Prolactin actions

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Abstract

Molecular genetics and other contemporary approaches have contributed to a better understanding of prolactin (PRL) actions at the cellular and organismal levels. In this review, several advances in knowledge of PRL actions are highlighted. Special emphasis is paid to areas of progress with consequences for understanding of human PRL actions. The impacts of these advances on future research priorities are analyzed.

Key Words
- parathyroid and bone
- lactation
- female reproduction
- skin

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Introduction

The goal of this review is to provide an analytical update on the biology of prolactin (PRL) actions, with a bias toward its effects in mammals, especially humans. The signal transduction mechanisms that underlie these biological actions will be reviewed in summary, but the focus will be on physiology and pathophysiology in the whole body context. We will highlight topics, both new and not new, where there is the potential for making important discoveries about PRL actions.

A little more than 15 years have passed since knockout mouse models of PRL, PRL receptor (PRL-R), and Stat5A were published in 1997. Developed independently, these models showed a remarkable degree of phenotypic concordance (Horseman *et al.* 1997, Liu *et al.* 1997, Ormandy *et al.* 1997). The genetic models provided strong proof of the main PRL signaling pathway, which had been discovered biochemically just 3 years earlier (Campbell *et al.* 1994, Sidis & Horseman 1994, Standke *et al.* 1994, Wakao *et al.* 1995). The coincidental publication of these mouse projects provided a powerful opportunity to reassess and re-imagine PRL research. A few intervening years provides a valuable perspective from which to view how our knowledge about PRL has been advanced by the 'knockout' era, though many old and new questions remain.

Inasmuch as mouse models dominate biomedical research today, it is useful to step back and reflect on the abundant scope of biology, and ultimately on how this abundance impacts our knowledge of human biology and medicine. PRL was identified biologically by observing mammary gland secretion after injecting rabbits with extracts of pituitary glands from cattle (Stricker & Grueter 1928); and it was biochemically purified from cattle pituitary extracts using a quantitatively precise bioassay based on the stimulation of crop milk secretion in pigeons (Riddle *et al.* 1933). Subsequent studies have demonstrated PRL to be secreted from the pituitary glands of the various vertebrate classes, from fishes through mammals (Bern & Nicoll 1968). Today, new tools for gene sequencing, expression analysis, and gene editing (knockout, and other forms of mutagenesis) promise to reopen the research scope of future biologists beyond the constraints imposed by heavy reliance on mouse models.

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Evolution

Despite the clear connection of PRL with lactation in mammals, its primitive functions predate the origin of mammalian lactation (Bern & Nicoll 1968). PRL has been said to encompass a greater diversity of physiological actions than other pituitary hormones, which indicates that, unlike that of other pituitary polypeptides, PRL did not become coupled to any single physiological effect in nonmammals. With the origin of definitive mammals, PRL became linked, inextricably, to mammary gland development and lactation.

The lack of a consistent physiological role for PRL in nonmammalian vertebrates provokes the question of a primitive function of PRL, which would explain its retention during pre-mammalian evolution. Notwithstanding the variety of actions attributed to PRL, a common thread is its association with the postmating phase of reproductive cycles. The pituitary gonadotropins drive gonadal growth, steroidogenesis, gametogenesis, and ovulation in all vertebrate species, although the exact details have been subject to myriad specializations. Having produced and distributed the gametes, species are left with organizing their postmating reproductive functions according to a vast array of reproductive strategies. In every species where the postmating reproductive functions have been studied in any detail, PRL plays a pivotal role. The obvious examples are forms of lactation that have evolved independently in fish, birds, and mammals. Other common functions of PRL during the postmating phase are behavioral changes (broodiness, suppression of aggression), metabolic adaptations, migrations, and seasonal gonadal suppression (Bern & Nicoll 1968, Bole-Feysot *et al.* 1998). PRL, seen in this way, is the reproductive hormone that takes over where the gonadotropins leave off, driving reproductive functions that are segregated, both in time and type, from those of the pre-mating phase.

The integument is the focal point of numerous PRL actions in nonmammalian vertebrates (Nicoll 1980, Foitzik *et al.* 2009), and the organ that best epitomizes each of the vertebrate classes as having specific integumentary appendages (scales, feathers, hair, and glands). The mammary glands are also integumentary appendages, although their exact origins are lost to the vagaries of the fossil record. In spite of those limitations, Oftedal has constructed a compelling scenario in which mammalian ancestors (early Synapsids) produced increasingly specialized glandular secretions in their ventral skin, which nourished and protected the eggs and offspring. Modern monotremes (egg-laying platypus and echidna) have legitimate mammary glands but lack the elaborate ducts and nipples of marsupial and placental mammals, so they deliver their milk along specialized clusters of hairs (Oftedal 2002*a*,*b*, 2012).

Signal transduction

The discovery of the pathway from PRL through its receptor (PRL-R) and the tyrosine kinase Jak2, leading to activation of the key transcription factor, Stat5, was a major advance in understanding PRL actions. Cloning and sequencing of the PRL-R might have provided a hint about PRL signaling (Boutin *et al.* 1988, Edery *et al.* 1989), but these structures did not implicate any of the pathways known at the time. The first direct evidence for a definitive PRL signaling mechanism, which would come to be known as the Jak–Stat pathway, was the discovery that PRL-induced tyrosine phosphorylation and DNA-binding activity of proteins that were biochemically related to components of interferon and interleukin signaling (Sidis & Horseman 1994, Standke *et al.* 1994). At about the same time, it was shown that PRL activated the Jak2 tyrosine kinase (Campbell *et al.* 1994). Based on these findings, it was clear that PRL shared the signal transduction pathway used by a variety of cytokines and growth factors (Horseman & Yu-Lee 1994). Subsequent identification of 'mammary gland factor' as a new member of the 'Stat' family, crystallized the picture of the core PRL signal transduction pathway (Wakao *et al.* 1995), which was ultimately validated by the knockouts.

Since these discoveries, the pathway has been clarified with many details, but these have only added to the explanatory power of the Jak–Stat model of PRL signaling. In fact, there appear to be no physiological actions of PRL that are independent of Jak–Stat activation. In addition to activating transcription via Stat phosphorylation and translocation, Jak2 activation recruits an array of networked signal transduction molecules (Radhakrishnan *et al.* 2012), which modulate PRL signaling in complex and poorly understood ways. Readers are referred to the following, as well as other excellent reviews on Jak–Stat and PRL signaling (Watson & Burdon 1996, Darnell 1997, Hennighausen *et al.* 1997, Edery *et al.* 2001, Rawlings *et al.* 2004, Brooks 2012, Radhakrishnan *et al.* 2012).

One important consequence of understanding PRL signal transduction has been attempts to develop PRL-R antagonists. In related work, discovery of a growth hormone (GH) antagonist ultimately led to the synthesis and therapeutic use of an antagonistic-modified GH that is used to treat acromegaly (Somavert, generically pegvisomant) (Muller *et al.* 2004). Three different PRL-R

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antagonists have been developed. The first, S179D-hPRL, was based on the antagonistic activity of phosphorylated PRL (Chen *et al*. 1998). The other two were based on the GH antagonist precedent, and knowledge of hormone: receptor stoichiometry and kinetics (Goffin *et al*. 1994, 1996, Chen *et al*. 1999). The literature on PRL antagonists has been reviewed by others (Kuo *et al*. 1998, Goffin *et al*. 2005, Walker 2007, Bernichtein *et al*. 2010), so we will only summarize the findings here, and analyze them in general terms.

The Walker Laboratory discovered that rat PRL was phosphorylated in a conserved serine (Ser179 in the human sequence), and that the phosphorylated PRL antagonized the proliferative response of Nb2 cells to PRL (Chen *et al*. 1998). Targeted mutation of the phosphorylation site to an acidic residue (glutamate or aspartate, S179E- and S179D-hPRL, respectively) resulted in recombinant molecules that mimicked, to different degrees, the antagonistic activity of phosphorylated PRL (Chen *et al*. 1998, Kuo *et al*. 1998, Lorenson & Walker 2001). The properties of S179D hPRL have been shown to be complex, behaving as an antagonist in some assays, and an agonist in others (Walker 2007).

Using the successful GH antagonist as a precedent, plus structural knowledge about receptor:ligand interactions, two groups developed and tested similar PRL analogs (Goffin *et al*. 1994, 1996, Chen *et al*. 1999). These antagonists were based, conceptually, on the accepted receptor-binding model, in which PRL (or GH) binds two receptors for each ligand (Wells & de Vos 1996). Ligation of this 1:2 complex results in a productive conformation so that Jak2, Stat5, and other signaling molecules are activated. In the case of GH, transgenic expression of GH mutated at a critical glycine (G120R in hGH) resulted in dwarf mice (Chen *et al*. 1990, 1991). The equivalent residue in hPRL (G129) was modified (Goffin *et al*. 1994, 1996, Chen *et al*. 1999) and has been tested extensively for biochemical and biological properties in various systems. This modified hPRL and an additional mutant in which the N-terminal nine amino acids were deleted (Δ1-9-G129R (Bernichtein *et al*. 2003, Goffin *et al*. 2003) have provided a wealth of information on the receptor binding theory for PRL, reviewed in Goffin *et al*. (2005). Recently, a separate group has identified mutations in binding site 1 that improved the potency of G129R hPRL (Liu *et al*. 2011), the effect of combining these site 1 mutations with Δ1-9-G129R in hPRL has not yet been tested.

Notwithstanding the complexities of the literature on PRL antagonists, it is worth recalling the old admonishment that 'the proof of the pudding is in the eating'. The successful GH antagonist (pegvisomant) was built on the early observation, *in vivo*, that a mutant GH inhibited the canonical physiological effect of GH, resulting in dwarf mice (Chen *et al*. 1990, 1991). The similar experiment with the PRL antagonists would test whether expression of the putative PRL antagonist would inhibit the canonical effect of PRL (mammopoiesis and lactation). Such experiments, as far as one can tell, have not been done or reported. An interesting *in vivo* experiment has been recently reported, in which expression of Δ1-9-G129R-hPRL inhibited prostate tumors that were induced by overexpression of PRL (Rouet *et al*. 2010). The study is limited by its obvious tautology in that the phenotype being inhibited had been created artificially by expressing PRL.

Ablation and replacement experiments for a modern age

Classic experimental approaches rested on the simple logic of eliminating and supplementing the hormone action. In the physiology realm, such studies used 'ablation' and 'replacement' by surgical, biochemical, and pharmacological techniques. Genetics classically relied on accidental 'loss-of-function' and 'gain-of-function' mutations. The ability to intentionally engineer the genome ushered in the astoundingly productive marriage of genetics and physiology through 'genetic ablation and replacement' approaches. The most widely used molecular genetic approaches for engineering the mammalian genome obviously are transgenic and gene disruption (knockout) approaches, especially in mice. In the case of PRL and PRL-R, targeted gene disruptions produced, for the most part, clear and consistent pictures of PRL actions *in vivo*. Most obvious were effects in the females: defective mammary gland development and complete female infertility (Horseman *et al*. 1997, Ormandy *et al*. 1997). Males were fertile, and with minor exceptions normal under laboratory conditions (Steger *et al*. 1998).

Two laboratories have recently used classic approaches to gather new information on PRL actions in recent years. In one case the Goffin Laboratory, in Paris, has identified gain-of-function variants of the human PRL-R (Bogorad *et al*. 2008, Courtillot *et al*. 2010). In the other, the Rui Laboratory, in Philadelphia, used a large-scale tissue array method to identify rat tissues that respond acutely to injected PRL (LeBaron *et al*. 2007).

The Goffin group hypothesized that the PRL-R might be involved in some women with proliferative breast disease. Based on this hypothesis, they analyzed PRL-R sequences in women with multiple fibroadenomas (MFA). They identified a variant (I146L) in four out of 74 MFA patients, which was absent in the control group of 170

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subjects. This mutation conferred constitutive activity on the PRL-R, as measured in several bioassays (Bogorad *et al.* 2008). A second larger cohort identified the same variant (I146L) in both MFA patients and controls, and also identified an additional variant with constitutive activity (I176V) (Courtillot *et al.* 2010). Thus, although it is unclear whether the ligand-independent activity of these receptors causes any pathology (proliferative breast disease, or other), these variants represent an important research advance for studying PRL actions.

The Rui group used an approach dubbed 'cutting-edge matrix assembly' (CEMA) to monitor the activation of the PRL, GH, erythropoietin (EPO), and granulocyte-macrophage colony-stimulating factor (GMCSF) pathways (LeBaron *et al.* 2007). These hormones have different physiological roles, but share (at least partially) common signal transduction mechanisms. The CEMA approach allowed this group to simultaneously observe Stat5 activation in the cell types contained within 40 separate tissues. Within these tissues, they observed 35 PRL-responsive cell types, 32 GH-responsive cell types, and 22 both PRL and GH-responsive cell types. Responses to EPO and GMCSF were more restricted, being apparent primarily in hematopoietic tissues (bone marrow, spleen). This method provided much needed information that is complementary to the gene knockout approaches, because it identified tissues that responded to levels of hormone above basal secretion. In the case of males, which were largely unaffected by PRL deficiency (Steger *et al.* 1998), several male tissues responded robustly to elevated PRL. These included the epididymi, seminal vesicles, preputial glands, and prostate glands (LeBaron *et al.* 2007). These findings reinforce other studies that have pointed to PRL as a potential stimulus for changes in male reproductive organs, particularly the prostate glands (Nevalainen *et al.* 1997, Goffin *et al.* 2011). While basal levels of PRL are not essential in nonstressed males under laboratory conditions, it is clear that male tissues respond to PRL through the known Jak–Stat signaling pathway. Therefore, because a variety of stimuli induce its secretion in nonlaboratory environments, PRL may regulate male tissues in subtle but important ways.

Humanized PRL mice

A new level of PRL ablation and replacement was attained in a recent study from our laboratories in which the human PRL gene, including all of its known regulatory elements, was used to replace the mouse PRL gene (humanized PRL mice) (Christensen *et al.* 2013). There are fundamental physiological differences between human and rodent PRL, which are most obvious in female reproduction. For example, the rodent corpus luteum is dependent on pituitary PRL, whereas the human corpus luteum is not (Risk & Gibori 2001).

The PRL gene in humans and other primates contains an alternative promoter, 5.8 kbp upstream of the pituitary transcription start site, which drives expression of PRL in a variety of tissues (Berwaer *et al.* 1994, Gellerson *et al.* 1994, Semprini *et al.* 2009). Extrapituitary PRL seems to be critically important for human reproduction. A recent study has shown that decidual PRL expression was impaired or absent in specimens obtained from women who had undergone spontaneous miscarriage. Inflammatory cytokines were elevated in tissues from women with impaired PRL, suggesting that extrapituitary PRL is critical for tolerance of the human fetus (Garzia *et al.* 2013). This evidence in humans is consistent with findings by Bao *et al.* (2007), showing that decidual PRL preserves gestation by downregulating the production of IL6 and 20α-hydroxysteroid dehydrogenase.

Using a bacterial artificial chromosome cloning system, a large fragment of human genomic DNA that includes the PRL gene was inserted into the mouse genome (Christensen *et al.* 2013), and these mice were intercrossed with PRL knockout (PRL-KO) mice (Horseman *et al.* 1997). Human PRL completely rescued the reproductive defects previously documented in PRL-KO females. Moreover in the context of the mouse, human pituitary PRL responded to known physiological regulators (dopamine and estrogen), both *in vitro* and *in vivo*.

The expression of human PRL was examined using this recombinant mouse model. Table 1 shows tissues that express the human and mouse PRL genes in ordinary laboratory conditions. Several reproductive tissues, both female and male, express human, but not mouse, PRL; and nonreproductive tissues that express human PRL included the kidneys, thymus, and spleen. An initial study to evaluate the regulation of extrapituitary human PRL gene expression, an inflammatory challenge (LPS) was used to induce PRL expression in the spleen (Semprini *et al.* 2009, Christensen *et al.* 2013). This humanized PRL model will provide a valuable resource for studying human PRL physiology and the roles of PRL in human pathologies, such as breast disease, infertility, autoimmunity, and prostate disease.

Mammary gland growth and differentiation

We will highlight three aspects of PRL action in the mammary glands that have been advanced by discoveries

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Table 1 Expression of mouse and human PRL in various tissues. Mice were engineered to express human PRL (hPRL) from a transgene that includes a large segment of human chromosome 6, which includes all the known regulatory elements for human PRL. These mice also expressed the endogenous mouse PRL (mPRL) from the endogenous gene. Tissues that expressed detectable mRNA (RT-PCR) are marked (+) and those without detectable mRNA are marked (−). The apparent level of expression varied from tissue-to-tissue, but that is not reflected here. Table from data originally published in Christensen *et al.* (2013)

Tissue	hPRL	mPRL
Anterior pituitary gland	+	+
Mammary gland (random cycling, virgin)	+	−
Uterus (random cycling, virgin)	+	−
Ovary (random cycling, virgin)	+	−
Prostate gland	+	−
Testis	+	−
Thymus gland	+	−
Spleen	+	−
Fat (abdominal)	−	−
Lung	−	−
Heart	−	−
Liver	−	−
Kidney	+	−
Stomach	−	−
Intestine (duodenum/jejunum)	−	−
Skeletal muscle (quadriceps)	−	−

in recent years. These include the mechanisms responsible for PRL-induced mammary epithelial proliferation, the role of local PRL expression in the mammary glands, and control of lactation-associated calcium mobilization.

Genes for the obvious mammary-related hormones and receptors (PRL, PRL-R, ERα, PR) have been knocked out, as well as the machinery downstream of PRL-R: Jak2 and Stat5 (Lubahn *et al.* 1993, Lydon *et al.* 1995, Horseman *et al.* 1997, Liu *et al.* 1997, Ormandy *et al.* 1997, Wagner *et al.* 2004, Walker & Korach 2004). The mammary phenotype of ERα KO mice is a severe deficiency in ductal growth (Lubahn *et al.* 1993). The PR-KO mice showed full development of the primary dichotomous branching ducts, but no lateral branching, and very limited sprouting of alveolar buds (Lydon *et al.* 1995). PRL and PRL-R knockout mice had identical mammary phenotypes, consisting of a lack of side branching, which could be rescued by exogenous progesterone (P_4), and no alveologenesis or lactation, which could not be rescued by P_4 (Horseman *et al.* 1997, Ormandy *et al.* 1997, Brisken *et al.* 1999, Vomachka *et al.* 2000). These phenotypes were consistent with 'pre-knockout' literature and illustrated that that the mammary glands develop in a stepwise fashion under the control of reproductive hormones that drive the growth of the primary ductal tree (estrogen), secondary lateral branches (P_4), ultimately leading to growth and differentiation of the alveolar sacs that synthesize milk (PRL).

A better understanding of how PRL drives mammary epithelial proliferation has been important for understanding the PRL actions (Brisken *et al.* 2002, Srivastava *et al.* 2003). Perhaps the most important lessen from these recent discoveries is that PRL-induced mammary proliferation is entirely mediated by indirect mechanisms. This feature contrasts the growth-stimulating actions of PRL with those of ordinary growth factors such as epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor (IGF), which plug directly into classical mitogen transduction mechanisms (i.e., mitogen-activated protein kinases, MAPK, etc.) (Pearson *et al.* 2001). The main mechanism by which PRL induces mammary epithelial proliferation is via induction of RANKL in a synergistic relationship with P_4 (Fata *et al.* 2000, Cao *et al.* 2001, Srivastava *et al.* 2003, Baxter *et al.* 2006, Schramek *et al.* 2010). IGF2, also induced by PRL, accelerates alveolar growth, but is ultimately dispensable (Brisken *et al.* 2002, Hovey *et al.* 2003). Because of its central role, the regulation and functional role of mammary RANKL warrants detailed discussion.

RANKL, its biological receptor RANK, and decoy receptor osteoprotegerin are members of the tumor necrosis factor (TNF) and TNF receptor families respectively (Wada *et al.* 2006, Blair *et al.* 2007). Binding to RANK initiates a signaling cascade that activates NF-κB, MAPKs, and protein kinase B/AKT. The mammary gland phenotype of RANKL-KO mice was discovered because these mice failed to lactate, resulting in death of their pups. When examined, the mammary glands were underdeveloped, lacking alveolar growth (Fata *et al.* 2000). This phenotype was very reminiscent of those earlier reported in mice with knockouts of PRL, PRL-R, or Stat5A (Horseman *et al.* 1997, Liu *et al.* 1997, Ormandy *et al.* 1997). The pathway downstream of RANKL in controlling alveolar proliferation was clarified by the knockout of the NF-κB regulatory kinase IKKα. These mice failed to activate NF-κB and upregulate cyclin D1 in the mammary epithelium, and the IKKα-KO phenotype was rescued by overexpression of cyclin D1, placing NF-κB signaling upstream of cyclin D1 in alveolar proliferation (Cao *et al.* 2001, Cao & Karin 2003).

In ovariectomized PRL-KO mice, replacement with both P_4 and PRL strongly stimulated RANKL expression, and this combination of hormones also induced RANKL in cultured primary mammary epithelial cells (Srivastava

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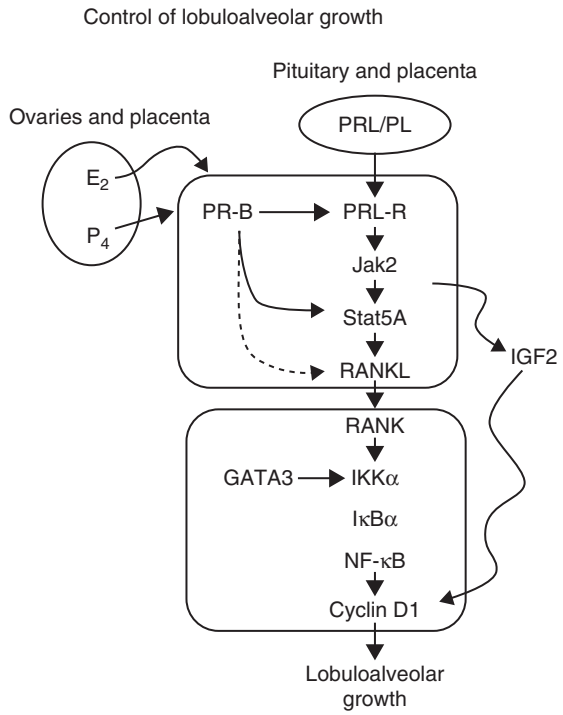

Figure 1
Control of mammary gland lobuloalveolar growth. Hormones from the pituitary gland, placenta, and ovaries converge on mammary epithelial cells that express receptors for estrogen, progesterone (esp. PR-B), and PRL. Activation of receptor-positive cells induces two mediators, IGF2, which acts as a diffusible autocrine–paracrine mitogen, and RANKL, which acts as a juxtacrine mitogen for neighboring cells. The Zn-finger transcription factor GATA3 is permissive for lobuloalveolar growth by inducing IKKα.

et al. 2003). The induction of RANKL by P_4 and PRL was authentically synergistic, since neither hormone was effective alone. The hormone combinations that induced RANKL also induced alveolar proliferation *in vivo*. Stat5A preferentially mediated induction of RANKL by PRL, whereas Stat5B appeared to not be involved (Srivastava *et al*. 2003). Given that Stat5A and Stat5B are equally effective for most target genes, the preferential control of RANKL through Stat5A is likely to be important for the selective deficiency of alveolar proliferation caused by Stat5A knockout (Liu *et al*. 1997, 1998). Transgenic expression of RANKL caused precocious and arbitrary development of lobuloalveoli in virgin mice, confirming, *in vivo*, that RANKL can substitute for PRL and P_4 during mammary gland growth (Fernandez-Valdivia *et al*. 2009).

Synergistic induction of RANKL by PRL + P_4 is limited to 'hormone-sensing' cells, which express ER, PR, and PRL-R. These cells are scattered throughout the alveolar epithelium and their neighbors express RANK, and cyclin D1 is induced in the neighboring RANK-positive cells (Grimm *et al*. 2002, Ismail *et al*. 2003, Mulac-Jericevic *et al*. 2003). These findings show that PRL induces mammary epithelial proliferation via juxtacrine ligation of RANKL and RANK. RANKL induces proliferation of progenitor cells, which go on to differentiate into milk-producing luminal cells and possibly also into new hormone-sensing cells (Fig. 1).

Two additional factors, Elf5 and Wip1, are important for guiding the cellular responses of PRL-sensitive mammary epithelial cells. Like many other factors, a role of Elf5 (an Ets-family transcription factor) in mammary development was discovered when a lactation defect appeared in knockout mice (Zhou *et al*. 2005, Choi *et al*. 2009). Elf5 is important for determining the alveolar secretory lineage phenotype, and is ultimately expressed in the milk-producing cells, but not in the hormone-sensing cells (Harris *et al*. 2006, Oakes *et al*. 2006, 2008, Lee *et al*. 2011, 2013). Elf5 is induced by PRL and locked into a positive feedback loop with Stat5, so that each transcription factor induces the expression of the other (Choi *et al*. 2009, Yamaji *et al*. 2009, Lee & Ormandy 2012; Fig. 1). This relationship between Stat5 and Elf5 can be conceived of being a mechanism that supports the explosive growth of differentiating mammary epithelium before lactation. The interruption of this positive feedback loop after weaning may also be involved in collapse and remodeling of the glandular tissue, but there does not appear to have been any specific study to test this possibility (Fig. 2).

Although fully differentiated milk-producing cells require Elf5, the hormone-sensing cells require Wip1,

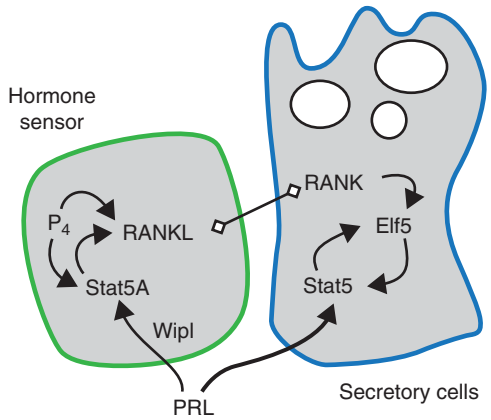

Figure 2
Interactions between hormone-sensing and secretory cells in the alveolar epithelium. Low levels of PRL (thinner arrow) are able to activate hormone-sensing cells and induce RANKL. Higher levels of PRL (thicker arrow) are required to stimulate differentiation of secretory cells. Growth and differentiation of the secretory epithelium are mediated by indirect and direct actions respectively.

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a PP2C family Ser/Thr protein phosphatase (Zhu & Bulavin 2012). Lack of Wip1 resulted in poor alveolar growth, traceable to deficient RANKL and IGF2 induction by PRL in hormone-sensing cells. Wip1 increased the sensitivity of hormone-sensing cells to PRL, so that Stat5A is activated at virgin and early pregnancy levels of PRL (before elevated placental lactogen). Erk activation downstream of ErbB-2 (Her2/neu) was also potentiated by Wip1 (Tarulli *et al*. 2013). The effects of Elf5 and Wip1 illustrate how cell context is an important determinant of PRL action during mammopoiesis and lactogenesis.

We want to return to the role of extrapituitary PRL. Although primates have a more exaggerated extrapituitary PRL system, expression of PRL in various somatic tissues has been reported several times in rodents (Ben-Jonathan *et al*. 1996), and a recent study has shown that rodent PRL may be transcribed from a nonclassical promoter in extrapituitary tissues (Emera *et al*. 2012). Two studies have shown that locally expressed PRL is physiologically important in the mammary glands of mice during *postpartum* secretory activation (Naylor *et al*. 2003, Chen *et al*. 2012). The first study showed that PRL-KO mammary epithelium failed to undergo a final round of proliferation on *postpartum* day 1, immediately before secretory activation (Naylor *et al*. 2003). In the second study (Chen *et al*. 2012), it was shown that PRL expression in mammary epithelial cells was induced by the Pten–Akt pathway during late pregnancy and early lactation. Activation of Akt, or suppression of Pten, caused precocious secretory differentiation of mammary epithelium. In the absence of Akt signaling, there was no induction of local PRL expression during the peripartum. These results indicate that even in rodents, where the extrapituitary PRL system is only rudimentary compared with primates, local PRL provides specific target organs, such as the mammary glands, a mechanism to enhance their PRL response at critical times.

Parathyroid hormone-related peptide induction during lactation

Parathyroid hormone-related peptide (PTHrP) is secreted in large quantities from the lactating mammary glands, causing bone resorption and other physiological effects (Wysolmerski 2012). Because PTHrP secretion is lactation-specific, PRL is implicated in its regulation, but until recently the mechanisms that induce PTHrP were unknown. Bone mobilization is essential during lactation because of the large amount of calcium that is exported into milk. If the flux from the bone calcium pool were not stimulated during lactation, blood calcium would become unstable because of hysteresis in the normal calcium homeostasis pathways.

Recent studies have shown that serotonin (5-HT) controls the expression and secretion of PTHrP in the mammary glands (Hernandez *et al*. 2012). The mammary gland 5-HT system was discovered by identifying PRL-induced genes through gene expression profiling (Matsuda *et al*. 2004). The synthesis of 5-HT in mammary epithelial cells is stimulated by dilation of the mammary gland alveoli in response to PRL-induced secretion. Consequently, 5-HT synthesis is elevated during pregnancy and lactation, and is further increased during milk stasis, when milk is not removed (Matsuda *et al*. 2004). Induction of PTHrP by 5-HT is mediated by 5-HT2B receptors, which are G-protein coupled (Gq/11) (Hernandez *et al*. 2012). While 5-HT and PTHrP are ultimately dependent on PRL, it is important to emphasize that this is an indirect relationship, mediated by dilation of the alveoli. The mammary gland 5-HT system and its control of bone mobilization are reviewed in more detail elsewhere (Horseman & Hernandez 2013).

PRL actions on skin appendages other than mammary gland

Given the close evolutionary and developmental relationships between the mammary glands and other skin appendages, it comes as no surprise that recent studies have provided substantial evidence that PRL plays important roles in the skin. These effects have been reviewed recently (Foitzik *et al*. 2009), so they will be only briefly considered here. In PRL-R-KO mice the hair cycle is disrupted such that molting occurred earlier and there was a reduced duration of the telogen phase (Craven *et al*. 2001, 2006). Both PRL-R and PRL have been detected in human hair follicles and skin glands, and human PRL has been associated with skin pathologies such as psoriasis and alopecia (Foitzik *et al*. 2009). Humanized PRL mice are likely to be an important tool for studying the potential role of PRL in human skin. These mice have been observed to undergo patchy hair loss without any obvious extrinsic cause or skin pathology (unpublished).

16K PRL: fascinating pathophysiology

A proteolytic fragment of the full-length 23 kDa PRL polypeptide, termed 16K PRL, was discovered in the 1980s (Mittra 1980*a*,*b*, Clapp 1987). Generation of 16K PRL actually involves multiple cathepsin D-sensitive cleavage

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sites in the long loop connecting the third and fourth α-helices. Reduction of the disulfide bridge connecting the N- and C-termini releases several closely related active N-terminal peptides (Piwnica *et al*. 2004). Further studies have shown that 16K PRL is a potent anti-angiogenic peptide and these activities have been termed as 'vasoinhibins' (Ferrara *et al*. 1991, Clapp *et al*. 1993, 2006, Lee *et al*. 1998). These fragments do not bind to the conventional PRL-R (Clapp & Weiner 1992). None of the known physiological effects of PRL have yet been attributed to 16K PRL, and the close correspondence of phenotypes in the PRL-KO and PRL-R-KO mice suggests that 16K PRL is not required for normal physiological actions of PRL.

Recent studies have pointed to 16K PRL as being a causal factor in peripartum cardiomyopathy (Hilfiker-Kleiner *et al*. 2007, 2012, Yamac *et al*. 2010, Dalzell *et al*. 2011). Peripartum cardiomyopathy is an uncommon but challenging pathology, and there may be multiple etiologies involved. Stat3 deficiency increased cathepsin D activity in the myocardium, leading to enhanced production of 16K PRL during the peripartum when PRL levels are high. As a consequence, the cardiac capillary network was impaired, leading to cardiomyopathy (Hilfiker-Kleiner *et al*. 2007). A causal role of 16K PRL in human peripartum cardiomyopathy was established by treating women at high risk of peripartum cardiomyopathy with bromocryptine to inhibit PRL secretion after delivery (Hilfiker-Kleiner *et al*. 2007, 2012, Dalzell *et al*. 2011).

The link between PRL and cardiomyopathy presents a very interesting example of pathophysiology, in which the high levels of PRL during the peripartum interacts with enhanced proteolytic activity, possibly related to oxidative stress (Hilfiker-Kleiner *et al*. 2007, 2012, Yamac *et al*. 2010). This combination of events converts PRL into one or more molecules that damage the tissue. The peripartum may be unique in exposing tissues to both PRL and active protease levels that are sufficient to generate 16K PRL in amounts that cause these effects. In contrast, hyperprolactinemia from prolactinomas has not been associated with cardiomyopathy or other obvious 'vasoinhibitory' disease, so a high PRL level, *per se*, does not appear to be sufficient to lead to pathologically elevated 16K PRL.

Summary and conclusions

Mouse genetic models and other experimental approaches have contributed importantly to a better understanding of PRL actions. Although the phenomenon of extrapituitary PRL expression has been known for quite some time, in recent years a few experiments have been able to show, *in vivo*, that locally expressed PRL is physiologically important. This is true even in rodents, which express PRL in fewer tissues, and at lower levels than do primates. A new model system the expresses human PRL under the control of human regulatory elements expresses PRL in a wide variety of tissues, and may finally bring real clarity to the roles of non-pituitary PRL expression.

We have gained a much clearer understanding of the cell biology and molecular signaling by which PRL controls lobuloalveolar proliferation and differentiation. Epithelial growth is driven by the combined effects of P_4 and PRL, which induce a juxtacrine RANKL signal that induces alveolar growth. The Elf5 transcription factor plays a key role in driving the differentiation of secretory epithelium and supporting its proliferation.

One PRL target organ that has not received enough attention is the integument, and particularly the hair follicle. The humanized PRL mice may provide new ways to address this target. Another area of particular human clinical relevance is the apparent involvement of PRL, specifically proteolytic fragments of PRL, in the etiology of peripartum cardiomyopathy. Here again, it may be possible to address this pathology and the mechanisms underlying in humans through the use of humanized PRL mice.

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

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Author contribution statement
N D H and K A G conceived of and authored the manuscript.

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