REVIEW

New concepts in prolactin biology

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Abstract

Human prolactin (PRL) is currently viewed as a hormone of pituitary origin, whose production (i.e. serum levels) is controlled by dopamine, whose biological actions relate exclusively to lactation and reproductive functions, for which any genetic disorder is yet to be identified, and whose unique associated pathology is hyperprolactinemia. Both experimental studies and human sample/cohort-based investigations performed during the past decade have considerably widened our perception of PRL biology: i) there are now strong epidemiological arguments supporting the fact that circulating PRL is a risk factor for breast cancer, ii) in addition to the endocrine hormone, locally produced PRL has been documented in several human tissues; there is increasing evidence supporting the tumor growth potency of local PRL, acting via autocrine/paracrine mechanisms, in both rodent models, and human breast and prostate tumors, iii) the first functional germinal polymorphisms of the PRL receptor were recently identified in patients presenting with breast tumors, which involve single amino acid substitution variants exhibiting constitutive activity, iv) human PRL analogs have been engineered, which were shown in experimental models to down-regulate the effects triggered by local PRL (competitive antagonism) or by the constitutively active receptor variants (inverse agonism). The aim of this review is to discuss these novel concepts in PRL biology, including their potential pathophysiological outcomes.

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Classical view

Prolactin (PRL) was discovered 80 years ago (Stricker & Grueter 1928, Riddle *et al*. 1933). In Endocrinology textbooks, PRL is defined as a pituitary-secreted polypeptide hormone which was named for its stimulatory action on lactation. Various animal models, including mice deficient for PRL (Horseman *et al*. 1997) or for its receptor (PRLR; Ormandy *et al*. 1997) have unambiguously confirmed this issue; these studies also shed light on the essential role of the PRLR/STAT5 pathway in mammopoiesis (Liu *et al*. 1997). In addition, these germline models of permanent PRLR signaling failure were very useful to reveal or confirm the other multiple physiological actions modulated by PRL, including actions on behavior and on the brain in general, metabolism, immune responses, and electrolyte balance (for reviews, Bole-Feysot *et al*. (1998), Dorshkind & Horseman (2000) and Ben Jonathan *et al*. (2008)). Despite the fact that almost 300 functions or targets could be identified for this hormone in various species, the question remains open as to which of them are really relevant in humans. One of the difficulties preventing a clear understanding is that no genetic disease related to the genes encoding human PRL or its receptor has been identified yet; therefore we lack a definitive clinical model of isolated PRL deficiency that could be used to identify the functions that depend on, or are modulated by PRL. It should be noted that even its main functions, i.e. actions on mammary gland development, are in fact poorly documented in humans, and our understanding is mainly from lessons learned using animal models.

Hyperprolactinemia is currently the only PRL-related pathology of concern to clinicians, which probably reflects our lack of knowledge of this 'old' hormone. Pathological hyperprolactinemia is defined as circulating PRL levels above normal range, which occurs in conditions other than pregnancy and lactation, when physiological hyperprolactinemia occurs. The major cause of pathological hyperprolactinemia involves tumors of pituitary lactotroph cells (prolactinomas), the main source of PRL in the organism. Pathological hyperprolactinemia results in galactorrhea and oligo/amenorrhea in women, impotence in men, and loss of libido and infertility in both sexes (Molitch *et al*. 1997). For 85–90% of patients, this condition is efficiently cured using synthetic analogs of dopamine, the physiological negative

regulator of PRL production by lactotrophs (Molitch 2003). In addition to normalized circulating PRL levels, which solves systemic effects of hyperprolactinemia, such treatments also reduce the volume of the pituitary tumor. Antipsychotic medications are also known to cause moderate hyperprolactinemia (Knegtering *et al.* 2003, Hummer & Huber 2004, Goffin *et al.* 2006). This is in agreement with the fact that their mechanism of action involves blockade of dopamine D2 receptors. Dopamine agonist therapy is usually not advisable for treating associated symptomatic hyperprolactinemia, although this approach has been used successfully in some instances (Molitch 2005*a*).

The role of PRL in mammary cancer was suggested several decades ago, mainly based on observations involving animal models (Welsch & Nagasawa 1977). However, as true for the multiple physiological functions attributed to PRL based on genetically modified models (Bole-Feysot *et al.* 1998), the relevance of extrapolating the tumorigenesis data obtained in animal models to humans has always been questioned. One of the reasons is that studies involving human patients failed to be conclusive. First, initial studies have shown that treatment using bromocriptine, the prototype dopamine agonist, did not improve the condition of advanced breast cancer patients despite reduction of their circulating PRL levels (Bonneterre *et al.* 1988, Anderson *et al.* 1993). Second, epidemiological studies performed during the 80's and 90's were unable to reach unified conclusions regarding any correlations between circulating PRL levels and breast cancer risk (for reviews, Clevenger *et al.* (2003) and Tworoger & Hankinson (2008)). Although these therapeutic trials and epidemiological studies involved only a small number of subjects to be conclusive, the take-home message was negative, which largely contributed to nourish the doubts regarding the potential involvement of PRL in human tumorigenesis. As a consequence, based on our current understanding of PRL pathophysiology, dysfunctions of this hormonal system are limited to prolactinomas and downstream hyperprolactinemia, which is no longer considered as a major problem in human health.

The past 10 years or so have been very rich in experimental and clinical reports that have considerably modified this classical view of PRL pathophysiology. The aim of this article is to overview four major advances in the field (Fig. 1):

- First, large-scale epidemiological studies have been performed that unambiguously reveal that high-normal circulating levels of PRL increase breast cancer risk.
- Second, locally produced PRL has been documented in various tissues, especially in human species, providing the molecular basis for an autocrine/paracrine mechanism of action for this hormone, classically considered as an endocrine factor; in addition, functional studies using animal models, and expression studies of human samples, brought strong arguments supporting the role of local PRL in breast and prostate tumorigenesis.
- Third, the only gain-of-function variants of the PRLR were reported very recently in patients presenting with
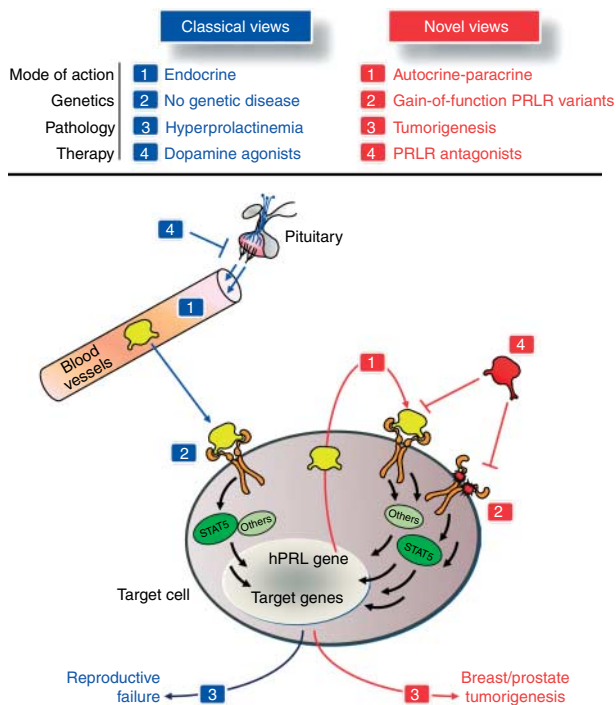
Figure 1 Novel views in PRL biology. The four aspects of PRL biology that are discussed in this article are schematized. The classical view is represented in blue, while novel concepts are shown in red. The latter should be considered as additional to the classical view, and do not substitute for principles that have been validated over the years. As the main intracellular cascade downstream the PRLR, STAT5 is the only one represented; for others, see references in text. Amplification of local mechanisms triggering the PRLR (autocrine/paracrine PRL and gain-of-function PRLR variants) is symbolized by the larger number of arrows.

breast tumors, further supporting the participation of increased PRLR signaling in human tumorigenesis.
- Fourth, a novel class of compounds with therapeutic potential to target PRLR signaling, namely competitive PRLR antagonists, have been developed and characterized in a wide series of experimental set-ups. Such molecules are candidates when dopamine agonists are intrinsically inefficient.

Recent advances in the field of PRL research

Circulating PRL as a risk factor for breast cancer

Classical view There is no clear evidence for an association between PRL levels and breast (or other tissues) cancer risk. Thus, hyperprolactinemia remains the unique clinical pathology associated with PRL.

Discussion In order to determine whether high PRL levels constitute a risk factor for cancer, conventional wisdom would suggest looking at hyperprolactinemic patients.

However, we are not aware of any large-scale study that focused on cancer occurrence in patients presenting with prolactinoma, and even less in those hyperprolactinemic patients whose PRL levels could not be normalized after dopamine agonist treatment (Molitch 2003). Actually, the only available data refers to a small cohort of 67 women presenting with prolactinomas, for which no increased incidence rate of malignancy, including breast cancer, could be demonstrated compared with controls (Popovic *et al.* 1998). A prospective, case–controlled study involving 20 men with prolactinoma investigated the possible risk for prostate hypertrophy (Colao *et al.* 2004). While experimental models have indicated that hyperprolactinemia correlates with benign prostate hyperplasia and hypertrophy in rodents (see below; Van Coppenolle *et al.* 2001, Kindblom *et al.* 2003), the study by Colao *et al.* suggested the opposite in humans. Although these data may be viewed as puzzling, they are not so surprising if one remembers that hypogonadism is frequently observed in hyperprolactinemic patients, therefore lower exposure to estrogens and androgens may partially mask the eventual increased risk associated with high PRL levels (Tworoger & Hankinson 2008).

The vast majority of epidemiological data that are currently available involve comparisons between high versus low PRL levels in normoprolactinemic subjects. The epidemiological data related to PRL and breast cancer risk have been exhaustively reviewed in a recent article (Tworoger & Hankinson 2008). In this paragraph, we will only address the major conclusions of the most relevant studies; the reader is invited to refer to the excellent above-mentioned review for additional details and references. The numerous studies can be stratified into three groups: retrospective case–controlled studies, prospective case–controlled studies of small sample size, and prospective case–controlled studies of large sample size.

More than 20 retrospective case–controlled studies most often including small cohorts have been published within the past 4 decades (for references, see Tworoger & Hankinson (2008)). Unfortunately, the bottom line remained inconsistent, as roughly half of them reported an associated risk with PRL levels, while the others achieved the opposite conclusion. Some of these studies are of concern regarding the methodology used, e.g. PRL levels were often assessed after breast cancer was diagnosed and/or treated, which could not reflect pre-disease exposure to the hormone. Globally, the take-home message from these studies is inconsistent.

The number of prospective studies is smaller. Those involving small sample size (maximum 71 cases) found at best a moderate risk, which could not be validated due to the limited number of subjects included (for references, see Tworoger & Hankinson (2008)). Two large prospective studies used a nested case–control design in the Nurses' Health Studies (NHS and NHS II). The combination of these two studies achieved 492 cases for pre-menopausal women (and 1001 controls), which represents nearly 80% of published pre-menopausal cases (Tworoger *et al.* 2007). There was an overall 40% increase in breast cancer risk for women with PRL levels in the highest versus lowest quartile of normal range (P trend$=0·05$). With respect to post-menopausal women, NHS and NHS II studies combined 915 cases with 10-year follow-up, which again represents nearly 80% of published cases for post-menopausal women. There was a 30% increase in breast cancer risk (P trend$=0·01$). One of the main take-home messages from these studies is that PRL association with breast cancer risk did not differ depending on menopausal status, invasive versus localized cancer, or ductal versus lobular cases (Tworoger & Hankinson 2008). In contrast, there was a clear difference with respect to estrogen receptor (ER) status, as risk reached 1·6 when considering only ER-positive breast cancer (0·9 for ER negative), while it remained independent of sex steroid levels. One of the methodological concerns regarding epidemiological studies involving single PRL measurements is reliability (reproducibility), as PRL levels are known to be modestly stable within a woman over time. Reproducibility studies have been conducted within NHS and NHS II studies, by collecting three samples over 3 years in a subset of 113 women (Tworoger *et al.* 2006, 2007). After correction, the relative risk for breast cancer increased to 1·7 for all cases, and to 2·1 for ER-positive cases, which is very similar to the risk associated with estradiol (Key *et al.* 2002).

With respect to prostate cancer, data is much more sparse than for breast cancer. The largest study published to date involved the Northern Sweden Health and Disease Cohort study, which involved follow-up of nearly 30 000 men, including 144 subjects diagnosed with prostate cancer (Stattin *et al.* 2001). This study concluded that there was no correlation between PRL levels and cancer risk.

Novel views There is now clear evidence that high-normal circulating PRL levels increase breast cancer risk in both pre- and post-menopausal women. The exact mechanism underlying this increased risk remains to be clarified. Beyond proliferative effects – which are not so marked using *in vitro* proliferation assays of classical breast cancer cell lines such as MCF-7 or T-47D – the various roles of PRL in breast cancer are currently being deeply investigated. It has been proposed that PRL may promote mammary tumor initiation via the JAK2/STAT5 signaling pathway, but as this cascade also maintains cell differentiation, it may also contribute to suppress invasive characteristics (Nevalainen *et al.* 2004, Wagner & Rui 2008, Tran *et al.* 2010). Otherwise, the concept is emerging that PRL could favor cell motility and confer resistance to chemotherapy, and thereby contribute to metastasis dissemination (for reviews Clevenger *et al.* (2003) and Lapensee & Ben Jonathan (2010)). Such diverse effects may be mediated by distinct signaling cascades. Future investigations are obviously required to better understand the role of this hormone in breast cancer, which could restore the interest (and perhaps determine the timing) of using anti-PRL drugs targeting either pituitary PRL production or PRLR activation in subsets of patients. Finally, the positive

association between circulating PRL levels and tumorigenesis does not exclude other mechanisms that might be involved in tumorigenesis, especially with respect to the prostate cancer for which no such association was identified. One such mechanism involves locally produced PRL.

PRL, not only an endocrine hormone

Classical view PRL is viewed as a classical endocrine hormone, produced by pituitary lactotroph cells, and delivered to its numerous target tissues via the blood circulation.

Discussion In addition to circulating PRL acting via endocrine routes, there is clear evidence that several human tissues also express PRL. This is true for the mammary gland, the prostate, the skin, the decidua, the brain, some immune cells, adipocytes, and several others (for review, Ben-Jonathan *et al.* (1996)). As the PRLR is also expressed in these tissues, co-expression of both partners suggests the existence of an autocrine–paracrine loop of action, which could be demonstrated using various functional approaches (see below). Even if such a mechanism is supposed to occur in many tissues, our understanding of its impact in physiological (i.e. non-pathological) conditions remains very limited. For example, local PRL was proposed to play a role in i) mammopoiesis, based on experiments using PRL-deficient mice (Naylor *et al.* 2003), ii) nociception, based on experiments using rat trigeminal sensory neurons (Diogenes *et al.* 2006), or iii) dermatological bioregulation, based on experiments using organ-cultured human hair follicles (Foitzik *et al.* 2009, Ramot *et al.* 2010). Even if these examples are not aimed at being exhaustive, the paucity of knowledge of local PRL contrasts with the pleiotropic actions usually linked to the endocrine hormone (Bole-Feysot *et al.* 1998). There are at least two reasons for that:

First, although extrapituitary PRL has been occasionally reported in animals, it appears to be much more commonly observed in human species; therefore, rodent models have in general proven to be less suitable to address the physiological role of local PRL. This particular feature presumably reflects species-specific mechanisms of *PRL* gene regulation, which includes the existence of two distinct promoters in human, but not in rodent *Prl* gene (for reviews, Ben-Jonathan *et al.* (1996) and Baudhuin *et al.* (2002)). In addition to the classical 'pituitary' promoter, a second promoter, referred to as 'extrapituitary' promoter, was identified ∼5·8 kb upstream of the initiation site of human *PRL* gene. Interestingly, comparative genomic analysis of this region revealed that the extrapituitary promoter occurs within a retroviral long terminal repeat-like transposable element that could also be identified in the *PRL* gene of some monkeys, but not of dog, mouse or rat (Gerlo *et al.* 2006). The transcriptional regulation of the extrapituitary promoter remains poorly known. According to initial studies performed using lymphocytes and decidual cells (Gellersen *et al.* 1994),

PRL expression in non-pituitary tissues has been classically considered to be dopamine and Pit-1 (listed as POU1F1 in the MGI Database) independent. Various factors have been proposed to regulate *PRL* gene expression in extrapituitary sites, e.g. insulin, transforming growth factor-β or progesterone in adipose tissue (Zinger *et al.* 2003, Hugo *et al.* 2008). However, a common regulator to all non-pituitary sites, if it exists, is yet to be identified. A very elegant humanized transgenic rat model was recently generated, in which expression of bioluminescent (luciferase) and fluorescent (GFP) reporters were put under the transcriptional control of *PRL* gene promoters (Semprini *et al.* 2009). This model should be very useful for identifying stimuli regulating extrapituitary expression of PRL *in vivo*. It may be misleading to consider that the dichotomy of promoter usage in pituitary versus other sites is absolute. Indeed, while *PRL* mRNA transcribed from the extrapituitary promoter is ∼150 bp longer compared with that transcribed from the pituitary promoter, both mRNA were shown to occur in various human breast/prostate cancer cell lines or biopsies (Shaw-Bruha *et al.* 1997, Dagvadorj *et al.* 2007). Moreover, it has been shown that in the SK-BR-3 human mammary tumor cell line, the sole pituitary promoter was active, despite of the absence of Pit-1 in mammary cells (Manfroid *et al.* 2005). Whatever our still limited understanding of PRL promoter usage, the rareness of extrapituitary *PRL* gene expression in rodents led to the fact that local PRL escaped (almost) all non-genetically modified animal models (see below).

Second, although *PRL* mRNA was identified in various human non-pituitary tissues/cell lines (Reynolds *et al.* 1997, Shaw-Bruha *et al.* 1997, Touraine *et al.* 1998, Dagvadorj *et al.* 2007), the protein itself is much more tricky to detect (Ginsburg & Vonderhaar 1995). In cell cultures, it is produced at very low yields, with no evidence of pulsatility or of secretory granules, and it is rapidly diluted in culture medium. It is possible that within a tissue, the limited volume of extracellular space allows the protein to locally reach higher concentrations, which is currently unknown. In the current state of the art, any specific feature which could distinguish pituitary from extrapituitary PRL is yet to be identified. Therefore, although PRL produced by peripheral tissues is classically assumed to remain within its production site, its eventual contribution to the pool of circulating PRL has been impossible to evaluate. It has been reported that hypophysectomized breast cancer patients regained near-normal PRL levels within several weeks of surgery (Lachelin *et al.* 1977) suggesting that, in some circumstances, extrapituitary sources may contribute to circulating PRL levels. In the absence of known negative regulator of extrapituitary PRL production, this issue remains impossible to assess (Ben-Jonathan *et al.* 1996). In conclusion, extrapituitary PRL is very difficult to identify by other means than immunohistochemical analyses of human biopsies, which at best allows semi-quantitative evaluation of its production level (Nevalainen *et al.* 1997, Reynolds *et al.* 1997, McHale *et al.* 2008). These limitations clearly hamper reliable tracking of time- or state-dependent

fluctuations of its expression, which currently precludes our understanding of its functional role in non-pathological conditions.

The functional activity of extrapituitary PRL has been mainly addressed using experimental models related to tumorigenesis. The first investigations involved interference with PRL secretion or action in human cancer cells. This was performed using breast or prostate cancer cell lines treated using PRL neutralizing antibodies (Ginsburg & Vonderhaar 1995) or receptor antagonists (Dagvadorj et al. 2007; see section on 'Anything beyond dopamine agonists'), or by generating PRL-deficient breast cancer cells from parental MCF-7 cells expressing PRL (Brockman et al. 2002, Schroeder et al. 2002; for reviews, Clevenger et al. (2003) and Goffin et al. (2005)). All these studies pointed to the proliferative/anti-apoptotic role of autocrine/paracrine PRL. As mentioned, due to the rareness of extrapituitary PRL expression in rodents, knockout strategies were rarely used to address functional issues (Naylor et al. 2003). Actually, animal models generated to date all involved the opposite strategy, i.e. the generation of tissue-specific PRL transgenic models aimed at recreating the autocrine/paracrine loop found in human tissues. Prostate-specific PRL transgenic mice were reported to develop precocious prostate hypertrophy (Kindblom et al. 2003). Ongoing studies in our laboratory show that these benign tumors developed to invasive adenocarcinomas in 18–20-month animals, highlighting the oncogenic potency of locally produced PRL. Similar findings were reported for virgin mammary-specific PRL transgenic females, which first displayed various pre-neoplastic lesions before eventually developing ER-positive and ER-negative adenocarcinoma at a median age of 16 months (Rose-Hellekant et al. 2003). When mammary expression of human PRL was driven by the whey acidic protein promoter (WAP, a milk protein gene), meaning onset of transgene expression occurred when the gland is already far in the differentiation process, various benign anomalies were reported, but no carcinoma (Manhes et al. 2006). These models of local PRL over-expression (for review, Arendt & Schuler (2008)) clearly demonstrated the tumor growth-promoting potency of locally produced PRL, which was reminiscent to that of circulating PRL based on the similar mammary and prostate phenotypes of transgenic mice over-expressing PRL systemically (Wennbo et al. 1997a,b).

As always, the question remaining is the extent to which these observations are relevant to human tumors. Recent investigations involved immunohistochemical analyses of PRL expression in mammary and prostate biopsies, where its expression levels were compared between cancer and healthy tissue or benign lesions (Li et al. 2004, McHale et al. 2008). Despite the difficulties linked to the use of this approach for quantification purposes, the level of PRL expression was clearly found to be higher in cancer for both tissues. For the prostate, a positive correlation with tumor grade (Gleason score) and STAT5 phosphorylation could also be established, which suggested the involvement of the PRLR/STAT5 pathway in prostate tumorigenesis. This hypothesis was nicely confirmed at the experimental level using models over-expressing or, in contrast, down-regulating STAT5 expression/activity in human prostate cancer tumors xenografted into immunodeficient mice (Dagvadorj et al. 2008). Finally, for the breast, upregulation of PRL labeling paralleled those of NEK3 and PIAS3, two regulators of PRLR signaling (McHale et al. 2008).

Novel views Arguments supporting the tumor growth-promoting potency of local PRL in humans are emerging. Beyond increased local expression of the hormone, some (but not all) authors have also reported concomitant upregulation of PRLR expression in breast and prostate tumors (Mertani et al. 1998, Touraine et al. 1998, Leav et al. 1999, Gill et al. 2001). This strengthens the idea that amplification of the autocrine/paracrine loop may be one of the mechanisms underlying the participation of local PRL in tumorigenesis. However, assessment of this hypothesis at the functional level has been persistently hampered due to the absence of drugs capable of blocking PRL synthesis in peripheral tissues. Alternative strategies involving neutralizing PRLR antibodies and even more PRLR antagonists have opened new areas of research in this field (see section on 'Anything beyond dopamine agonists'). Finally, although the involvement of local PRL in tumorigenesis has retained increasing attention within the past 15 years (Clevenger et al. 2003, Goffin et al. 2005), another mechanism potentially participating in local amplification of PRLR signaling in tumor contexts has recently emerged, and involves gain-of-function PRLR variants.

Genetic alterations of the PRLR gene

Classical view There is no pathology associated with genetic anomalies of *PRL* or *PRLR* genes.

Discussion In contrast to what is observed for all other pituitary hormones, no mutation of the *PRL* or *PRLR* genes has been identified yet. Isolated or familial PRL deficiency (hypoprolactinemia) and PRL resistance have been proposed as possible mechanisms responsible for puerperal alactogenesis observed in a few patients (Zargar et al. 1997, 2000, Saito et al. 2007). However, no investigation was conducted to support any genetic cause underlying these functional defects. Hypoprolactinemia is also observed in patients harboring mutation of transcription factors involved in pituitary ontogenesis, e.g. *Pit-1* (for reviews, Andersen & Rosenfeld (2001) and Davis et al. (2010)). Nevertheless, as more than a single cell lineage are affected by such mutations, the multiple hormonal deficiencies displayed by these patients do not provide a clear picture of those strictly dependent on PRL. Briefly, a human genetic model is currently lacking to highlight the involvement of PRL in pathophysiology.

Based on the fact that the breast is − and remains − the main target of PRL, search for mutations of the PRL system

(ligand and receptor) has been historically performed in breast cancer patients. The first report was conducted in 30 patients presenting with invasive ductal or lobular breast cancer (Glasow *et al*. 2001). No missense alteration of the *PRLR* gene was identified in patients nor in 20 healthy controls (the *PRL* gene was not evaluated). A second study included 38 invasive carcinomas and 100 healthy controls (Canbay *et al*. 2004). The *PRL* gene was not investigated in that study. One identical missense single nucleotide polymorphism (SNP) was observed in the *PRLR* gene of two patients, and none in that of the control subjects. This germinal variant encoded a receptor variant harboring one residue substitution in the extracellular domain (Leu substituted for Ile146; Fig. 2). No functional investigation was conducted on this variant, and the number of patients was too small to provide significant association with the disease. Vaclavicek *et al*. (2006) investigated the *PRL* and *PRLR* genes in 441 familial breast cancer cases and 522 age-matched controls. No increased risk was associated with individual non-coding SNPs in the *PRLR*, but the combination of *PRL* and *PRLR* risk haplotypes was associated with an increased relative risk of 2·6 (95% CI: 1·4–4·6, *P* trend=0·007). Finally, the Multi-Ethnic Cohort study involved 1615 breast cancer cases and 1962 control subjects (Lee *et al*. 2007). This comprehensive analysis covering both *PRL* (59 kb) and *PRLR* (210 kb) loci identified no significant association between common variations in these genes and breast cancer risk (or plasma PRL levels). The bottom lines of these four studies are that i) only one missense SNP was identified in the *PRLR* gene
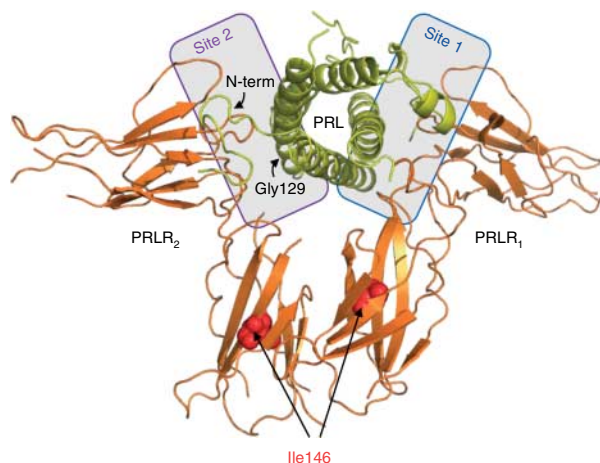
Figure 2 Crystal structure of the PRL–PRLR₂ complex. The structure of PRL (green) bound to two moieties of the PRLR extracellular domain (orange) has been solved recently (Broutin *et al*. 2010). This picture shows the two binding sites of PRL to its receptor. The two main features within site 2 are localized: Gly129 and the N-terminus. The mutation of these two structural components is required to obtain a pure PRLR antagonist (Del1-9-G129R-hPRL). Isoleucine 146 is also represented (red). Substitution of a leucine for Ile146 leads to constitutive activity without affecting ligand binding (see text).

(I146L), and none in the *PRL* gene, ii) at best, associations could be suggested for combined non-coding SNPs, and iii) in any case, none of the associations proposed were supported by functional investigations (e.g. gene expression, mRNA stability, protein properties, etc).

Beyond breast cancer, breast pathologies include a wide panel of benign breast disease (BBD). The etiology of BBDs is poorly known, especially for those presenting with rare features (Santen & Mansel 2005). In addition to the morbidity linked to the disease itself, BBDs represent an important risk factor for developing a cancer (Hartmann *et al*. 2005, Worsham *et al*. 2009). Multiple breast fibroadenoma (MFA) is a rare BBD of unknown etiology, which is defined by the presence of at least three lesions (adenomas) simultaneously in one breast (Hughes *et al*. 2000). We and others have demonstrated that the PRLR is expressed in mammary biopsies from various benign and malignant breast lesions (Clevenger *et al*. 1995, Mertani *et al*. 1998, Touraine *et al*. 1998, Gill *et al*. 2001). Therefore, based on the essential role of the PRL system in mammary gland morphogenesis and differentiation, we hypothesized that PRLR-triggered signaling cascades could participate in benign breast tumorigenesis, as already suggested for various growth factors (Courtillot *et al*. 2005). In such a context, we conducted a study aimed at identifying genetic anomalies of *PRL* and *PRLR* genes in a population of patients presenting with MFAs (Courtillot *et al*. 2010). We only focused on coding regions (exons) of these genes. While no missense SNP was identified in *PRL* gene, we found three germline, heterozygous missense SNPs in the *PRLR* gene (Courtillot *et al*. 2010). This result was particularly encouraging given the paucity of coding SNP reported in the literature and databases for this receptor. Two of them involved the extracellular domain (exon 5/I76V, exon 6/I146L), and one involved the intracellular domain (exon 10/E554Q). The latter was identified in one patient only. Otherwise, PRLR-I76V was identified in both patient and control populations, suggesting that it is a real polymorphism independent of the disease. More interestingly, the third one (PRLR–I146L; Fig. 2) was harbored by four patients (*n*=95), and none by the control subjects (*n*=194; *P*<0·01; Courtillot *et al*. 2010). Even more intriguing, one should note that two earlier studies focused on breast cancer (see above) also identified these SNPs in patients and not in control populations (Canbay *et al*. 2004, Vaclavicek *et al*. 2006). In these studies, however, no significant association was found between PRLR–I146L and the disease, which may be due to the low number of patients in which exon 6 was sequenced (*n*=38 and 23 respectively) and the low prevalence of this SNP (*n*=2 and 1 respectively, i.e. ∼5% of patient populations).

We characterized the functional impact of I146L substitution on PRLR properties, using several *in vitro* cell-based bioassays involving various readouts, that were specifically developed for testing human lactogens (Bernichtein *et al*. 2003*a*). We demonstrated that the sole I146L substitution was sufficient to convert the PRLR into a constitutively active

receptor (Bogorad *et al.* 2008). This means that in the absence of any PRL stimulation, PRLR–I146L triggers downstream signaling cascades (Stat5 and MAPK), which protects cells from apoptosis and/or stimulates their proliferation. These observations were confirmed using stably transfected MCF-7 breast cancer cells exhibiting the 'heterozygous' PRLR profile observed in the four MFA patients harboring one PRLR–I146L allele (Bogorad *et al.* 2008, Courtillot *et al.* 2010). The affinity of the PRLR for PRL was not affected by I146L substitution, and in most assays, PRL stimulation had an additive effect over PRLR–I146L constitutive activity, leading to higher levels of activation compared with cells expressing the wild-type receptor stimulated by equivalent PRL doses (Bogorad *et al.* 2008).

PRLR–I146L is the first ever reported functional mutation of the PRLR associated with a human disease. Interestingly, PRLR–I76V also displayed some level of constitutive activity, albeit to a much lesser extent than PRLR–I146L (Courtillot *et al.* 2010). In total, 15% of our MFA patients appeared to harbor one of these PRLR allelic variants exhibiting constitutive activity, strongly suggesting that enhanced PRLR signaling may be one of the molecular pathways involved in MFA etiology, and perhaps more globally, in breast pathogenesis. Interestingly, one of the four PRLR–I146L patients initially diagnosed with MFA developed a rapidly evolutive grade III invasive ductal carcinoma at the age 38, suggesting the possible involvement of this receptor variant in benign/malignant transition. Although one must remain very cautious not to over-interpret such a case report, this observation underlines the need for appropriate epidemiological and functional data supporting this hypothesis.

Novel views PRLR–I146L is the first functional genetic variant identified for the PRLR. Based on its gain-of-function properties, suspecting its involvement in breast tumorigenesis seems reasonable. Various investigations currently performed in our laboratory are aimed at better delineating the impact of PRLR–I146L in breast cancer, which includes the use of diverse breast cancer cell lines and the analysis of patient cohorts. Another challenge is to understand the molecular mechanisms underlying the gain-of-function properties of PRLR–I146L. The recently determined structure of the homodimerized rat PRLR extracellular domain bound to PRL will be a useful tool in awaiting the determination of PRLR–I146L three-dimensional structure (Broutin *et al.* 2010). The intrinsic PRL-independent activity of PRLR–I146L also implies the use of alternative approaches to dopamine agonists for down-regulating its downstream effects, which may become therapeutically relevant once its impact on breast cancer is better delineated. Compounds specifically targeting the receptor itself, or its major downstream signaling cascades, are necessary. PRLR antagonists are obvious candidates.

Anything beyond dopamine agonists?

Classical view Dopamine agonists are the unique compounds relevant to treat disorders linked to excess PRL levels.

Discussion There is no doubt that dopamine agonists (bromocriptine, cabergoline, etc.) are very efficient drugs to treat hyperprolactinemic patients presenting with prolactinoma (Molitch 2005*a*). However, as described above, such compounds are *per se* inappropriate to target locally produced PRL (whose expression is dopamine independent) or constitutively active receptors (whose basal activity is independent of PRL). At best, dopamine agonists could eventually prevent additive effects of endocrine and local mechanisms triggering PRLR signaling, which remains to be demonstrated experimentally. New strategies targeting the receptor itself, and not the central production of its ligand, have been a field of intense investigations during the past decade.

One of the pioneering reports published by the group of Vonderhaar showed the ability of neutralizing anti-PRL antibodies to reduce proliferation of breast cancer cell lines *in vitro* (Ginsburg & Vonderhaar 1995). However, to the best of our knowledge, preclinical development of anti-PRL monoclonals is yet to be reported. In contrast, the development of modified PRLR ligands based on the PRL core has been very productive. Three types of compounds can be distinguished: competitive PRLR antagonists (that target the PRLR only, prototype is Del1-9-G129R–hPRL; Bernichtein *et al.* 2003*b*), chimeric antagonists (bifunctional molecules targeting two receptors and/or exhibiting increased half life; Zhang *et al.* 2002), and alternative agonists (that selectively activate downstream PRLR targets, prototype is S179D-hPRL; Walker 2007). The various types of antagonists have been recently reviewed (Goffin *et al.* 2005, 2007, Tallet *et al.* 2008), therefore we will only focus on data recently obtained using Del1-9-G129R–hPRL, the sole pure PRLR antagonist reported to date.

The development of this compound started in our laboratory in the nineties, through the engineering of G129R-hPRL variant, in which glycine 129 was replaced by a sterically hindering arginine residue (Goffin *et al.* 1994; Fig. 2). This substitution was designed to alter the functionality of so-called binding site 2 of human PRL, which was later confirmed using surface plasmon resonance analyses demonstrating that any substitution of this conserved residue prevented interaction between Gly129 hPRL mutants and the soluble extracellular domain of the PRLR (Jomain *et al.* 2007). Despite the fact that it exhibited antagonistic properties, G129R-hPRL also displayed residual agonism that was more easily detectable in some assays than others (Bernichtein *et al.* 2003*a*, Goffin *et al.* 1996, 2005). Using both structural and functional approaches, we identified the N-terminus of the molecule as responsible for this residual agonism. Hence, deletion of nine N-terminal amino acids led to a compound displaying pure antagonistic properties (Bernichtein *et al.* 2003*b*, Jomain *et al.* 2007, Broutin *et al.* 2010).

The properties of Del1-9-G129R–hPRL have been characterized by several collaborators using a wide panel of bioassays, involving various cell types of mouse, rat, or human origin (for review, Tallet *et al.* (2008)). First, all studies published so far confirm the absence of residual agonism of this analog. Accordingly, we generated transgenic mice expressing this antagonist systemically, and we have failed to observe any 'hyperprolactinemic phenotype', which contrasted with transgenic mice expressing the first generation antagonist G129R–hPRL (Tallet *et al.* 2008 and unpublished data). With respect to antagonistic properties, the ability of Del1-9-G129R–hPRL to inhibit the actions mediated by exogenous/circulating (co-injected) PRL has been widely demonstrated, providing the antagonist was added in molar excess over the agonist to compensate its lower affinity for the PRLR (for reviews, Goffin *et al.* (2005) and Tallet *et al.* (2008)). Inhibition of the effects induced by locally produced growth factor is a more tricky challenge. *In vitro*, Del1-9-G129R–hPRL was shown to antagonize the survival effect mediated by autocrine PRL/STAT5 cascade in prostate cancer cell lines (Dagvadorj *et al.* 2007). Its ability to counteract the effects induced by local PRL has also been demonstrated in other cell types, including sensory neurons (Diogenes *et al.* 2006), human uterine fibroblasts (Eyal *et al.* 2007), and more recently in human hair follicles (Ramot *et al.* 2010). In these assays, however, it is difficult to state whether the antagonist achieved complete inhibition of the pathways/effects specifically activated by autocrine PRL. This issue was partly solved using a transgenic approach. Double transgenic mice expressing the antagonist systemically, and PRL only in the prostate, were recently generated (V Rouet, RL Bogorad, C Kayser, K Kessal, C Genestie, A Bardier, DR Grattan, B Kelder, JJ Kopchick, PA Kelly & V Goffin, unpublished observations). This model showed that all the phenotypes linked to prostate tumorigenesis induced by autocrine PRL compared to wild-type littermates (i.e. prostate hypertrophy, histological defects, and PRLR signaling) were reduced or even abolished in double transgenics, assessing the anti-tumor properties of the antagonist *in vivo*. To our knowledge, this is the first molecule demonstrating the capacity to functionally counteract the pro-tumor actions triggered by local PRL.

Del1-9-G129R–hPRL was also tested in cell systems expressing the constitutively active receptor PRLR-I146L. A single dose of this PRL analog was shown to down-regulate PRLR phosphorylation and STAT5 signaling (significant inhibition lasted up to 24 h), which resulted in reduced proliferation over 3 days of both Ba/F3 and MCF-7 cells stably expressing PRLR-I146L (Bogorad *et al.* 2008). This indicates that in this specific case, the 'antagonist' Del1-9-G129R–hPRL actually acts as an 'inverse agonist' toward this gain-of-function receptor. The molecular (structural) mechanism underlying this effect, as well as the one leading to PRLR-I146L constitutive activity, is currently unknown.

Novel views Although the impact of autocrine/paracrine PRL and of gain-of-function PRLR variant(s) in human pathophysiology remains to be more precisely defined, their independence to dopamine regulation supports the need for developing alternative targeting strategies. Data recently accumulated using Del1-9-G129R–hPRL antagonist make the latter a good candidate to counteract the undesirable actions potentially mediated by these 'local' mechanisms of PRLR signaling amplification (Goffin *et al.* 2006). However, progress is needed to demonstrate first, the ability of Del1-9-G129R–hPRL to inhibit the phenotypes induced by PRLR-I146L *in vivo* (themselves to be identified), and second, the long-term anti-tumor potency of Del1-9-G129R–hPRL toward human tumors, in order to complete ongoing studies involving animal models. Engineering of long-acting version of this pure antagonist is an immediate challenge. As recently discussed (Goffin *et al.* 2006), PRLR antagonist therapy is not anticipated to have adverse effects, as the absence of PRLR in mice has not shown any sign of strong metabolic disorder, toxicity or lethality. We should remain vigilant, however, since animal models may not fully reflect the outcome of PRL blockade in humans. This is especially true with respect to autocrine PRL, which may exert specific functions that are currently unknown or under-estimated in humans. Since the level of inhibition exerted by competitive antagonists is directly proportional to their concentration relative to the natural ligand, appropriate doses should be evaluated to not achieve complete receptor blockade if required.

Conclusions

PRL pathophysiology is currently viewed as a minor issue in public health. This is due to the fact that i) dopamine analogs have proven to be very efficient in controlling hyperprolactinemic patients, ii) investigations of the involvement of this hormone in human tumorigenesis failed to provide any clear picture, leading clinicians to forget about it, and iii) clinical, epidemiological and therapeutic studies have exclusively focused on endocrine (systemic) PRL, which more and more appears to represent only a part of this hormonal system. Indeed, as discussed in this article, two novel mechanisms that are acting locally – i.e. independent of pituitary PRL – have been recently added into the global PRL scheme (Fig. 1): autocrine/paracrine PRL and gain-of-function PRLR variants. The impact of these novel mechanisms in human pathophysiology must be better understood, as only descriptive reports have been published to date, which suggest their participation in tumorigenesis, but certainly do not prove any causal link. The development of molecules of proven efficacy to target these pathways is of concern to move forward. PRLR antagonists are candidates, but others also exist. The proof-of-concept demonstrating the ability of PRLR antagonists to inhibit the pro-tumor actions triggered by autocrine PRL and/or by PRLR-I146L has been obtained using experimental systems. Now, these data need to be validated using human cell xenograft approaches, as these two

novel mechanisms seem to be more specific to this species. Although PRL-transfected breast cancer cells have been used in the past to demonstrate the growth-promoting effect of locally over-expressed PRL in xenografts (Liby *et al.* 2003), there is currently no unified cell model used for targeting autocrine/paracrine PRL actions at preclinical stages of therapeutic molecule development. This is even more true for PRLR–I146L, which is yet to be identified in any cell line classically used in PRL biology.

Tumorigenesis of PRL target tissues may not be the unique pathophysiological context in which inhibitors of PRLR activation may be of interest. Despite the acknowledged efficacy of dopamine agonists for treating prolactinoma-associated hyperprolactinemia, dopamine resistance accounts for 10–15% of the cases. In such patients, therapeutic strategies include increased doses of the same dopamine analog, use of alternative ones, surgery or radiotherapy (Molitch 2005*b*). While all above strategies target the pituitary tumor, PRLR antagonist could be viewed as alternative means to treat systemic consequences of hyperprolactinemia by targeting the PRLR in periphery. This would of course imply that they do exert detrimental effect on the volume of pituitary tumors, which is currently unknown. Probably more than dopamine-resistant prolactinomas, medication-induced hyperprolactinemia has become of major concern for clinical endocrinologists. This condition is classically observed in patients treated for psychiatric disorders using blockers of dopamine D2 receptors. Hence, dopamine agonist therapy is not suitable for treating associated symptomatic hyperprolactinemia. The availability of compounds blocking the PRLR without affecting pituitary D2 receptors may be helpful to improve the condition of such patients. Effective PRLR blockade should indeed restore the physiological functions imbalanced by hyperprolactinemia, including fertility which is often the concern.

Declaration of interest

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