen receptor expression, Trends Endocrinol. Metab. 14 (2003) 118–123.
[17] S. Kleis-SanFrancisco, A. Hewetson, B.S. Chilton, Prolactin augments progesterone-dependent uteroglobin gene expression by modulating promoter-binding proteins, Mol. Endocrinol. 7 (1993) 214–223.
[18] T.G. Muldoon, Prolactin mediation of estrogen-induced changes in mammary tissue estrogen and progesterone receptors, Endocrinology 121 (1987) 141–149.
[19] C.J. Ormandy, A. Camus, J. Barra, D. Damotte, B. Lucas, H. Buteau, M. Edery, N. Brousse, C. Babinet, N. Binart, P.A. Kelly, Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse, Genes Dev. 11 (1997) 167–178.
[20] W.P. Bocchinfuso, J.K. Lindzey, S.C. Hewitt, J.A. Clark, P.H. Myers, R. Cooper, K.S. Korach, Induction of mammary gland development in estrogen receptor-α knockout mice, Endocrinology 141 (2000) 2982–2994.
[21] M. Edery, W. Imagawa, L. Larson, S. Nandi, Regulation of estrogen and progesterone receptor levels in mouse mammary epithelial cells grown in serum-free collagen gel cultures, Endocrinology 116 (1985) 105–112.
[22] J. Bonneterre, J.P. Peyrat, R. Beuscart, A. Demaille, Biological and clinical aspects of prolactin receptors (PRL- R) in human breast cancer, J. Steroid Biochem. Mol. Biol. 37 (1990) 977–981.
[23] L.J. Murphy, L.C. Murphy, E. Vrhovsek, R.L. Sutherland, L. Lazarus, Correlation of lactogenic receptor concentration in human breast cancer with estrogen receptor concentration, Cancer Res. 44 (1984) 1963–1968.
[24] C.J. Ormandy, R.E. Hall, D.L. Manning, J.F.R. Robertson, R.W. Blamey, P.A. Kelly, R.I. Nicholson, R.L. Sutherland, Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer, J. Clin. Endocrinol. Metab. 82 (1997) 3692–3699.
[25] B. Rae-Venter, T. Nemoto, S.L. Schneider, T.L. Dao, Prolactin binding by human mammary carcinoma: relationship to estrogen receptor protein concentration and patient age, Breast Cancer Res. Treat. 1 (1981) 233–243.
[26] M. Ben David, J.L. Wittliff, M. Fekete, T. Kadar, S. Biran, A.V. Schally, Lack of relationship between the levels of prolactin receptors and steroid receptors in women with breast cancer, Biomed. Pharmacother. 42 (1988) 327–334.
[27] N. Waseda, Y. Kato, H. Imura, M. Kurata, Prognostic value of estrogen and prolactin receptor analysis in human breast cancer, Jpn. J. Cancer Res. 76 (1985) 517–523.
[28] S. De Placido, C. Gallo, F. Perrone, A. Marinelli, C. Pagliarulo, C. Carlomagno, G. Petrella, M. D'Istria, G. Delrio, A.R. Bianco, Prolactin receptor does not correlate with oestrogen and progesterone receptors in primary breast cancer and lacks prognostic significance. Ten year results of the Naples adjuvant (GUN) study, Br. J. Cancer 62 (1990) 643–646.
[29] C. Reynolds, K.T. Montone, C.M. Powell, J.E. Tomaszewski, C.V. Clevenger, Expression of prolactin and its receptor in human breast carcinoma, Endocrinology 138 (1997) 5555–5560.
[30] S. Shafie, S.C. Brooks, Effect of prolactin on growth and the estrogen receptor level of human breast cancer cells (MCF-7), Cancer Res. 37 (1977) 792–799.
[31] R.A. Adan, J.J. Cox, J.P. van Kats, J.P. Burbach, Thyroid hormone regulates the oxytocin gene, J. Biol. Chem. 267 (1992) 3771–3777.
[32] N.E. Cooke, D. Coit, J. Shine, J.D. Baxter, J.A. Martial, Human prolactin cDNA structural analysis and evolutionary comparisons, J. Biol. Chem. 256 (1981) 4007–4016.
[33] R.P.C. Shiu, H.P. Elsholtz, T. Tanaka, H.G. Friesen, P.W. Gout, C.T. Beer, R.L. Noble, Receptor-mediated mitogenic action of prolactin in a rat lymphoma cell line, Endocrinology 113 (1983) 159–165.
[34] K.D. O'Neal, L. Yu-Lee, Differential signal transduction of the short, Nb2, and long prolactin receptors. Activation of interferon regulatory factor-1 and cell proliferation, J. Biol. Chem. 269 (1994) 26076–26082.
[35] T. Tanaka, R.P. Shiu, P.W. Gout, C.T. Beer, R.L. Noble, H.G. Friesen, A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum, J. Clin. Endocrinol. Metab. 51 (1980) 1058–1063.
[36] J.L. Brockman, M.D. Schroeder, L.A. Schuler, Prolactin activates the cyclin D1 promoter via the JAK2-STAT pathway, Mol. Endocrinol. 16 (2002) 774–784.
[37] J.B. Kline, H. Roehrs, C.V. Clevenger, Functional characterization of the intermediate isoform of the human prolactin receptor, J. Biol. Chem. 274 (1999) 35461–35468.
[38] P.A. Kelly, J. Djiane, M.-C. Postel-Vinay, M. Edery, The prolactin/growth hormone receptor family, Endocrinol. Rev. 12 (1991) 235–251.
[39] C.J. Ormandy, R.L. Sutherland, Mechanisms of prolactin receptor regulation in mammary gland, Mol. Cell Endocrinol. 91 (1993) C1–C6.
[40] Z.Z. Hu, L. Zhuang, M.L. Dufau, Prolactin receptor gene diversity: structure and regulation, Trends Endocrinol. Metab. 9 (1998) 94–102.
[41] P. Webb, G.N. Lopez, G.L. Greene, J.D. Baxter, P.J. Kushner, The limits of the cellular capacity to mediate an estrogen response, Mol. Endocrinol. 6 (1992) 157–167.
[42] L. Altucci, R. Addeo, L. Cicatiello, S. Dauvois, M.G. Parker, M. Truss, M. Beato, V. Sica, F. Bresciani, A. Weisz, 17β-Estradiol induces cyclin D_1 gene transcription, $p36^{D1}$- $p34^{cdk4}$ complex activation and $p105^{Rb}$ phosphorylation during mitogenic stimulation of G_1-arrested human breast cancer, Oncogene 12 (1996) 2315–2324.
[43] O.W.J. Prall, B. Sarcevic, E.A. Musgrove, C.K.W. Watts, R.L. Sutherland, Estrogen-induced activation of cdk4 and cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-cdk2, J. Biol. Chem. 272 (1997) 10882–10894.

[44] B. Perillo, A. Sasso, C. Abbondanza, G. Palumbo, 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence, Mol. Cell Biol. 20 (2000) 2890–2901.
[45] M. Razandi, A. Pedram, E.R. Levin, Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer, Mol. Endocrinol. 14 (2000) 1434–1447.
[46] P. Bouchard, Progesterone and the progesterone receptor, J. Reprod. Med. 44 (1999) 153–157.
[47] M.D. Planas-Silva, J.L. Donaher, R.A. Weinberg, Functional activity of ectopically expressed estrogen receptor is not sufficient for estrogen-mediated cyclin D1 expression, Cancer Res. 59 (1999) 4788–4792.
[48] B.T. Zafonte, J. Hulit, D.F. Amanatullah, C. Albanese, C. Wang, E. Rosen, A. Reutens, J.A. Sparano, M.P. Lisanti, R.G. Pestell, Cell-cycle dysregulation in breast cancer: breast cancer therapies targeting the cell cycle, Front. Biosci. 5 (2000) D938–D961.
[49] D.M. Barnes, C.E. Gillett, Cyclin D1 in breast cancer, Breast Cancer Res.Treat. 52 (1998) 1–15.
[50] S. Cory, J.M. Adams, The Bcl2 family: regulators of the cellular life-or-death switch, Nat. Rev. Cancer 2 (2002) 647–656.
[51] L. Hennighausen, G.W. Robinson, Signaling pathways in mammary gland development, Dev. Cell 1 (2001) 467–475.
[52] T.N. Seagroves, J.M. Rosen, Knockout and transgenic mouse models that have contributed to the understanding of normal mammary gland development, in: M. Matzuk, C.W. Brown, T.R. Kumar (Eds.), Contemporary Endocrinology: Transgenics in Endocrinology, Humana Press, Inc., Totowa, N.J., 2001 pp. 205–229.
[53] T.A. Rose-Hellekant, L.M. Arendt, M.D. Schroeder, K. Gilchrist, E.P. Sandgren, L.A. Schuler, Prolactin induces ERα-positive and ERα-negative mammary cancer in transgenic mice, Oncogene 22 (2003) 4664–4674.
[54] T.K. Bera, S. Hwang, S.M. Swanson, R.C. Guzman, M. Edery, S. Nandi, In situ localization of prolactin receptor message in the mammary glands of pituitary-isografted mice, Mol. Cell. Biochem. 132 (1994) 145–149.
[55] J. Djiane, P. Durand, Prolactin-progesterone antagonism in self regulation of prolactin receptors in the mammary gland, Nature 266 (1977) 641–643.
[56] Y. Mizoguchi, J.Y. Kim, J. Enami, S. Sakai, The regulation of the prolactin receptor gene expression in the mammary gland of early pregnant mouse, Endocr. J. 44 (1997) 53–58.
[57] G.A. Jahn, M. Edery, L. Belair, P.A. Kelly, J. Djiane, Prolactin receptor gene expression in rat mammary gland and liver during pregnancy and lactation, Endocrinology 128 (1991) 2976–2984.
[58] J. Djiane, P. Durand, P.A. Kelly, Evolution of prolactin receptors in rabbit mammary gland during pregnancy and lactation, Endocrinology 100 (1977) 1348–1356.
[59] N. Genty, J. Paly, M. Edery, P.A. Kelly, J. Djiane, R. Salesse, Endocytosis and degradation of prolactin and its receptor in Chinese hamster ovary cells stably transfected with prolactin receptor cDNA, Mol. Cell. Endocrinol. 99 (1994) 221–228.
[60] J. Djiane, L.M. Housebine, P.A. Kelly, Correlation between prolactin-receptor interaction, down-regulation of receptors, and stimulation of casein and deoxyribonucleic acid biosynthesis in rabbit mammary gland explants, Endocrinology 110 (1982) 791–795.
[61] J. Djiane, P.A. Kelly, L.-M. Houdebine, Effects of lysosomotropic agents, cytochalasin B and colchicine on the “down-regulation” of prolactin receptors in mammary gland explants, Mol. Cell. Endocrinol. 18 (1980) 87–98.
[62] W.E. Simon, V.G. Pahnke, F. Holzel, In vitro modulation of prolactin binding to human mammary carcinoma cells by steroid hormones and prolactin, J. Clin. Endocrinol. Metab. 60 (1985) 1243–1249.
[63] K. Liby, B. Neltner, L. Mohamet, L. Menchen, N. Ben-Jonathan, Prolactin overexpression by MDA-MB-435 human breast cancer cells accelerates tumor growth, Breast Cancer Res. Treat. 79 (2003) 241–252.
[64] E.D. Galsgaard, J.H. Nielsen, A. Moldrup, Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells—prolactin and growth hormone activate one of the rat PRLR gene promoters via STAT5a and STAT5b, J. Biol. Chem. 274 (1999) 18686–18692.
[65] Z.Z. Hu, L. Zhuang, J. Meng, C.H. Tsai-Morris, M.L. Dufau, Complex 5′ genomic structure of the human prolactin receptor: multiple alternative exons 1 and promoter utilization, Endocrinology 143 (2002) 2139–2142.
[66] Z.Z. Hu, L. Zhuang, J.P. Meng, M.L. Dufau, Transcriptional regulation of the generic promoter III of the rat prolactin receptor gene by C/EBPβ and Sp1, J. Biol. Chem. 273 (1998) 26225–26235.
[67] I. Olazabal, J. Munoz, S. Ogueta, E. Obregon, J.P. Garcia-Ruiz, Prolactin (PRL)-PRL receptor system increases cell proliferation involving JNK and AP-1 activation: inhibition by glucocorticoids, Mol. Endocrinol. 14 (2000) 564–575.
[68] M.B. McAlexander, L. Yu-Lee, Sp1 is required for prolactin activation of the interferon regulatory factor-1 gene, Mol. Cell. Endocrinol. 184 (2001) 135–141.
[69] R. Nanbu-Wakao, Y. Fujitani, Y. Masuho, M. Muramatu, H. Wakao, Prolactin enhances CCAAT enhancer-binding protein-β (C/EBPβ) and peroxisome proliferator-activated receptor gamma (PPARgamma) messenger RNA expression and stimulates adipogenic conversion of NIH-3T3 cells, Mol. Endocrinol. 14 (2000) 307–316.
[70] J.-M. Boutin, M. Edery, M. Shirota, C. Jolicoeur, L. Lesueur, S. Ali, D. Gould, J. Djiane, P.A. Kelly, Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells, Mol. Endocrinol. 3 (1989) 1455–1461.
[71] K.K. Kaulsay, H.C. Mertani, J. Tornell, G. Morel, K. Lee, P.E. Lobie, Autocrine stimulation of human mammary carcinoma cell proliferation by human growth hormone, Exp. Cell Res. 250 (1999) 35–50.
[72] M.Y. Lorenson, A.M. Walker, Structure-function relationships in prolactin, in: N.D. Horseman (Ed.), Prolactin, Kluwer Press, Boston, 2001, pp. 189–217.
[73] K.A. Krown, Y.-F. Wang, A.M. Walker, Autocrine interaction between prolactin and its receptor occurs intracellularly in the 235-1 mammotroph cell line, Endocrinology 134 (1994) 1546–1552.
[74] C.V. Clevenger, S.W. Altmann, M.B. Prystowsky, Requirement of nuclear prolactin for interleukin-2 stimulated proliferation of T-lymphocytes, Science 253 (1991) 77–79.
[75] Y.-P. Rao, D.J. Buckley, M.D. Olson, A.R. Buckley, Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells, J. Cell Physiol. 162 (1995) 266–276.
[76] H.S. Wiley, M.F. Woolf, L.K. Opresko, P.M. Burke, B. Will, J.R. Morgan, D.A. Lauffenburger, Removal of the membrane-anchoring domain of epidermal growth factor leads to intracrine signaling and disruption of mammary epithelial cell organization, J. Cell Biol. 143 (1998) 1317–1328.
[77] M.T. Keating, L.T. Williams, Autocrine stimulation of intracellular PDGF receptors in v-sis- transformed cells, Science 239 (1988) 914–916.
[78] R.N. Re, The intracrine hypothesis and intracellular peptide hormone action, BioEssays 25 (2003) 401–409.