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2. Target Validation

a) Validation table

Prolactin receptor (Prlr)	Hyperprolactinemia.	1	(1-5)
	Breast Cancer	2	(6-11)
	Prostate cancer	2	(12-17)
	Telogen effluvium and hirsutism	3	(18-21)

	Female fertility	4	(22, 23)
	Metabolism and growth	4	(10, 24-26)
	Osteoporosis	4	(27)
Male fertility	4	(17)	

(b) Implicated Diseases

The prolactin receptor (Prlr) has been implicated in a myriad of human diseases (28). The evidence for this involvement is variable in strength and so the approach taken here is to concentrate on those disorders for which strong evidence has been obtained using transgenic and knockout mouse models. A caveat here is that modulation of prolactin (Prl) or Prlr levels produces systemic endocrine disruption and so we have confined our detailed examination to those examples where systemic endocrine disruption has been demonstrated to play no part in the observed phenotype, indicated by a validation level of 2 and 3. Where endocrine disruption in transgenic and knockout animals has not been excluded as a contributing factor we have used a level of 4. In the case of fertility, metabolism and growth, endocrine regulation by Prl is central to its effects and so at least a portion of the consequent endocrine disruption in knockout models represents a valid and direct action of Prl. We have not detailed the many examples where non-genetic animal and cell-based

models reveal biology that may impinge on a specific disease process and the reader is referred to detailed reviews of these areas (28-30).

Hyperprolactinemia

Hyperprolactinemia is a common endocrine disease causing reproductive defects such as amenorrhea, infertility, or galactorrhea/gynecomastia and impotence. Osteoporosis is also observed (5). Hyperprolactinemia may result from the pharmacological use of dopamine antagonist neuroleptics, from pathologies that impinge on the dopamine control of Prl secretion, and most frequently from adenomas of the Prl-secreting pituitary lactotrophs. In the latter case large tumours may cause secondary effects such as headache and visual disturbance. Hyperprolactinemia is currently the only indication for which ablation of prolactin action is used in the clinic. Treatment by dopamine agonism using improved analogues of bromocriptine such as pergolide, cabergoline or quinagolide is usually successful in reducing Prl levels, shrinking and controlling tumour mass and restoring fertility. Between 10-20% of patients exhibit resistance to dopamine agonists (4). These patients are treated by trans-sphenoidal surgery or radiotherapy only when infertility is a problem or visual disturbance occurs. This group may benefit from the future development of drugs that modulate signalling by the Prlr, in an analogous way to blockade of the growth hormone receptor in acromegalia using Pegvisomant. In light of the growing body of evidence regarding the potential carcinogenic (see below) and osteoporotic effects of elevated Prl it may no longer be wise to allow hyperprolactinemia to go undetected and untreated. The actual rate of elevated and hyperprolactinemia in the population is unknown.

Breast Cancer

The very large and prospective Nurses Health Studies I and II (http://www.channing.harvard.edu/nhs/) demonstrated that top quartile serum Prl (PRL) conferred a higher relative risk (2.03 fold 95% CI 1.24-3.31 p=0.01) of developing breast cancer compared to women with bottom quartile serum Prl (8), with ER+ PR+ tumours having an increased relative risk of 1.78 (95% CI, 1.28-2.50; P-trend < 0.001) compared to ER-tumours (0.76 95% CI, 0.43-1.32; P-trend=0.28) (31). Clinical studies using bromocriptine in advanced breast cancer showed no effect (32,

33). These observations have led to two hypotheses regarding Prl action for which experimental support has been provided. Firstly advanced breast cancer may have lost sensitivity to Prl, suggesting that Prl may act during the early stages of carcinogenesis (10). Secondly the mammary epithelial cell produces Prl via a second promoter not sensitive to dopamine, allowing for autocrine or paracrine stimulation of cancer cell proliferation to continue in the presence of low serum Prl (34).

Prolactin is a complete carcinogen in rodent models of breast cancer (35). More recent transgenic strategies provide further proof and insight. Models that increase serum Prl levels result in mammary cancer. For example, transgenic mice that over-express human growth hormone (hGH), which binds both Prlr and growth hormone receptors, develop mammary carcinoma while mice that over-express the growth hormone receptor-restricted ligand bovine GH do not (36). Over-expression of rat Prl using the lipocalin promoter to drive expression predominantly in mammary epithelium produces oestrogen receptor positive tumours at a higher rate than other mouse mammary cancer models (11). Prl knockout mice expressing the polyoma middle-T antigen oncogene develop tumours significantly later (37). Using the SV40 T transgene a similar delay in tumour onset is seen in Prlr knockout animals (10) via increased cell proliferation in very early hyperplastic lesions, which was lost as they progressed to invasive carcinoma. Here the effect of Prl was demonstrated to occur via the mammary epithelial cell Prlr and not via systemic effects on other hormones like oestrogen. This result indicates that anti Prl or Prlr therapies may be most beneficial in preventing the progression of preneoplastic lesions to carcinoma. Advances in imaging technology have led to these pre-cancerous lesions being more frequently detected via micro calcification, usually at multifocal sites and so presenting a treatment dilemma; watchful waiting for evidence of progression to carcinoma, with attendant risk of metastasis, or prophylactic radical mastectomy.

Prostate Cancer

Prolactin is trophic for the normal prostate, where it operates with androgens. Greater castration-induced prostate regression occurs with additional hypophysectomy (38), androgen replacement after castration restores prostate weight less effectively with hypophysectomy (39, 40), and pituitary grafts reduce the rate and

extent of prostate regression induced by castration (41) via androgen-independent mechanisms (42). Prl is capable of producing stromal hyperplasia and intraepithelial dysplastic features with long exposure (12, 13). Prl can also operate directly; Prl and the Prlr are expressed in human and rodent prostate (14) by the epithelial cells with a weak signal in the fibro-muscular stroma (43). *In vitro* Prl is mitogenic for cultured prostate epithelial cells (44) and in organ cultures normal morphology was best maintained by the addition of androgen and Prl (45). Expression in the prostate of both Prl and the Prlr is increased by androgen treatment *in vivo*, while Prlr level is also increased by Prl (15, 46). Prl has been viewed as an autocrine/paracrine growth factor (14, 15), or a survival factor (16) for prostate epithelial cells. Transgenic expression of Prl in the mouse prostate results in massive enlargement, stromal hyperplasia, ductal dilation and focal epithelial dysplasia (13). Knockout of the Prlr in animals expressing the SV40T oncogene in the prostate caused a reduction in intraepithelial neoplasia and prevented tumour formation (17).

Clinical investigation has shown that serum Prl levels in men with prostate disease are generally not different from age-matched controls when measured at presentation (47) or up to 13 years prior to diagnosis (48). During oestrogen, anti-oestrogen, anti-androgen or GnRH analogue therapy for prostate cancer, Prl levels can increase and are predictive of poor prognosis (49). This has lead to 11 reported small uncontrolled clinical trials of adjuvant bromocriptine during antiandrogen therapy; some of which report increased response rates when bromocriptine is used (50), despite the enrolment of late stage patients where tumour Prlr levels can be diminished or lost (51), and where the potential autocrine/paracrine effects of Prl produced by the prostate epithelium may operate. The use of a molecular mimic of phosphorylated Prl, S197D, successfully inhibited tumour initiation and the growth of human DU145-derived tumours in nude mice (52). Thus Prl may play a role in escape from androgen-dependence and anti Prl therapy may prove useful as an adjuvant to anti androgen therapy.

Telogen effluvium and hirsutism

The hair follicle cycle is comprised of a period of active hair shaft elongation followed by involution of the follicle and a quiescent state during which the hair shaft

is retained by the follicle. Re entry of the follicle into the active state results in the production of a new hair, which in many species including humans ejects the old hair. This process is hormonally regulated, by systemic and local factors that form a zone of follicle reactivation that moves across the skin. The hair follicle expresses the Prlr in a cycle dependent manner and is only sensitive to Prl at specific cycle points (18-21). In Prlr knockout mice the timing of the first cycle of follicle reactivation- the first moult, occurs at a younger age, the resulting hair is longer and coarser, and the sexual dimorphism in these characteristics is lost (20). Importantly when Prlr knockout hair follicles and skin are transplanted to normal host animals the same effects occur, demonstrating direct Prl action on the hair follicle and discounting a systemic hormonal disruption as the cause (18). The human hair follicle is sensitive to Prl, opening the opportunity to pharmacologically regulate this pathway in cases of telogen effluvium and hirsutism.

Endocrine disruption

Modulation of Prl alters systemic hormone levels. In mice hyperprolactinemia causes increased androgen levels (12). Null mutation of Prl or Prlr in mice (23, 53) causes reduced levels of oestrogen, progesterone, and PTHRP (54). This central regulatory role of Prl produces a caveat for the remaining disease indications in Table 1; some of the effects may be directly attributable to Prlr and some may be due to the altered levels of other hormones. For this reason they are listed at level 4 and are not discussed further.

C. Disease Models.

Target	Model	Disease	Reference	Source
Prlr	Prlr knockout mice	Hyperprolactinemia Breast Cancer Prostate Cancer Hair disorders	(23)	Jackson Laboratory 600 Main Street Bar Harbor, Maine 04609 USA Paul Kelly Centre de Recherche Croissance et Signalisation Inserm U845 Faculté de Médecine Paris Descartes - Site Necker 156 rue de

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Prl	Prl knockout	Hyperprolactinemia Breast Cancer Prostate Cancer Hair disorders	(53)	Nelson Horseman. Department of Molecular and Cell Physiology University of Cincinnati Ohio USA
Prl	Prl transgenic Prostate specific	Prostate cancer	(12, 55)	Jan Tornell Sektionen för fysiologi, Medicinaregatan 11 Endokrin Box 434 405 30 GÖTEBORG SWEDEN
Prl	Prl transgenic Mammary specific	Mammary cancer	(11)	Linda Schuller Department: Comparative Biosciences, School of Veterinary Medicine 4354B Veterinary Medicine Bldg, 2015 Linden Dr., Madison, WI USA
Prl	Prl transgenic Whole body expression	Hyperprolactinemia	(12)	Jan Tornell Sektionen för Fysiologi, Medicinaregatan 11 Endokrin Box 434 405 30 GÖTEBORG SWEDEN
Prl	Pituitary transplant	Hyperprolactinemia		

Prlr S2	Transgenic expressing a short form of the Prlr		(56-58)	Paul Kelly

The Prlr (23) and Prl (53) knockout models have defined the actions of Prl and are the major experimental models available. Both are constitutive in all tissues from birth and so suffer from the usual range of caveats that apply to this approach. There are no knockout models using a floxed approach to provide an inducible knockout.

The prostate (55) and mammary specific (11) transgenic models of forced Prl expression produce dysplasia and tumours respectively. The mammary model is the only mouse model that produces high frequency of oestrogen receptor positive cancer. The prostate model has not been combined with an oncogene to determine if the kinetics of carcinogenesis are altered- the most likely outcome would be decreased latency to tumourigenesis due to local prostate expression of Prl.

The Prl whole body transgenic (12) resulted from an attempt to create an inducible model of Prl expression using the metallothionine promoter. No inducible lines were found but a line exhibiting constitutive Prl over-expression was derived. This provides a model of hyperprolactinemia. Hyperprolactinemia can be easily produced experimentally by pituitary transplantation to the kidney capsule or mammary fat pad, which removes hypothalamic dopamine inhibition of pituitary Prl secretion in the transplant. Transgenic over expression of the Prlr short forms, both in wild type and a Prlr knockout background provides insight regarding the relative activity of these isoforms (56-58).

3. Drugs and Biotherapeutics.

a. Current Status

All current artificial modulators of the Prlr are based on amino acid substitutions using the natural ligands for the Prlr. The natural ligands of the Prlr in humans and other primates are GH, Prl, and during pregnancy placental lactogen. In non-Primates

GH does not bind to the Prlr. A single ligand molecule dimerises two Prlr molecules, similar to other cytokine receptors, but the mechanism is not fully defined. Whether the receptor is pre-dimerised, or dimerised by ligand binding, and how this alters receptor and ligand conformation, is contentious (29).

The prototypic inhibitor of the Prlr was discovered and patented (US00418561 1989) by John Kopchick and colleagues, who replaced bovine growth hormone Gly120 with large residues such as arginine (G120R) or lysine (G120K) to produce an antagonist of the growth hormone receptor due to disruption of interaction between ligand Gly120 and receptor Trp104. Genentech followed with a similar approach using human growth hormone (US6800740 1995) that was patented (US6429186 1994) for use in breast cancer (59) and for antagonism of the Prlr by the co-administration of zinc (WO05058232). A further 8 mutations and pegylation produced the drug Pegvisomant (Somavert®), used for the treatment of human growth hormone excess (60). Pegvisomant, due to the extra 8 mutations, does not antagonise or agonise the Prlr and so there is currently no drug approved for human use that acts as a Prlr antagonist.

The homologous Prl mutant to hGH-G120R is hPrl-G129R and it is unpatented due to early poor results (61). The ability of these mutant ligands to antagonise the Prlr has been extensively characterised and mixed antagonism and agonism has been described. The mutation causes a 10-fold reduction in affinity, so a complicating feature is the large concentrations required to overcome endogenous Prl levels. The agonistic activity of hPrl-G129R can be removed by deletion of 9 N-terminal amino acids, a strategy developed by comparison to ovine placental lactogen and patented (WO03057729) by Vincent Goffin, Paul Kelly and colleagues. The problem of lower affinity remains but a more pure antagonism is produced (29, 62-64).

Brooks and colleauges have taken a different approach, patenting (US6995244 2003) Prl mutations that do not interfere with ligand-receptor binding, but instead interfere with the hypothesised conformational change in Prl that may be induced by initial then subsequent ligand binding to two receptor molecules (61).

Walker and colleagues have made molecular mimics of phosphorylated Prl, hPrl-S179D (US6890738 2003), based on observed antagonism of Prl action by phosphoprolactin (65). Controversy has raged between Goffin and colleagues, and Walker and colleagues regarding the mechanism of S179D action. The argument has turned on whether S179D is an agonist or antagonist of Prl action, complicated by assay details such as the ratio of background unphosphorylated Prl, tissue and species of origin, relative concentrations, hormone preparation etc. Generally S179D antagonises the biological effects of Prl in whole animal experiments, but this is not always reflected at the molecular level, where S179D exerts mixed agonism and antagonism in receptor interaction and activation assays. It now appears that S179D generates a qualitatively different signal from the Prlr compared to other ligands and that this response is context specific (65). Possible mechanisms include variable final receptor conformations causing differential activation of associated signalling pathways. Thus S179D, and indeed perhaps all mutants of Prl and GH, may be considered as context specific receptor activity modulators (SPRMs) analogous to the Specific Oestrogen Receptor Modulators (SERMs) such as Tamoxifen and Raloxifen (65). So the resolution of the SPRM controversy may also take a similar path to that which surrounded the SERMs.

Ligands and antibodies

Reagent	**Patent**	**Group**	**Company**	**Reference**
hGH-G120R +Zn	US6800740 1995 US6429186 1994	Fuh and Wells Clark et al..	Genentec	(66) (61)
bGH-120R	US00418561 1989	Kopchick et al.	Ohio University	(60)
hPrl-G129R	No	Goffin et al	INSERM U808	(64)

Pegvisomant Somavert®	US00418561 1989 and others.	http://www.somavert.com/	Pfizer	(60)
D1-9hPrl-G129R		Goffin et al	INSERM U808	(64)
hPrl-S179D	US6890738 2003	Walker et al	UC Riverside	(65)
hPrl mutant	US6995244 2003	Brooks et al		(64)
Anti Prlr antibody SPM213			AbCam	Catalogue number Ab17831
Anti Prlr antibody 1A2B1		Clevenger et al.	Zymed	Catalogue number 35-9200
Anti Prlr antibody-U5		Kelly et al.	AbCam	Catalogue number Ab2772
Prl ovine and human			Sigma	
Prl		http://www.humc.edu/hormones/	NIDDK	

b. Next frontiers.

Currently available Prlr modulators produce either no detectable signal from the receptor (Δ1-9-hPrl-G129R), or a modified signal (hPrl-S179D). Δ1-9-hPrl-G129R and hPrl-G229R display a 10 fold loss of affinity and all molecules have a very short serum half life of 15-30 minutes. Use of pegylation to increase the half life of Pegvisomant to 70 hours caused reduced potency, and so it is likely that this strategy would further decrease the potency of any Prlr modulators based on mutation of the natural ligand. Thus although approaches to date have made great progress in identifying key ligand mutations, it is clear that they need be developed further to

become therapeutics. Especially pressing is the restoration of binding affinity, which ideally should be an order of magnitude higher than endogenous ligands. Low affinity modulators can overcome endogenous ligands by 10-100 fold concentration excess, and if the modulator elicits no pathogenic signal from the receptor, or no signal at all, this may represent a feasible approach. Alternative strategies include the generation of antibodies that block ligand activation of the Prlr, a strategy that has proved successful for a number of cell surface receptors. More distant is the development of small molecules capable of interfering with ligand binding, with receptor dimerisation, or with the associated signalling machinery. Defining and then targeting effectors down-stream of the Prlr provides a third approach that may offer the added advantage of selective ablation or stimulus of a specific Prl action, while maintaining others.

4. Function and localisation

a. In homeostasis.

There are a number of different protein isoforms of the Prlr that are produced via alternative splicing and post translational processing. These isoforms contain various deletions of the motifs responsible for ligand binding, membrane localisation and association with members of the signalling cascade such as Jak2 or MapK (Figure 1). Multiple receptor isoforms provide the potential for complex modulation of signalling via homodimer competition for ligand, and via heterodimerisation of the various isoforms and differentially associated signalling molecules. In Primates this may extend to the GHR via ligand-induced PRLR/GHR heterodimerisation (67). The major outputs include the Jak-Stat, MapK, Akt and Rac pathways to modulate cell proliferation, differentiation motility and survival (6, 28, 29).

b. In Disease

How the various signalling events that can be generated by the Prlr relate to disease remains to be defined.

5. Characteristic structural features.

Figure 1 shows the structural features and conserved sequence motifs of the Prlr relative to the mouse and human gene structure. The structural features are organised into discrete exons, reflecting the molecular evolution of the cytokine receptor family (68). Receptor diversity is generated via alternate exon splicing to produce receptors with different intracellular motifs, and thus interactions with the Jak/Stat pathway, the MapK pathway via Fak and the Akt pathway via Src (6, 29). The major signalling dichotomy exists between the short form that activates MapK alone and the long form that activates all three pathways. The Prlr has not been crystallised but the homologous GHR provides a model (60), and Prl has been the subject of intense structural study (30). The receptors may exist as pre-dimerised molecules or solo molecules that sequentially bind site 1 and then site 2 of the ligand, and so undergo a conformational change that results in activation via the close apposition of associated kinases (61).

Modulation of the activity of the Prlr has focussed primarily on the ligand binding site and in particular on mutation of site 2 of Prl, as binding here is thought to be the key step in receptor activation. Specific modulation of the signalling response of the receptor may be possible via this means, as indicated by the effects of S179D. A high affinity receptor-blocking pure-antagonist or a binding site blocking monoclonal antibody could be used to promote complete ablation of signalling. Other approaches to modulating the Prlr include generating small molecules molecules that can specifically interrupt the association of one or more signalling molecules with the receptor or that prevent the formation of receptor dimers, presumed to involve the membrane proximal regions. Elucidation and targeting of the down stream effectors of the transcriptional and other actions of the Prlr may also provide a future therapeutic strategy.

In summary the Prlr mediates the detrimental effects of hyperprolactinemia, which is treated in the clinic using dopamine agonists with high but not complete success. Raised prolactin levels play a role in the aetiology of breast and possibly prostate cancer. The first action of prolactin in these cancers may be to hasten progression of preneoplastic lesions to carcinoma, via effects on cell proliferation. Prototype Prlr modulators have been developed and so anti prolactin receptor therapy may find a place as a preventative measure, especially as imaging technology improves the

discovery of these early lesions. A subset of cancers may retain sensitivity to prolactin and so anti prolactin receptor therapy may also have a role as an adjuvant to other anti-hormonal agents.

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7. Figures

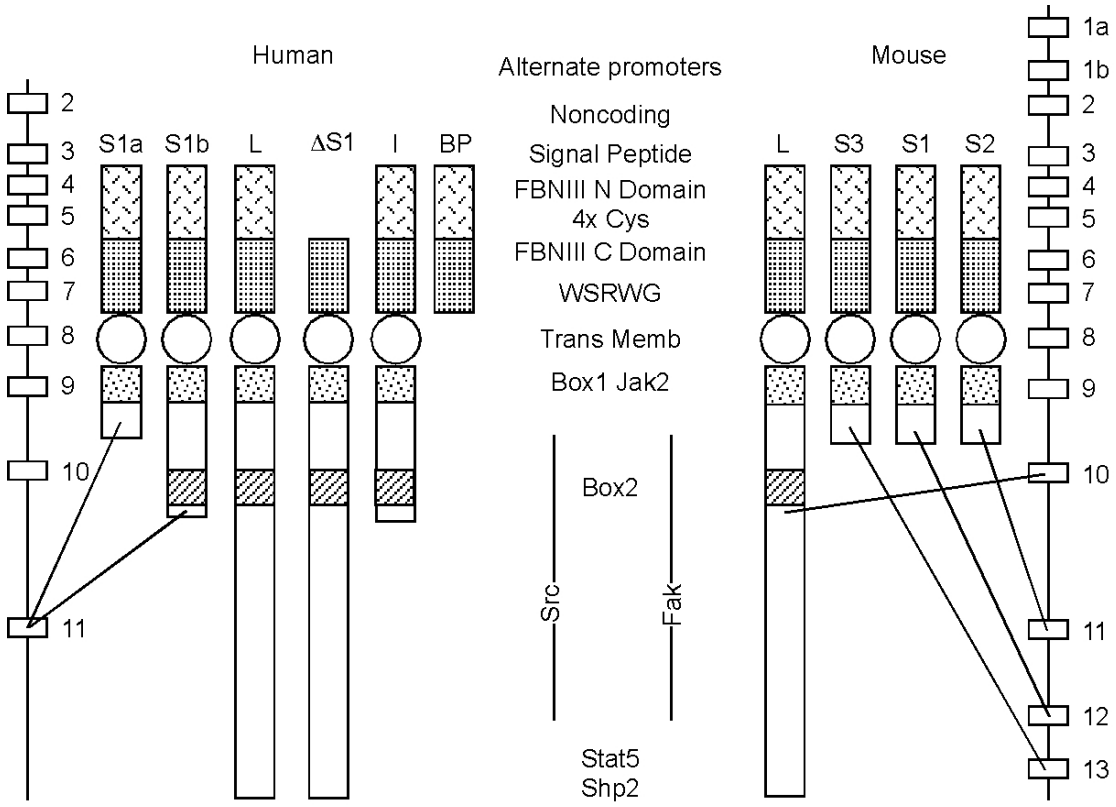

Figure 1. Prlr structural motifs and genomic organization.

Exons of the prolactin receptor gene (numbered boxes) of the human (left hand side) and mouse (right hand side) are shown beside their protein product so that the indicated structural features (variously shaded shapes and middle text) correspond horizontally with the exon(s) that encode them. Alternative splicing of exons 10-13 produces different proteins, indicated by lines, and produces multiple protein products designated as short (S) long (L), intermediate (I) or binding protein (BP). Human S1b is produced by variable splicing of exon 11 with prematurely terminated exon 10. Human long and delta S1 use exon 10 while human intermediate shows a frame shift transcription of exon 10. Human BP is produced by protease activity. Alternative splicing of mouse exons 10-13 produces the four Prlr isoforms shown. Tissue specific alternate promoter usage is common.