*Journal of Dairy Research* (2005) **72** Special Issue 58–65. © Proprietors of Journal of Dairy Research 2005
doi:10.1017/S0022029905001196 Printed in the United Kingdom


# Autocrine prolactin as a promotor of mammary tumour growth

Caroline Manhes, Vincent Goffin*, Paul A Kelly and Philippe Touraine

Université Paris Descartes, Faculté de Médecine, Necker, Inserm, Unit 584, 75730, Paris cedex 15, France

**Keywords:** Prolactin, autocrine, mammary tumour, antagonist, dopamine.

## Prolactin and the mammary gland

Prolactin (PRL) plays a key role in normal growth, development and differentiation of the mammary gland. Indeed, strong evidence suggests that the development of alveolar cells requires not only oestradiol and progesterone, but also PRL. *In vitro*, PRL has mitogenic activity on normal mouse mammary epithelial cells (reviewed in Das & Vonderhaar, 1997). *In vivo*, PRL also seems to be involved in such proliferative activity, although it is more difficult to distinguish the role of PRL from the influence of the hormonal milieu (Das & Vonderhaar, 1997). This physiological role of PRL in lobular development of the mammary gland is supported by results obtained from mice deficient for PRL (Horseman et al. 1997) or for its receptor (PRLR) (Ormandy et al. 1997). Although the infertility of females homozygous for the deletion of the PRLR gene ($PRLR^{-/-}$) can be partially reversed by restoring progesterone levels close to normal, their mammary gland fails to differentiate during pregnancy, leading to lactation failure (Binart et al. 2000). In addition, heterozygous mice ($PRLR^{+/-}$), who have half normal receptor levels, show impaired mammary gland development and fail to lactate following their first pregnancy, clearly indicating that signals mediated by the PRL/PRLR interaction have to achieve a certain level to permit mammary gland differentiation and lactation (Kelly et al. 2002). Since the pioneering work of Topper (Topper, 1970), who observed that PRL was necessary to induce casein synthesis, our understanding of the mechanism of such induction has greatly expanded. PRL appears to be the primary hormone involved in this activity, although other hormones such as insulin and glucocorticoids are also required for lactation. Since the cloning by our group of the cDNA encoding the PRLR (Boutin et al. 1988), the mechanisms by which PRL induces lactation have been deciphered at the molecular level, especially since specific effectors of signal transduction were clearly identified as mediators of PRL functions, e.g., Stat5a and Stat5b genes (Liu et al. 1997; Udy et al. 1997). The complete absence of lactation in $Stat5a^{-/-}$ mice confirmed the major role of such proteins in PRL transduction. Therefore, PRL can be considered as a hormone involved in both normal mammary gland proliferation and differentiation.

*For correspondence; e-mail: goffin@necker.fr

## Pathophysiology of PRL

In contrast to what is observed for other pituitary hormones, no mutation of the PRL gene (or of the PRL receptor) has been described yet, so that there is no clinical model to evaluate clearly the consequences of the absence of PRL actions in humans. Although hypoprolactinaemia is a very rare pathology, such a human model could be helpful in understanding the vast number of biological actions that have been attributed to PRL (Bole-Feysot et al. 1998). However, patients harbouring Pit-1 mutations (the main regulator of PRL gene transcription in the pituitary (Freeman et al. 2000)), suffer from combined hormonal deficiencies (lactotrope, thyrotrope and somatotrope axes are all impaired), and are therefore not very informative with respect to PRL failure *per se* (Brue et al. 1997). Isolated hypoprolactinaemia was recently described in patients unable to lactate, a phenotype correlated with low, or even undetectable PRL levels (Zargar et al. 1997; 2000). Unfortunately, no information was reported regarding the state of mammary gland development. A genetic origin of hypoprolactinaemia was suspected (but not demonstrated) for these patients, since very low PRL levels were also detected in one of the mothers (Zargar et al. 1997). Hyperprolactinaemia is a frequent disease, especially in women. Excess of PRL secretion leads to galactorrhoea, failure of menstrual cycle (amenorrhoea) and sterility, which again emphasizes the close relationships between PRL, gonadotropic functions and mammary gland physiology. Whether hyperprolactinaemia results from pituitary adenomas or is idiopathic, it is efficiently treated by synthetic analogues of dopamine, which is the physiological negative regulator of PRL synthesis and secretion in the pituitary (Ben-Jonathan et al. 1996; Molitch, 2002). The involvement of PRL in auto-immune diseases, such as Systemic Lupus Erythematosus (SLE), has also been suggested, based on the observation that the disease is accentuated *post partum*, when PRL levels are elevated to maintain lactation (Touraine & Kelly, 1997). Nevertheless, PRL levels are not necessarily abnormally

elevated in patients suffering from SLE (McMurray et al. 1994; Pauzner et al. 1994; Neidhart 1996), and the possible correlation between circulating PRL levels and the severity of the disease remains controversial (Jara et al. 1992; Buskila et al. 1996). Finally, although treatment using bromocriptine, a dopamine agonist, sometimes leads to improvement, additional studies are required to confirm this observation (Walker, 2001).

In summary, the implication of PRL in human diseases is mainly based on two criteria: first, the correlation between PRL levels and the pathology of interest; and second, when these data are available, the efficiency of dopaminergic analogues to treat the pathology. Based on these criteria, hyperprolactinaemia is the only pathology unanimously recognized as related to PRL.

## PRL and mammary tumour: from mice to humans

For more than three decades, PRL has been suspected of involvement in tumour proliferation. As the main target tissue of PRL, the breast has been used as the prototype model to investigate the tumour growth-promoting potency of PRL. Interestingly, conclusions similar to those described below have also been reported for various models of prostate tumour, which emphasizes that the growth-promoting effects of PRL are not limited to the mammary gland. Given the focus of *Journal of Dairy Research* on the mammary gland, the arguments for PRL involvement in prostate tumours will not be developed in this article.

Figure 1 shows that recombinant human PRL stimulates the proliferation of normal human mammary epithelial cells. These experiments were performed using primary cultures established from tissue freshly harvested from young women undergoing mammary reduction. The growth-promoting activity of hPRL is reflected in the dose-dependent increased expression of the proliferation antigen PCNA and of the cell cycle protein cyclin D1. As reported for breast cancer cell lines (Brockman et al. 2002; Acosta et al. 2003), MAP kinases Erk 1 and 2 were also activated. Thus, PRL is intrinsically a mitogen for normal mammary cells. This proliferative activity has also been clearly demonstrated in tumour contexts, using classical breast cancer cell lines (Vonderhaar, 1998; Goffin et al. 1999; Vonderhaar, 1999; Llovera et al. 2000a; Clevenger et al. 2003). Various animal models have further strengthened the pro-tumour role of PRL *in vivo*, e.g., by demonstrating the growth-promoting action of exogenous PRL on spontaneous or carcinogen-induced mammary tumours (Nandi et al. 1995; Ben Jonathan et al. 2002), or the growth-inhibiting action of dopamine agonists in similar models (Welsch & Nagasawa, 1977; Welsch et al. 1977). More recently, transgenic mice over-expressing systemic PRL were generated; these animals develop mammary neoplasia, which appears approximately at the middle of lifespan (Wennbo et al. 1997). In PRL-deficient mice

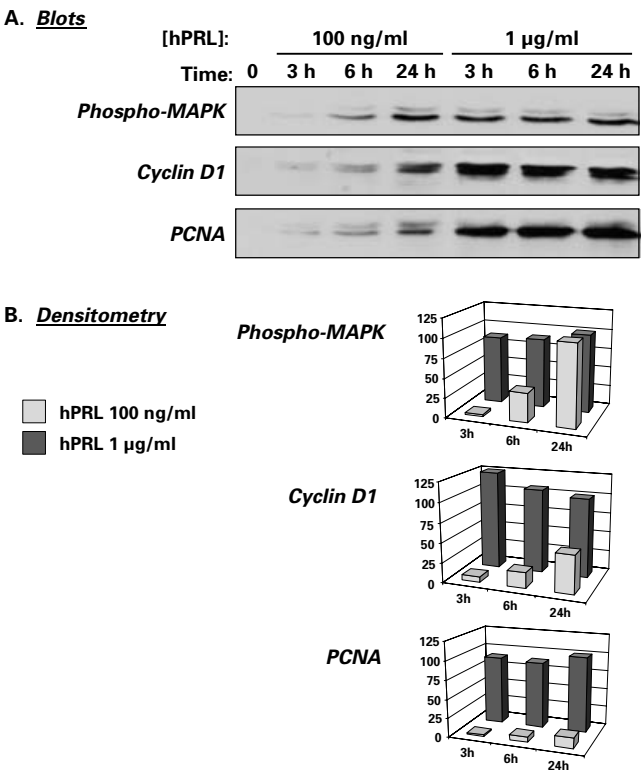


**Fig. 1.** Human PRL stimulates the proliferation of normal human mammary epithelial cells. Primary cultures of mammary epithelial cells were established from tissue freshly harvested from young women undergoing mammary reduction, as earlier described (Malet et al. 1991). Two hundred thousand cells (per well of 24-well plates) were treated with recombinant hPRL (100 ng/ml or 1 µg/ml) for the times indicated. Cell lysates were obtained as previously described (Llovera et al. 2000a). Thirty µg of proteins were separated on 10% SDS-PAGE, then transferred onto nitrocellulose membranes that were blotted using anti-phospho MAP kinase antibody (1:1000, Cell Signaling), anti-cyclin D1 (1:500, Santa-Cruz Biotechnology) and anti-PCNA (1:500, Dako), and revelation was performed using appropriate HRP-conjugated anti-immunoglobulin antibodies. **A.** The action of hPRL on mammary cells is assessed by the activation of the MAP kinase pathway. The growth-promoting activity of hPRL is reflected in the dose-dependent increase of expression of the proliferation antigen PCNA, which correlates up-regulation of cyclin D1 expression. **B.** This panel represents densitometric analysis of the blots shown in panel A (performed using The Kodak Digital Science™ 1D software). Band intensities were normalized for each antigen, taking as 100% the value obtained after 24 h under stimulation with 1 µg/ml. Using 100 ng/ml hPRL, it is clear that the activation of MAP kinase pathways preceeds the up-regulation of cyclin D1 expression, which itself preceeds that of PCNA, although this does not establish a link between these pathways. Concentration of 1 µg/ml induces maximal effects in all the conditions tested.

(knockout), the appearance of virus-induced mammary tumours was delayed (Vomachka et al. 2000), arguing for the permissive role of PRL in tumour growth. Accordingly, human mammary tumour cell lines xenografted into

https://doi.org/10.1017/S0022029905001196 Published online by Cambridge University Press

immunodeficient mice grow more rapidly when they over-express hPRL after transfection (Liby et al. 2003). Thus, PRL appears to be a pro-tumour factor in a wide variety of models (Ahonen et al. 1999; Clevenger et al. 2003). Multiple mechanisms could be involved, since PRL acts on cell division (Biswas & Vonderhaar, 1987; Llovera et al. 2000a; Schroeder et al. 2002), cell survival (anti-apoptotic effect) (Chen et al. 1999; Beck et al. 2002; Perks et al. 2004), and cell motility (Maus et al. 1999); (reviewed in Clevenger et al. 2003). Finally, given the major role of steroid hormones (oestrogens) in mammary tumour development, whether PRL and steroid hormones act in concert to promote tumour growth was also investigated. It was shown that receptors for oestradiol (ER), progesterone (PR) and PRL are mutually regulated in human mammary tumour cell lines (Ormandy et al. 1997), suggesting that synergic effects between these hormones can be suspected in the context of tumour growth. Finally, it must be noted that the pro-tumour action of PRL does not require the presence of steroids, since the proliferative role of PRL is also observed in oestrogen receptor-negative (ER−) mammary cell lines (Fuh & Wells, 1995; Llovera et al. 2000a), which is in agreement with the fact that mammary tumours appearing in PRL transgenic mice are not necessarily ER+ (Rose-Hellekant et al. 2003).

Although these numerous experimental data argue convincingly for the tumour growth-promoting potency of PRL, one must be careful in making straightforward transposition to the human context, since the two classical criteria to assess the PRL-dependence of a given disease (see above) are ambiguous with respect to breast cancer. Indeed, in the few clinical trials involving breast cancer patients, dopamine agonists showed a lack of effect in terms of long-term survival (reviewed in Clevenger et al. 2003). Although these studies can be criticized for their methodology or for the small number of patients evaluated, they are often cited as reference articles in the absence of any other studies. Moreover, epidemiological studies investigating PRL levels as a possible risk factor for the development of human breast cancer are somewhat limited, and for most, conclusions were ambiguous (Clevenger et al. 2003). Only two recent epidemiological studies involving the Nurse Health Cohort (>30 000 women) indicated a correlation between PRL levels and breast cancer risk in post-menopausal women. The first study correlated PRL levels in the higher quartile of the normal range with an increased risk (by a factor of 2) of developing breast cancer compared with the lower quartile (Hankinson et al. 1999) and the second study showed that this risk mainly affected ER+ tumours (relative risk of 1·78, 95% confidence interval 1·28–2·5) (Tworoger et al. 2004). Unfortunately, we are not aware of any large-scale study investigating whether the risk of developing breast cancers is increased in hyperprolactinaemic patients, or in those patients whose PRL levels could not be normalized after treatment with a dopamine agonist.

## Autocrine PRL and mammary tumor: from humans to mice

All these observations appear to lead to a contradiction. Whereas the tumour growth-promoting action of PRL is well demonstrated in several experimental models (including those involving human cells), the relationship between breast tumours and PRL levels, or any beneficial action of dopamine agonists, remains fragile at best in humans. This controversy led several laboratories, including ours, to suspect that other parameters should be considered in accurately evaluating the involvement of PRL in human breast cancer (Clevenger & Plank, 1997; Das & Vonderhaar, 1997; Goffin et al. 1999).

Ten years ago, Vonderhaar and colleagues demonstrated that PRL is synthesized by human mammary cell lines, and that neutralizing anti-PRL antibodies inhibits cell proliferation (Ginsburg & Vonderhaar, 1995). These observations were the first to suggest the existence of an autocrine-paracrine loop stimulating cell proliferation in mammary tumour cells, which was recently confirmed by generating PRL-deficient breast cancer cell lines (Brockman et al. 2002; Schroeder et al. 2002). At the same time, Clevenger and colleagues demonstrated that co-expression of hPRL and of its receptor was not restricted to breast cancer cell lines (which are usually considered as highly derived models) but was also observed in human mammary tumour biopsies (Clevenger et al. 1995; Reynolds et al. 1997). Expression of hPRL and of its receptor is also detected in normal mammary tissue, which indicates that the autocrine-paracrine loop is physiologically relevant, and cannot *per se* explain the pro-tumour action of local hPRL (Touraine et al. 1998). The presence of the hPRLR (mRNA, protein) was detected in 98% of mammary tumour biopsies (Reynolds et al. 1997; Touraine et al. 1998). Our quantitative analysis indicated that although PRLR mRNA expression is highly variable from one patient to another, it was always higher in tumour compared with normal tissue; this suggests that the tumour cells could be more sensitive/responsive to the action of (autocrine) PRL (Touraine et al. 1998). Although the increased expression of the receptor in tumours was not confirmed in some other studies (Mertani et al. 1998), gene expression profiling recently identified the PRLR as one of the genes over-expressed in subsets of breast cancer patients (Bertucci et al. 2002). To the best of our knowledge, quantitative analysis of hPRL expression in breast tumour (*v.* normal tissue) has not been reported.

In summary, these studies suggest that the growth-promoting action of autocrine PRL in breast tumours might, at least in part, involve over-expression of the PRL receptor. In addition, a very recent study from Dufau's group indicated that the ratio between short and long isoforms of the human PRLR was lower in tumours, suggesting that not only the total amount of PRLR, but also the nature of the isoforms expressed, could differ in pathological states (Meng et al. 2004). Since short hPRLR

isoforms were reported to act as dominant-negative against the long isoform (Hu et al. 2001; Trott et al. 2003), their lower expression leads to the hypothesis that PRL sensitivity could be increased in breast tumours. Human PRL activates many signalling pathways in target cells, leading to cell proliferation (Bole-Feysot et al. 1998). Recent reports indicate that signalling pathways activated by autocrine PRL cross-talk with others, e.g., by activating the ErbB2 receptor which also stimulates proliferation (Yamauchi et al. 2000). Finally, autocrine PRL, but not exogenous PRL, was shown to induce expression of ERalpha which, in turn, increases cell responsiveness to oestrogens (Gutzman et al. 2004). All these data provide evidence that the autocrine/paracrine loop involving PRL participates in the proliferation of breast tumours (Clevenger & Plank, 1997; Vonderhaar, 1998; Goffin et al. 1999; Llovera et al. 2000b; Clevenger et al. 2003).

One major difference between mouse models and human tissues is that PRL is permanently expressed (i.e., independently of physiological status) in human breast, whereas it is at best restricted to gestation/lactation in mouse mammary tissue (Clevenger et al. 2003). Accordingly, a recent study involving transplantation of wild-type or PRL-deficient mouse mammary epithelium into immunodeficient mice showed reduced proliferation at the end of pregnancy in the latter, arguing for the proliferative role of autocrine PRL in a normal context (Naylor et al. 2003). To mimic the human situation in animal models, mammary-specific transgenic mice were generated recently by Schuler's group (Rose-Hellekant et al. 2003). Expression of the PRL coding sequence was under the control of the hormonally non-responsive promoter from neu-related lipocalin (NRL). NRL-PRL transgenic virgin females display various developmental abnormalities of the mammary gland, including mammary intraepithelial neoplasias and invasive neoplasms. PRL increased proliferation in morphologically normal alveoli and ducts, as well as in tumour lesions. Interestingly, both ER+ and ER− tumours were reported, indicating the complexity of PRL actions. The occurrence of a neoplastic mammary phenotype in these mice is reminiscent of what was reported earlier in transgenic mice expressing systemic PRL under the control of the ubiquitous metallothionein promoter (Wennbo et al. 1997), which further demonstrates the pro-tumour potency of autocrine PRL *in vivo*. Our laboratory, in collaboration with the group of JJ Kopchick, has recently developed a transgenic model in which the hPRL transgene is controlled by another mammary-specific promoter, namely that from the milk protein WAP (whey acidic protein). This recombinant system provides not only a mammary-specific overexpression of hPRL, but also a time-dependent expression, which encompasses the last third of gestation and lactation. It may be noticed that weak expression of WAP-driven transgenes has been reported in some tissues other than the mammary gland, such as pituitary or pancreas (Pittius et al. 1988); this possibility is under investigation with respect to our model. Interestingly, sporadic over-expression of PRL in the mammary gland leads to various functional and morphological phenotypes of this tissue. Histological analysis of young and mid-age animals (<1 year) identified the appearance of various lesions diagnosed as benign tumours, with abnormalities aggravating with successive pregnancies. We are awaiting the availability of older animals to determine whether these benign tumours evolve towards malignant tumours, as reported for the above-mentioned transgenic animals in which expression of the PRL transgene is permanent (Wennbo et al. 1997; Rose-Hellekant et al. 2003). If not, this may indicate that the development of mammary cancer requires long-term (permanent) exposure to elevated PRL levels. Alternatively, the difference of mammary phenotypes between these models might also relate to the intrinsic characteristics of the experimental models used by the investigators, e.g., the genetic background. Obviously, detailed analysis of this new animal model will help elucidate the actions of autocrine PRL in the mammary gland. In particular, the development of PRL-induced benign mammary tumours might be an interesting model to understand better the development of similar pathologies in humans, whose aetiology remains completely unknown (Hughues, 2000; Ryska et al. 2001).

## Inhibition of autocrine PRL in mammary tumours

In humans, the autocrine/paracrine loop can be proposed as a possible explanation for the discrepancy between the weighty experimental arguments and the lack of clinical evidence with respect to the involvement of PRL in breast cancer. Indeed, autocrine PRL is secreted into its local environment and is thus assumed to not (or only very modestly) contribute to circulating PRL levels. This parameter has been totally ignored in epidemiological studies, which only take into account serum PRL levels. Moreover, since dopamine does not regulate PRL synthesis in extrapituitary tissues (Ben-Jonathan et al. 1996), dopamine analogues used in clinical trials involving breast cancer patients presumably failed to affect autocrine PRL expression. The hypothesis that local PRL participates in the proliferation of breast tumours has become a major axis of research in many well established laboratories in the field (Ben Jonathan et al. 2002; Llovera et al. 2000b; Clevenger et al. 2003; Gutzman et al. 2004). Although a number of highly respected articles have been published in the last decade, the relative contribution of local *v.* systemic PRL to the growth of breast tumours *in vivo* remains unknown. Thus, the autocrine mechanism of action still needs more study to be better understood, and most importantly, to be definitely accepted as a relevant factor involved in the promotion of tumour growth in humans. This latter objective has been hindered by the absence of any therapeutic molecule able to inhibit the expression of PRL in tissues other than the pituitary. In

https://doi.org/10.1017/S0022029905001196 Published online by Cambridge University Press

humans, but not in animals, the PRL gene is regulated at the transcriptional level by two distinct promoters (Ben-Jonathan et al. 1996; Baudhuin et al. 2002). Conventional wisdom has linked the proximal promoter to hPRL expression in the pituitary (involving Pit-1 as major activating transcription factor and dopamine as major negative regulator) and the more distal promoter to extrapituitary hPRL expression (independent of Pit-1 and dopamine). Such a dichotomy of promoter usage is probably a simplified view of what really occurs *in vivo* (Shaw-Bruha et al. 1997). In addition, although various hormones, peptides or neurotransmitters were evaluated for their ability to regulate PRL synthesis in extrapituitary sites, none of them seems to exert a major role on PRL production similar to that of dopamine in the pituitary (Ben Jonathan et al. 2002).

The increasing evidence that locally produced, perhaps even more than pituitary-produced PRL, might play a key role in promoting tumour growth, encouraged the search for alternative strategies to counteract this proliferative effect. One such approach is the development of specific antagonists, acting at the level of PRL receptor activation, instead of PRL production. Based on the mechanism of activation of the PRLR by homodimerization (Fig. 2A), we designed some time ago the first prototypes of receptor antagonists, by introducing steric mutations (substitute Arg for Gly 129) within the second binding site of the hormone to its receptor (Fig. 2B). The development and characterization of so-called G129R-hPRL analogue has been extensively described (Kinet et al. 2001; Goffin et al. 2003). Although acting as an antagonist in many systems, this analogue maintains residual agonistic activity in sensitive bioassays, as well as *in vivo*. We then developed second generation anatgonists by deleting the 9 N-terminal residues in G129R-hPRL, generating so-called delta1–9G129R-hPRL. This new hPRL analogue was shown to be devoid of residual agonistic activity, thereby acting as a pure antagonist in various routine bioassays (proliferation of lymphoid cells, reporter-gene assay, etc; Bernichtein et al. 2003b; Goffin et al. 2003). With the perspective to develop a potential drug for the treatment of breast cancer, it is extremely important to demonstrate that delta1–9-G129R-hPRL also acts as a pure antagonist on mammary tumour cells. To that end, we used mouse mammary tumour (MMT) cells. As shown in Fig. 3 (panel A), recombinant hPRL induces the proliferation of these cells at low concentrations, with the typical bell-shaped curve observed in various bioassays, which reflects PRL self-antagonism at higher PRL concentration (Bernichtein et al. 2003a). As expected, MMT cells do not express endogenous mouse PRL (Fig 3B). Therefore, we could generate stable clones expressing WT hPRL or delta1-9-G129R-hPRL, without interference with endogenously produced mouse PRL. A clone transfected using an ''empty'' vector was also generated as a control. The expression level of autocrine hPRL (or antagonist) was assessed by immunoblotting (Fig. 3C), and by measuring the concentration of

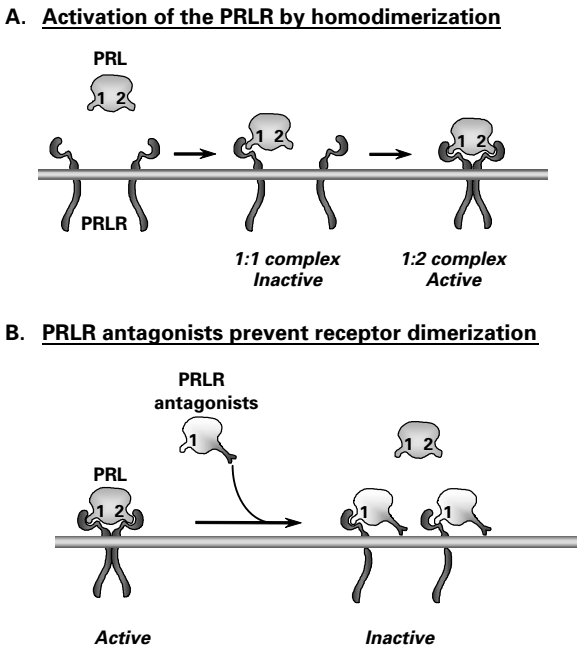


**Fig. 2.** Mechanism of action of PRLR antagonists. **A.** The PRL receptor is assumed to be activated in two steps: first, one molecule of PRL binds to one molecule of receptor through its binding site 1, leading to an inactive 1 : 1 complex. Then, this intermediary complex binds to a second molecule of receptor through the hormone binding site 2, leading to the formation of an active trimeric 1 : 2 complex. **B.** In PRLR antagonists, binding site 2 is hindered by a steric mutation (e.g., Arg substituted for Gly in G129R-hPRL antagonists). Therefore, such analogues are unable to induce PRLR dimerization, and in competition experiments, they prevent PRL to activate its receptor.

PRL in conditioned media, using a human PRL-specific ELISA (Bernichtein et al. 2003b). Good correlation was obtained between band intensities in immunoblots and ELISA measurements. The biological activity of secreted hPRL was demonstrated by its ability to stimulate the proliferation of PRL-responsive Ba/F03 cells expressing the human PRLR (Bernichtein et al. 2003a) whereas the antagonist failed to have any effect, even at the highest concentration that we could analyse (Fig. 3D). Finally, the intrinsic proliferation rates of MMT clones were estimated (Fig. 3E). In good agreement with panel A, which shows that self-antagonism occurs at low hPRL concentration (30 ng/ml), the clone secreting the highest amount of hPRL (clone 7) exhibited lower proliferation rate compared with clone 10. In contrast, the proliferation rates of clones expressing delta1–9-G129R-hPRL was at best equivalent to that of control cells. Since it is known that very low amounts of hormone produced locally elicit biological responses, MMT cell proliferation can be considered as a very sensitive bioassay (Bernichtein et al. 2003a). These data are thus in good agreement with previous findings showing that delta1–9-G129R-hPRL is devoid of any residual agonistic activity.

https://doi.org/10.1017/S0022029905001196 Published online by Cambridge University Press

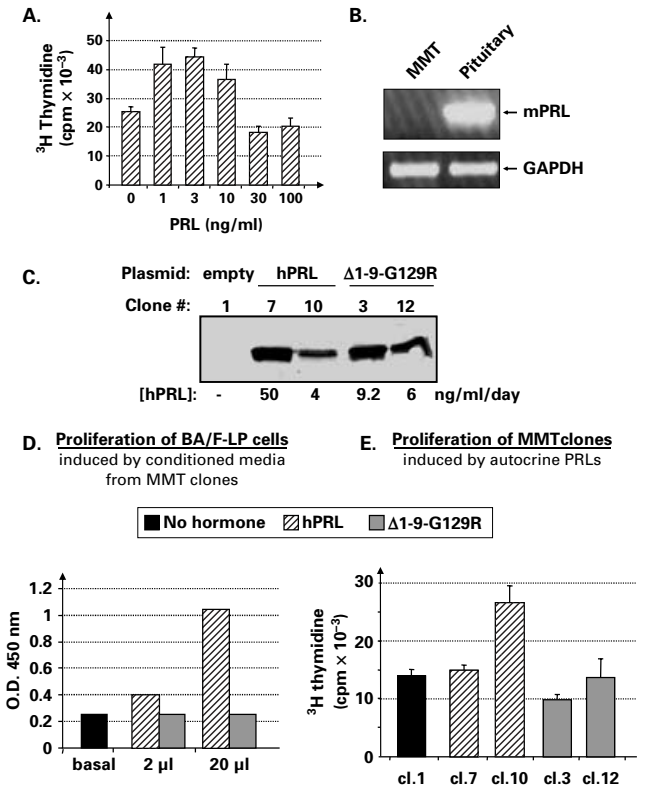


**Fig. 3.** Effect of autocrine PRL (wild type or antagonist) on the proliferation of mouse mammary tumour (MMT) cells. **A.** Cell proliferation (20 000 MMT cells per well in a 24-well plate) was assessed by a classical method of thymidine incorporation, using 1 µCi $^3$H-thymidine per ml for 4 h (performed in quadruplicate). Exogenous recombinant hPRL induces the proliferation of MMT cells at low concentrations, with typical self-antagonistic effect (bell-shaped curve) (Bernichtein et al. 2003a). **B.** RT-PCR using specific primers in mouse PRL coding sequence assessed that MMT cells do not express endogenous mouse PRL; GAPDH was used as positive control of the reaction. **C.** Parental MMT cells were transfected using plasmids encoding WT hPRL or delta1–9-G129R-hPRL, and stable clones were selected after antibiotic selection as previously described (Bernichtein et al. 2003a). A control clone transfected using the parental pCDN3 eukaryotic vector (''empty'' vector) was also generated as a control. The expression of autocrine hPRL (WT or antagonist) by the various clones was confirmed by immunoblotting (using polyclonal anti-hPRL from DAKO), and the concentration of PRL expression in conditioned media was determined using a hPRL-specific ELISA, as reported before (Bernichtein et al. 2003b). **D.** The biological activity of secreted hPRL was determined by testing the ability of conditioned media to stimulate the proliferation of Ba/F03 cells expressing the human PRLR; cell proliferation was monitored using the Wst-1 colourimetric assay (optical absorbance at 450 nm), following routine protocols earlier reported (Bernichtein et al. 2003a). Whereas conditioned medium containing hPRL induced cell proliferation dose-dependently, no effect could be obtained for delta1–9-G129R-hPRL. **E.** Finally, the intrinsic proliferation rates of the various MMT clones were estimated by $^3$H-thymidine incorporation as indicated above (10 000 cells per well were plated in a 24-well plate, and incubated for 24 h in 1 ml of DMEM 0·1% fetal bovine serum). With respect to clones producing hPRL, proliferation rates were inversely correlated with the amount of PRL secreted (clones 7 and 10). None of the clones expressing delta1–9-G129R-hPRL proliferated more rapidly than control cells.

The next challenge is to confirm that these PRL antagonists are potent anti-tumour molecules in long-term studies, which will be performed by analysing double transgenic mice expressing autocrine PRL and systemic PRL antagonists, mimicking the situation of patients treated with the PRLR antagonist, administered by injection. These studies are ongoing in our group.

The authors are grateful to Christine Kayser for excellent technical assistance, Catherine Malet for help with primary cultures of normal human mammary epithelial cells, and Drs John Kopchick and Bruce Kelder for the generation of transgenic mice.

## References

**Acosta JJ, Munoz RM, Gonzalez L, Subtil-Rodriguez A, Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Lazaro-Trueba I & Martin-Perez J** 2003 Src mediates prolactin-dependent proliferation of T47D and MCF7 cells via the activation of focal adhesion kinase/Erk1/2 and phosphatidylinositol 3-kinase pathways. *Molecular Endocrinology* **17** 2268–2282

**Ahonen TJ, Harkonen PL, Laine J, Rui H, Martikainen PM & Nevalainen MT** 1999 Prolactin is a survival factor for androgen-deprived rat dorsal and lateral prostate epithelium in organ culture. *Endocrinology* **140** 5412–5421

**Baudhuin A, Manfroid I, Van De WC, Martial JA & Muller M** 2002 Transcription of the human prolactin gene in mammary cells. *Annals of the New York Academy of Sciences* **973** 454–458

**Beck MT, Peirce SK & Chen WY** 2002 Regulation of bcl-2 gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R. *Oncogene* **21** 5047–5055

**Ben Jonathan N, Liby K, McFarland M & Zinger M** 2002 Prolactin as an autocrine/paracrine growth factor in human cancer. *Trends in Endocrinology and Metabolism* **13** 245–250

**Ben-Jonathan N, Mershon JL, Allen DL & Steinmetz RW** 1996 Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. *Endocrinology Reviews* **17** 639–669

**Bernichtein S, Jeay S, Vaudry R, Kelly PA, Goffin V** 2003a New homologous bioassays for human lactogens show that agonism or antagonism of various analogs is a function of assay sensitivity. *Endocrine* **20** 177–190

**Bernichtein S, Kayser C, Dillnerm K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA & Goffin V** 2003b Development of pure prolactin receptor antagonists. *Journal of Biological Chemistry* **278** 35988–35999

**Bertucci F, Nasser V, Granjeaud S, Eisinger F, Adelaide J, Tagett R, Loriod B, Giaconia A, Benziane A, Devilard E, Jacquemier J, Viens P, Nguyen C, Birnbaum D & Houlgatte R** 2002 Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. *Human MolecularGenetics* **11** 863–872

**Binart N, Helloco C, Ormandy CJ, Barra J, Clement-Lacroix P, Baran N & Kelly PA** 2000 Rescue of preimplantatory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* **141** 2691–2697

**Biswas R & Vonderhaar BK** 1987 Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. *Cancer Research* **47** 3509–3514

https://doi.org/10.1017/S0022029905001196 Published online by Cambridge University Press

**Bole-Feysot C, Goffin V, Edery M, Binart N & Kelly PA** 1998 Prolactin and its receptor: actions, signal transduction pathways and phenotypes observed in prolactin receptor knockout mice. *Endocrinology Reviews* **19** 225–268

**Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J & Kelly PA** 1988 Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. *Cell* **53** 69–77

**Brockman JL, Schroeder MD & Schuler LA** 2002 PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Molecular Endocrinology* **16** 774–784

**Brue T, Vallette S, Pellegrini-Bouiller I & Enjalbert A** 1997 Congenital multiple anterior pituitary hormone deficiencies. An approach of pituitary ontogenesis. *Annals of Endocrinology* **58** 436–450

**Buskila D, Lorber M, Neumann L, Flusser D & Shoenfeld Y** 1996 No correlation between prolactin levels and clinical activity in patients with systemic lupus erythematosus. *Journal of Rheumatology* **23** 629–632

**Chen WY, Ramamoorthy P, Chen N, Sticca R & Wagner TE** 1999 A human prolactin antagonist, hPRL-G129R, inhibits breast cancer cell proliferation through induction of apoptosis. *Clinical Cancer Research* **5** 3583–3593

**Clevenger CV, Chang WP, Ngo W, Pasha TM, Montone KT & Tomaszewski JE** 1995 Expression of prolactin and prolactin receptor in human breast carcinoma. *American Journal of Pathology* **146** 695–705

**Clevenger CV, Furth PA, Hankinson SE & Schuler LA** 2003 The role of prolactin in mammary carcinoma. *Endocrinology Reviews* **24** 1–27

**Clevenger CV & Plank TL** 1997 Prolactin as an autocrine/paracrine factor in breast cancer. *Journal of Mammary Gland. Biology and Neoplasia* **2** 59–68

**Das R & Vonderhaar BK** 1997 Prolactin as a mitogen in mammary cells. *Journal of Mammary Gland Biology and Neoplasia* **2** 29–39

**Freeman ME, Kanyicska B, Lerant A & Nagy G** 2000 Prolactin: structure, function, and regulation of secretion. *Physiological Reviews* **80** 1523–1631

**Fuh G & Wells JA** 1995 Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines. *Journal of Biological Chemistry* **270** 13133–13137

**Ginsburg E & Vonderhaar BK** 1995 Prolactin synthesis and secretion by human breast cancer cells. *Cancer Research* **55** 2591–2595

**Goffin V, Bernichtein S, Kayser C & Kelly PA** 2003 Development of new prolactin analogs acting as pure prolactin receptor antagonists. *Pituitary* **6** 89–95

**Goffin V, Touraine P, Pichard C, Bernichtein S & Kelly PA** 1999 Should prolactin be reconsidered as a therapeutic target in human breast cancer? *Molecular and Cellular Endocrinology* **151** 79–87

**Gutzman JH, Miller KK & Schuler LA** 2004 Endogenous human prolactin and not exogenous human prolactin induces estrogen receptor alpha and prolactin receptor expression and increases estrogen responsiveness in breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **88** 69–77

**Hankinson SE, Willett WC, Michaud DS, Manson JE, Colditz GA, Longcope C, Rosner B & Speizer FE** 1999 Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. *Journal of the National Cancer Institute* **91** 629–634

**Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E & Dorshkind K** 1997 Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO Journal* **16** 6926–6935

**Hu ZZ, Meng J & Dufau ML** 2001 Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. *Journal of Biological Chemistry* **276** 41086–41094

**Hughues LE** 2000 Fibroadenomas and related tumors. In: *Benign Disorders and Diseases of the Breast. Concepts and Clinical Management*, (Eds LE Hughues, RE Mansel & DJT Webster) pp. 73–94. London: WB Saunders

**Jara LJ, Gomez-Sanchez C, Silveira LH, Martinez-Osuna P, Vasey FB & Espinoza LR** 1992 Hyperprolactinemia in systemic lupus erythematosus: association with disease activity. *American Journal of Medical Science* **303** 222–226

**Kelly PA, Bachelot A, Kedzia C, Hennighausen L, Ormandy CJ, Kopchick JJ & Binart N** 2002 The role of prolactin and growth hormone in mammary gland development. *Molecular and Cellular Endocrinology* **197** 127–131

**Kinet S, Bernichtein S, Llovera M, Kelly PA, Martial JA & Goffin V** 2001 Molecular basis of the interaction between human prolactin and its membrane receptor: a ten year study. *Recent Research in Developmental Endocrinology* **2** 1–24

**Liby K, Neltner B, Mohamet L, Menchen L & Ben Jonathan N** 2003 Prolactin overexpression by MDA-MB-435 human breast cancer cells accelerates tumor growth. *Breast Cancer Research and Treatment* **79** 241–252

**Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A & Hennighansen L** 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes and Development* **11** 179–186

**Llovera M, Pichard C, Bernichtein S, Jeay S, Touraine P, Kelly PA & Goffin V** 2000a Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation. *Oncogene* **19** 4695–4705

**Llovera M, Touraine P, Kelly PA & Goffin V** 2000b Involvement of prolactin in breast cancer: redefining the molecular targets. *Experimental Gerontology* **35** 41–51

**Malet C, Gompel A, Yaneva H, Cren H, Fidji N, Mowszowicz I, Kuttenn F & Mauvais-Jarvis P** 1991 Estradiol and progesterone receptors in cultured normal human breast epithelial cells and fibroblasts: immunocytochemical studies. *Journal of Clinical Endocrinology and Metabolism* **73** 8–17

**Maus MV, Reilly SC & Clevenger CV** 1999 Prolactin as a chemoattractant for human breast carcinoma. *Endocrinology* **140** 5447–5450

**McMurray RW, Allen SH, Braun AL, Rodriguez F & Walker SE** 1994 Longstanding hyperprolactinemia associated with systemic lupus erythematosus: possible hormonal stimulation of an autoimmune disease. *Journal of Rheumatology* **21** 843–850

**Meng J, Tsai-Morris CH & Dufau ML** 2004 Human prolactin receptor variants in breast cancer: low ratio of short forms to the long-form human prolactin receptor associated with mammary carcinoma. *Cancer Research* **64** 5677–5682

**Mertani HC, Garcia-Caballero T, Lambert A, Gerard F, Palayer C, Boutin JM, Vonderhaar BK, Waters MJ, Lobie PE & Morel G** 1998 Cellular expression of growth hormone and prolactin receptors in human breast disorders. *International Journal of Cancer* **79** 202–211

**Molitch ME** 2002 Medical management of prolactin-secreting pituitary adenomas. *Pituitary* **5** 55–65

**Nandi S, Guzman RC & Yang J** 1995 Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proceedings of the National Academy of Sciences of the USA* **92** 3650–3657

**Naylor MJ, Lockefeer JA, Horseman ND & Ormandy CJ** 2003 Prolactin regulates mammary epithelial cell proliferation via autocrine/paracrine mechanism. *Endocrine* **20** 111–114

**Neidhart M** 1996 Elevated serum prolactin or elevated prolactin/cortisol ratio are associated with autoimmune processes in systemic lupus erythematosus and other connective tissue diseases. *Journal of Rheumatology* **23** 476–481

**Ormandy CJ, Hall RE, Manning DL, Robertson JF, Blamey RW, Kelly PA, Nicholson RI & Sutherland RL** 1997 Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer. *Journal of Clinical Endocrinology and Metabolism* **82** 3692–3699

**Pauzner R, Urowitz MB, Gladman DD & Gough JM** 1994 Prolactin in systemic lupus erythematosus. *Journal of Rheumatology* **21** 2064–2067

**Perks CM, Keith AJ, Goodhew KL, Savage PB, Winters ZE & Holly JM** 2004 Prolactin acts as a potent survival factor for human breast cancer cell lines. *British Journal of Cancer* **91** 305–311

**Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J & Gordon K** 1988 A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. *Proceedings of the National Academy of Sciences of the USA* **85** 5874–5878

**Reynolds C, Montone KT, Powell CM, Tomaszewski JE & Clevenger CV** 1997 Expression of prolactin and its receptor in human breast carcinoma. *Endocrinology* **138** 5555–5560

**Rose-Hellekant TA, Arendt LM, Schroeder MD, Gilchrist K, Sandgren EP & Schuler LA** 2003 Prolactin induces ERalpha-positive and ERalpha-negative mammary cancer in transgenic mice. *Oncogene* **22** 4664–4674

**Ryska A, Reynolds C & Keeney GL** 2001 Benign tumors of the breast with multinucleated stromal giant cells. Immunohistochemical analysis of six cases and review of the literature. *Virchows Archives* **439** 768–775

**Schroeder MD, Symowicz J & Schuler LA** 2002 PRL modulates cell cycle regulators in mammary tumor epithelial cells. *Molecular Endocrinology* **16** 45–57

**Shaw-Bruha CM, Pirrucello SJ & Shull JD** 1997 Expression of the prolactin gene in normal and neoplastic human breast tissues and human mammary cell lines: promoter usage and alternative mRNA splicing. *Breast Cancer Research and Treatment* **44** 243–253

**Topper YJ** 1970 Multiple hormone interactions in the development of mammary gland in vitro. *Recent Progress in Hormone Research* **26** 287–308

**Touraine P & Kelly PA** 1997 Physiologie de la prolactine et mécanismes d'action. In: *Médecine de la Reproduction* pp. 115–136 (Eds P Mauvais-Jarvis, G Schaison & P Touraine). Paris: Flammarion

**Touraine P, Martini JF, Zafrani B, Durand JC, Labaille F, Malet C, Nicolas A, Trivin C, Postel-Vinay MC, Kuttenn F & Kelly PA** 1998 Increased expression of prolactin receptor gene assessed by quantitative polymerase chain reaction in human breast tumors versus normal breast tissues. *Journal of Clinical Endocrinology and Metabolism* **83** 667–674

**Trott JF, Hovey RC, Koduri S, Vonderhaar BK** 2003 Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *Journal of Molecular Endocrinology* **30** 31–47

**Tworoger SS, Eliassen AH, Rosner B, Sluss P & Hankinson SE** 2004 Plasma prolactin concentrations and risk of postmenopausal breast cancer. *Cancer Research* **64** 6814–6819

**Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ & Davey HW** 1997 Requirement of Stat5b for Sexual dimorphism of body growth rates and liver gene expression. *Proceedings of the National Academy of Sciences of the USA* **94** 7239–7244

**Vomachka AJ, Pratt SL, Lockefeer JA & Horseman ND** 2000 Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth. *Oncogene* **19** 1077–1084

**Vonderhaar BK** 1998 Prolactin: the forgotten hormone of human breast cancer. *Pharmacological Therapy* **79** 169–178

**Vonderhaar BK** 1999 Prolactin involvement in breast cancer. *Endocrine-Related Cancer* **6** 389–404

**Walker SE** 2001 Treatment of systemic lupus erythematosus with bromocriptine. *Lupus* **10** 197–202

**Welsch CW, Lambrecht LK & Hassett CC** 1977 Suppression of mammary tumorigenesis in C3H/He mice by ovariectomy or treatment with 2-bromo-alpha-ergocryptine: a comparison. *Journal of the National Cancer Institute* **58** 1135–1138

**Welsch CW & Nagasawa H** 1977 Prolactin and murine mammary tumorigenesis: a review. *Cancer Research* **37** 951–963

**Wennbo H, Gebre-Medhin M, Gritli-Linde A, Ohlsson C, Isaksson OG & Tornell J** 1997 Activation of the prolactin receptor but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice. *Journal of Clinical Investigation* **100** 2744–2751

**Yamauchi T, Yamauchi N, Ueki K, Sugiyama T, Waki H, Miki H, Tobe K, Matsuda S, Tsushima T, Yamamoto T, Fujita T, Taketani Y, Fukayama M, Kimura S, Yazaki Y, Nagai R & Kadowaki T** 2000 Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. *Journal of Biological Chemistry* **275** 33937–33944

**Zargar AH, Masoodi SR, Laway BA, Shah NA & Salahudin M** 1997 Familial puerperal alactogenesis: possibility of a genetically transmitted isolated prolactin deficiency. *British Journal of Obstetrics and Gynaecology* **104** 629–631

**Zargar AH, Salahuddin M, Laway BA, Masoodi SR, Ganie MA & Bhat MH** 2000 Puerperal alactogenesis with normal prolactin dynamics: is prolactin resistance the cause? *Fertility and Sterility* **74** 598–600