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Prolactin Regulation of Mammary Gland Development

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Abstract Mammary morphogenesis is orchestrated with other reproductive events by pituitary-driven changes to the systemic hormone environment, initiating the formation of a mammary ductal network during puberty and the addition of secretory alveoli during pregnancy. Prolactin is the major driver of development during pregnancy via regulation of ovarian progesterone production (in many species) and direct effects on mammary epithelial cells (in all species). Together these hormones regulate two aspects of development that are the subject of intense interest: (1) a genomic regulatory network that integrates many additional spatial and temporal cues to control gene expression and (2), the activity of a stem and progenitor cell hierarchy. Amalgamation of these two aspects will increase our understanding of cell proliferation and differentiation within the mammary gland, with clear application to our attempts to control breast cancer. Here we focus on providing an over-view of prolactin action during development of the model murine mammary gland.

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Abbreviations

Blg	β-lactoglobulin
BrdU	5-bromo-2'-deoxyuridine
CALLA	membrane metallo-endopeptidase
Cdc6	cell division cycle 6 homolog (*S. cerevisiae*)
Cis	cytokine-inducible SH2-containing protein
dpc	days post-coitus
dpp	day post-partum
Er	estrogen receptor
Elf5	E74-like factor 5
Egf	epidermal growth factor
Egfr	epidermal growth factor receptor
Erbb4/ Her4	v-erb-b2 erythroblastic leukemia viral oncogene homolog 4/Hairy-related 4
FACS	fluorescence activated cell sorting
Fak	focal adhesion kinase
Gal	galanin
Gata3	GATA binding protein 3
GH	growth hormone
GnRH	gonadotropin-releasing hormone
Igf2	insulin growth factor 2
Jak2	Janus kinase 2
LH	leutinising hormone
Mcmd	mini chromosome maintenance deficient homolog (*S. cerevisiae*)
MEC	mammary epithelial cell
MMTV	mouse mammary tumor virus
MUC1	mucin 1
PgR	progesterone receptor
Prl	prolactin
Prlr	prolactin receptor

Rank	receptor activator of NF-κB
RankL	receptor activator of NF-κB-ligand
Socs	suppressor of cytokine signaling
Srebf/ Srebp1	sterol regulatory element-binding protein 1
Stat5	signal transducer and activator of transcription 5
TDLU	terminal ductal lobular units
Tgfβ	transforming growth factor beta
Wap	whey acidic protein

Introduction

Mammary glands are a defining feature of mammals—specialised milk producing sweat glands that allow the feeding of offspring. Hormonal signals elicited by the ovaries and pituitary gland co-ordinate the development of the mammary gland during puberty, however, it is the pituitary hormone prolactin (Prl) which plays a major role in driving the pregnancy and postpartum development of the mammary gland to produce successful lactation. Due to the limited availability of human breast tissue, the mouse mammary gland is the model in which Prl action is studied, as the basic biology and microscopic anatomy of the mammary glands of human and mice is similar [1].

The majority of mammary gland development occurs during puberty and pregnancy. Puberty in both the mouse and human is controlled by hormonal signals elicited by the hypothalamic–ovarian–pituitary axis. In the human, the first release of gonadotropin-releasing hormone (GnRH) from the hypothalamus signals the onset of puberty. GnRH stimulates the release of leutinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which in turn act upon the ovaries to promote the maturation of the ovarian follicles and the subsequent release of estrogen and progesterone from the corpus luteum [2]. The maintenance of the corpus luteum in the mouse requires both Prl and LH stimulation, whereas in the human, only LH is required for corpora leutea function—illustrating a difference in Prl function between mouse and human. In the mouse, estrogen, growth hormone and progesterone predominantly control the establishment and branching of the mammary ductal epithelium during puberty. During pregnancy, increased progesterone and prolactin (Prl) secretion results in development of the milk secreting lobuloalveoli. The effect of Prl on mammary gland development is both indirect, via modulation of the systemic hormone environment, and direct, via binding to Prl receptors (Prlrs) within the mammary epithelium [3, 4].

The mammary epithelium is composed of three main cell types, the ductal and alveolar luminal cells, and the surrounding contractile myo-epithelial cells. At birth, a rudimentary ductal anlage is present in the mouse, which extends isometrically until puberty. During this time, bulbous terminal end buds form at the ends of the immature ducts [5]. At puberty, the body cells within the terminal end buds proliferate, resulting in ductal elongation, and the terminal end buds bifurcate to form Y-shaped ductal branch points. Once the terminal end buds reach the periphery of the fat pad, or close proximity to other ducts, they differentiate into quiescent alveolar buds. Concomitantly, and additionally with each estrous cycle in the mouse, lateral buds emerge from the ducts, forming secondary and tertiary ductal side branches which also terminate in alveolar buds. Analogous development occurs in the human breast but with some differences. Multiple separate ductal networks extend from the nipple, as in some other species, and the alveoli are arranged into groups termed terminal ductal lobuloalveolar units, surrounded by a fibroblastic sheath. The process of ductal morphogenesis establishes a complex network of ducts and alveolar buds that remain relatively quiescent until a pregnancy occurs.

Alveolar morphogenesis is initiated in mice by coitus, providing nervous stimulation of pituitary prolactin secretion that also sustains ovarian progesterone secretion for up to 10 days, regardless of embryo implantation [6, 7]. These hormones induce rapid and global proliferation of epithelial cells within the ductal epithelium and developing alveoli, increasing both epithelial cell number and surface area [8]. During the second half of pregnancy the cells of the alveoli differentiate and polarise to form the secretory alveolar epithelium, capable of milk production and secretion during lactation. Progesterone withdrawal in both humans and mice brings about the onset of secretory activation, which is characterised by closure of tight junctions and the movement of milk and lipids into the alveolar lumina [8]. At weaning, the newly formed alveolar epithelium undergoes apoptosis, and along with extracellular matrix remodelling leaves the ductal framework in readiness for the next pregnancy. The stages of mammary gland development are schematically represented in Fig. 1.

The ability of the mammary epithelium in all species to undergo several rounds of proliferation, differentiation and apoptosis first led researchers to hypothesise the existence of a population of multipotent self-renewing mammary stem cells (For review see [9]). This hypothesis was supported by the ability of a small fragment of mouse mammary tissue to recapitulate a fully functional ductal tree when transplanted into a cleared mammary fat pad of another mouse [10]. The capacity of these mammary stem cells to generate a complete mammary outgrowth was maintained throughout the lifespan of the mouse, suggesting that mammary stem cells are long-lived [11]. Serial transplants that were clonally derived from the primary mammary outgrowth, suggested that mammary stem cells

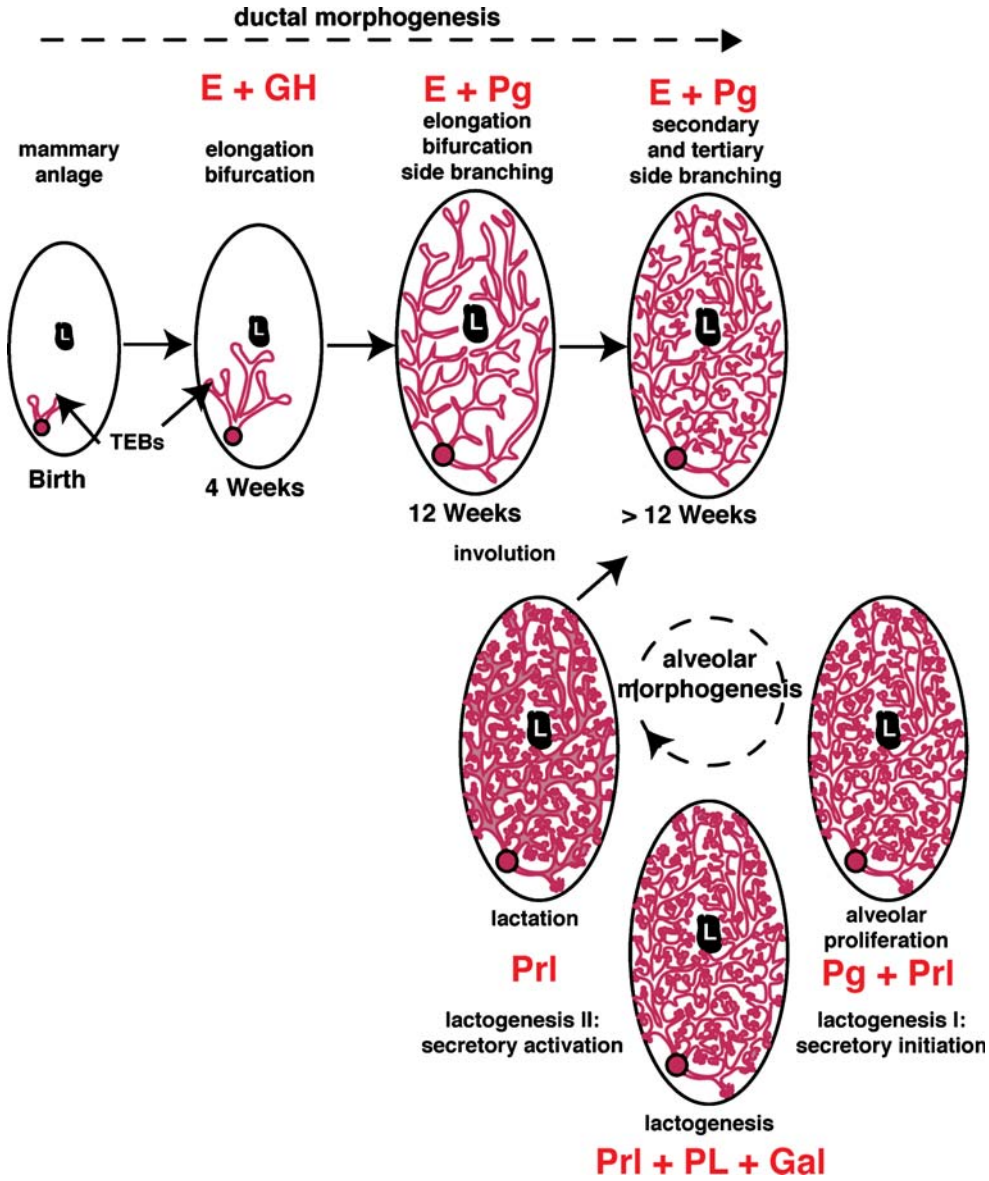

Figure 1 Stages of mammary gland development. At birth the mammary epithelium exists a rudimentary ductal tree comprised of only a small number of ducts. The epithelium grows isometrically until puberty when the terminal end buds (*TEBs*) undergo a rapid increase in proliferation in response to growth hormone (*GH*) and ovarian estrogen (*E*) resulting in ductal elongation and bifurcation, extending the epithelium throughout the fat pad. Ovarian progesterone (*Pg*) induces ductal side branches that sprout from the lateral surfaces of the ducts resulting in further ductal arborisation. This process is collectively termed ductal morphogenesis. Once the epithelium reaches the periphery of the fat pad, the TEBs regress to form alveolar buds. Coitus initiates prolactin (*Prl*) and progesterone secretion, which results in alveolar morphogenesis, and begins with a rapid increase in alveolar proliferation, which continues until mid-pregnancy. This is followed by alveolar differentiation, termed lactogenesis I (*secretory initiation*), which results in the expression of milk genes in a temporal order, and is regulated by Prl, placental lactogen (*PL*) and Galanin (*Gal*). The last phase of alveolar differentiation or Lactogenesis II (*secretory activation*) requires the withdrawal of progesterone, and results in milk and lipid movement into the alveolar lumens ready for lactation. Milk ejection maintains lactation, and after weaning the mammary gland undergoes rapid programmed cell death (*involution*), returning the gland to a near-virgin state.

were also self-renewing [12]. The existence of committed progenitor cell lineages was then hypothesised by limiting dilution mammary epithelial cell transplantation, which resulted in complete mammary outgrowths, as well mammary outgrowths with limited developmental capacity [13]. Thus it was hypothesised that the mammary epithelium is composed of a primary mammary epithelial stem cell, which gives rise to epithelial progenitor lineages that ultimately differentiate into the different mature cell types in the mammary epithelium [9].

More recently a self-renewing multipotent stem cell was isolated from a haemopoetic and endothelial depleted cell

population from the mouse mammary gland, based on increased expression of the cell surface markers CD24 (Heat stable antigen) and either CD29 (β1-integrin) or CD49f (α6-integrin) [14, 15]. With limited efficiency, single cells from this population were able to reconstitute a complete mammary ductal tree with full developmental capacity which could be serially transplanted [9], demonstrating the self-renewing potential of a subset of this population. Furthermore, CD24 positive populations with reduced expression of CD29, which were also cytokeratin-18 positive, could be further differentiated into lineage limited luminal progenitor cells and differentiated luminal epithelial cells based on the expression of CD61 (β3-integrin) [16]. This body of work confirmed the existence of both mammary stem and committed progenitor cells.

A putative population of mammary stem cells has also been identified in the human breast, however the markers used to define the human breast stem cells differ from those used to define murine mammary stem cells. MUC1, CALLA/CD10 and ESA have been used to define different populations of progenitor cells in the human mammary epithelium. $MUC1^{+}$ $CALLA^{-}$ ESA^{+} population enriches for luminal cell progenitors, whereas the $MUC1^{-/low}$ $CALLA^{low/+}$ ESA^{+} population can give rise to either luminal epithelium or basal/myoepithelium [17]. Therefore while the human and mouse mammary epithelial cell hierarchy may be similar in structure, the molecular basis for them has potentially diverged. The identification of genes that both characterise and regulate these stem and progenitor cells in both the mouse and human mammary glands is a subject of intense focus, as the longevity and proliferative capacity of these cells potentially implicates them in carcinogenesis [18–21]. The specification of alveolar cell fate from mammary stem/progenitor cells in both humans and mice is likely to be controlled by the regulatory networks that control alveolar morphogenesis, and thus those induced by the pituitary hormone Prl.

Mouse models of altered Prl signaling have revealed the essential functions of Prl during establishment of the post-pubertal ductal framework, and additionally the secretory alveolar epithelium during pregnancy [22–26]. To perform these functions, Prl signalling activates multiple regulatory networks within specific cellular compartments, ultimately resulting in the proliferation and differentiation of the secretory alveolar epithelium. The challenge to biologists today, is to integrate our expanding knowledge of Prl action and its downstream effectors, with the emerging information of the mammary cell hierarchy. In this review we will précis the role of Prl signaling in the regulation of mammary ductal and alveolar morphogenesis specifically in the mouse. We will then examine the current literature regarding the components of the genomic regulatory networks that exert Prl's effects, and provide evidence that various components of the Prl signaling pathway may be involved in the specification of mammary epithelial cell fate.

Prolactin, Prolactin Receptor and Prolactin Signaling

Prolactin is a 23 kDa polypeptide hormone produced by the lactotrophic cells of the anterior pituitary [27]. Prl is a member of a large family of related hormones including growth hormone and placental lactogen [28]. Prl is also produced at several extra-pituitary sites such as the mammary epithelium, placenta, uterus, brain and immune system (reviewed in [29, 30]). Pituitary Prl output is under hypothalamic control via a dopamine-based mechanism, including responses based on nervous stimulation such as the suckling reflex during lactation or the pseudo-pregnancy observed in rodents in response to coitus [7, 30, 31]. Extra pituitary control is regulated by local factors and utilises a different region of the prolactin gene promoter [32]. The neuropeptide galanin also regulates pituitary Prl output, as it modulates estrogen-induced lactotroph proliferation [33, 34], thus serum Prl levels are significantly reduced in galanin knockout mice [34]. Galanin also synergises with mammary-derived Prl by augmenting alveolar differentiation via a mammary epithelial cell autonomous mechanism [35]. Several forms of Prl exist, each with different post-translational modifications, which may be important for modulating Prl action [36]. Phosphorylation of Prl appears to be the most critical modification [37, 38], and has been reported to have both agonistic and antagonistic roles [39–41]. Treatment of mice with a molecular mimic of phosphorylated Prl (S179D) results in failed secretory activation and almost complete attenuation of Prl-induced gene transcription [41].

Prl binds the prolactin receptor (Prlr), a class I cytokine receptor superfamily member [42, 43]. A number of isoforms of the Prlr exist that differ in the length of their cytoplasmic tails [44, 45], which are the products of mutation in a rat cell line (intermediate form), alternative splicing in the mouse, and a mixture of alternate splicing and post translational modification in humans. The expression of the various isoforms of Prlr vary at different time points of mammary development and during the estrous cycle, implying a regulatory role for these isoforms during development [46, 47]. Prl, placental lactogen and primate growth hormones bind the Prlr [30, 48], which results in the activation of various signaling pathways including Jak2/Stat5 [49, 50], Shc/Grb2/Ras/Raf/Mek/MapK [51–54], PKB/PI3K [55, 56], Vav [57] and Bag1/Bcl2 [58]. The signaling cascades induced by Prl have been recently reviewed [59, 60].

It is generally accepted that Prlr dimerization occurs upon binding ligand binding [61–63]. However Prlr dimerization may also occur in the absence of ligand [64]. The kinase Jak2 is constitutively associated with the membrane proximal region of all isoforms of the Prlr [65, 66]. Activation of Prlr by ligand then results in the tyrosine phosphorylation and activation of Jak2 [67, 68], which in turn phosphorylates specific tyrosine residues in the long and intermediate, but not the short isoforms of the Prl [66, 69]. Stat5 is then recruited to the receptor and phosphorylated by Jak2 [70]. Phosphorylated Stat5 then dimerizes and translocates to the nucleus where it can activate the transcription of genes involved in alveolar morphogenesis [71], including the milk protein β-Casein [72]. The short isoform of the Prlr acts as a dominant negative for β-Casein transcription through heterodimerization [73, 74], but can activate MapK resulting in cellular proliferation [75]. Other transcriptional targets of this pathway will be discussed later in this review. Although Prl induced activation and nuclear translocation of Stat5 is accepted as the canonical Prl induced pathway involved in alveolar morphogenesis [76], there is some evidence to suggest that Prl signaling may occur independent of Stat5 activation. For example, Prl has been shown to activate nuclear localised Jak2 resulting in the phosphorylation of nuclear factor 1-C2 [77], important for the transcription of the milk gene carboxyl ester lipase [78]. The requirement for the various Prl signaling components during mammary morphogenesis has been demonstrated using genetic manipulation of these components in mouse models of mammary development [22–26].

Endocrine Prolactin is Required for Mammary Ductal Side Branching

The role of Prl during mammary gland morphogenesis has been investigated using Prl ($Prl^{-/-}$) and Prlr knockout ($Prlr^{-/-}$) mouse models, which lack functional copies of the Prl or Prlr genes respectively [23, 79]. Normal pre-pubertal isometric growth of the mammary rudiment is observed in both Prl and Prlr knockout mice, a process which appears to be hormone independent, and requires expression of parathyroid hormone-related protein (PTHrP) in the mammary bud, and the expression of its receptor (PTH1R) in the mammary mesenchyme [80–82]. Terminal end buds formed in both $Prl^{-/-}$ and $Prlr^{-/-}$ mice, which proliferated at puberty resulting in normal ductal elongation and bifurcation. Estrogen [83, 84], growth hormone [85], Epidermal growth factor (Egf) [86], Transforming growth factor beta (Tgfβ) [87–89] and Insulin growth factor (Igf2) [90] have all been shown to modulate terminal end bud proliferation during ductal outgrowth. Further, estrogen receptor (Er) [91] and epidermal growth factor receptor (Egfr) within the stroma [92] are required for pubertal ductal extension. More recently, evidence from mammary specific Erα conditional knockout mice, has demonstrated a cell autonomous role for epithelial Erα during ductal extension, and for modulating the expression of progesterone receptor in the mammary gland [93]. Thus, mammary epithelial ductal extension and bifurcation is under control of a number of factors including the ovarian steroids and growth factors, but does not require Prl or Prlr.

Ductal side branching was severely disrupted in $Prl^{-/-}$ and $Prlr^{-/-}$ mice, demonstrating the requirement of Prl and Prlr during this process. Once the terminal end buds in wildtype animals reach the periphery of the fat pad, they regress and differentiate into alveolar buds [94]. In contrast, the terminal end buds in the mammary glands of mature $Prl^{-/-}$ and $Prlr^{-/-}$ mice failed to regress and differentiate into quiescent alveolar buds [4, 25, 95]. These terminal end bud-like structures were much smaller and contained fewer apical cell layers compared to normal terminal end buds, suggesting that they were indeed quiescent, but had stalled at a stage intermediate between the highly proliferative structures and differentiated alveolar buds [4]. Furthermore, alveolar-like structures, which form throughout the entire epithelium in a mature mammary gland, were absent in mammary glands from $Prl^{-/-}$ and $Prlr^{-/-}$ mice, indicating that Prl is also required for this process.

Mammary transplantation was used to investigate whether these effects were mammary cell autonomous [4, 96]. Ductal side-branching was normal in $Prl^{-/-}$ and $Prlr^{-/-}$ mammary tissue which had been transplanted into a host with a normal endocrine system, suggesting that the effects of Prl signaling on this phase of ductal morphogenesis are indirect, and likely mediated via the systemic hormone environment [4]. Engraftment of Prl heterozygote ($Prl^{+/-}$) pituitaries restored ductal side branching and alveolar budding in $Prl^{-/-}$ mice, thus pituitary-derived Prl appears to be important for this process [95]. Prl and placental lactogen provide trophic support to the corpus luteum, resulting in estrogen and progesterone secretion [97]. This was further indicated in animals lacking the Prlr, which had significantly reduced serum progesterone levels [98]. Progesterone receptors in the mammary epithelium are required for ductal side branching, as demonstrated by progesterone receptor knockout mouse models and mammary transplantation [99–101]. The extent of mammary ductal side branching is also dependent on factors from the stroma, as indicated by mammary stromal–epithelial recombination experiments using different strains of mice [102]. Estrogen treatment of ovariectomised $Prl^{-/-}$ mice resulted in hypertrophy of the terminal end buds and increased ductal elongation, whereas progesterone treatment restored ductal side branching but not alveolar buds,

confirming that progesterone is responsible for mammary ductal side branching. Furthermore, prolonged progesterone exposure restored mammary ductal side branching in Prlr knockout mice [3, 103]. These data demonstrated that Prl binding to Prlr receptors in the corpus luteum, is necessary for progesterone mediated ductal side branching in virgin mammary epithelium [3]. Thus Prl acts indirectly via the pituitary–ovarian axis, to modulate the release of progesterone from the corpus luteum, which in turn binds to mammary epithelial progesterone receptors resulting in ductal side branching.

Mammary Prolactin Receptor is Essential for Alveolar – But Not Ductal – Morphogenesis

To examine the effects of loss of the Prl gene on pregnancy-associated mammary gland development, infertility observed in $Prl^{-/-}$ mice was rescued by progesterone pellet administration. Mammary lobuloalveolar development was morphologically indistinguishable to wildtype at 19 days post coitus (dpc), indicating that Prl is not required during alveolar morphogenesis [95]. Placental lactogen can bind and activate the Prlr [104, 105], providing an explanation for this observation. As the mammary epithelium is a source of extra-pituitary Prl, $Prl^{-/-}$ mammary epithelial transplants were performed to investigate a role for autocrine Prl [96]. Neither Prl produced by the mammary epithelium nor stroma was necessary for lobuloalveolar development, however a 2.8-fold decrease in the rate of proliferation in the alveolar epithelium was observed at parturition. Thus Prl is not necessary for alveolar morphogenesis and secretory activation, due to substitution by PL, but mammary derived Prl produced by the mammary epithelium regulates epithelial cell proliferation during the post-partum secretory activation phase. Knockout of the receptor for Prl revealed the essential role for this receptor–ligand system in mammary gland development.

$Prlr^{-/-}$ mice are infertile due to an implantation defect [79], therefore $Prlr^{-/-}$ mammary epithelium was transplanted into immune-compromised hosts, and alveolar morphogenesis was investigated [4]. Although ductal morphogenesis appeared normal in $Prlr^{-/-}$ mammary transplants, alveolar morphogenesis and milk secretion failed. The mammary epithelium of $Prlr^{-/-}$ mammary transplants failed to undergo both proliferation and differentiation phases of alveolar development. This was accompanied by a complete absence of lipid droplets in the alveolar cytoplasm, secretions within alveolar lumens and the expression of the milk protein β-Casein, all indicative of failed secretory activation [4]. Thus mammary Prlrs are essential for alveolar morphogenesis and secretory activation.

Jak2 and Stat5 knockout mice phenocopy failed lobuloalveolar development in $Prlr^{-/-}$ mammary glands, indicating their critical role in Prlr-mediated alveolar morphogenesis [24, 26]. Mammary specific knockouts of Jak2 and Stat5a have demonstrated that both of these genes have a mammary cell autonomous role during alveolar—but not ductal morphogenesis, and deletion of these genes results in failed alveolar proliferation and differentiation during pregnancy [22, 26, 106]. Thus Prlr signaling via Stat5/Jak2 within the mammary epithelium is essential for alveolar morphogenesis and milk secretion. Stat5 binds to consensus DNA binding sites (GAS elements) within the promoter region of target genes such as the milk protein encoding genes βCasein [71, 107, 108] and whey acidic protein (WAP) [109], and in turn induces their expression. Additional downstream mediators of Prl signaling will be discussed later in this review.

The Prolactin Receptor Heterozygote Mouse—Insights into Prl Actions in the Mammary Gland

Two thirds of Prlr heterozygous mice on a mixed 129SvPas/Ola or 129SvPas/C57Bl/6 background failed to lactate on their first pregnancy [25], which was associated with decreased expression of the Prlr [110], and reduced Stat5 phosphorylation [41]. Sufficient lactation was restored in mice by increasing the expression of the short form of the Prlr [110], suggesting that a critical threshold of Prlr expression is necessary for complete alveolar morphogenesis. However as the short form of the receptor lacks essential cytoplasmic tyrosine residues necessary for Jak2 phosphorylation and subsequent Prlr activation [66, 69], it is unknown how the short form was able restore lactation in these mice. Lobuloalveolar development was variously retarded in individual $Prlr^{+/-}$ animals, and the degree of alveolar development correlated with lactational performance [25]. The phenotype is therefore partially penetrant and is presumably dependent on allelic variants or haplotypes that segregate in a mixed genetic background, as breeding onto a pure C57Bl/6 background results in complete penetrance (Hennighausen L, Lindeman GJ personal communication).

Transcript profiling of lactating and non-lactating $Prlr^{+/-}$ mice on a 129SvPas/129OlaHsd background revealed insights into the possible Prlr-mediated mechanisms during alveolar morphogenesis [41]. Mammary glands taken from $Prlr^{+/-}$ mice that were able to lactate sufficiently had increased expression of genes involved in DNA replication machinery such as Cdc6, Mcmds 2–7, Proliferating cell nuclear antigen, Replicating factor Cs and Cyclin D1 involved in G1-S transition [41]. Thus, rescued lactation in 25% of Prlr mice, may be due Prl-mediated modulation of

cellular proliferation and mitosis within the mammary epithelium, resulting in increased alveolar surface area enabling sufficient lactation. Whether the downstream targets of Prlr signaling within mammary epithelial cells are directly responsible for these effects remain to be confirmed.

Keeping a Check on Prolactin-Signaling

Signaling via the Prlr-Jak2-Stat5 pathway culminates in the expression of milk protein genes including β-casein, which contains Stat5 responsive elements in its promoter [72]. Signaling via this pathway is highly regulated, with numerous enhancers and negative regulators acting to keep Prl signaling in check, as summarised in Fig. 2.

Mammary alveoli are surrounded by a specialised lamanin-rich extracellular matrix termed the basement membrane. Via signalling through its receptors – the integrins – the extracellular matrix plays a regulatory role in maintaining Prl signaling [111, 112]. Deletion of β1-integrin in mammary epithelial cells via Cre-recombinase under the control of the β-lactoglobulin (BLG) and Whey acidic protein (WAP) promoters resulted in failed lactation due to impaired epithelial cell proliferation, lobuloalveolar development and Stat5 phosphorylation [113, 114]. β1-integrin was demonstrated to acutely regulate Stat5 activation in response to Prl in primary mammary epithelial cells [114]. The mechanism by which β1-integrin acts to maintain Stat5 activation is not yet known, however it may be through activation of the Rho GPTase Rac1, as restoration of Rac1 expression in β1-integrin null mammary epithelial cells restores their capacity to differentiate in response to Prl [115].

Similar to β1-Integrin, the receptor tyrosine kinase, ErbB4, acts to maintain Stat5 activation in the mammary gland, and is essential for the maintenance of mammary epithelial cell differentiation during pregnancy. As such, deletion of ErbB4 in the mammary gland during pregnancy, specifically during the second phase of lactogenesis, resulted in defective alveolar differentiation, as indicated by a decrease in the expression of differentiation markers and an inability of these mice to lactate [116]. Proliferation was also affected by the loss of ErbB4 as demonstrated by a decrease in BrdU positive mammary epithelial cells in these glands [116]. In addition, phosphorylation of Stat5 was not detected at 17.5 dpc or 1 day post-partum (dpp) in the ErbB4-null glands [116]. To maintain the differentiated state, ErbB4 enhances Stat5 activation in mammary epithelial cells [116] via a direct interaction between the two molecules resulting in the phosphorylation of Stat5 [117, 118]. Thus, during the late stages of pregnancy, ErbB4 acts to enhance the transcriptional network induced by Stat5 by acting to preserve Stat5 in an activated state, thereby augmenting Prl signaling.

While β1-integrin and ErbB4 act to enhance Prl signaling, four members of the suppressors of cytokine signaling family

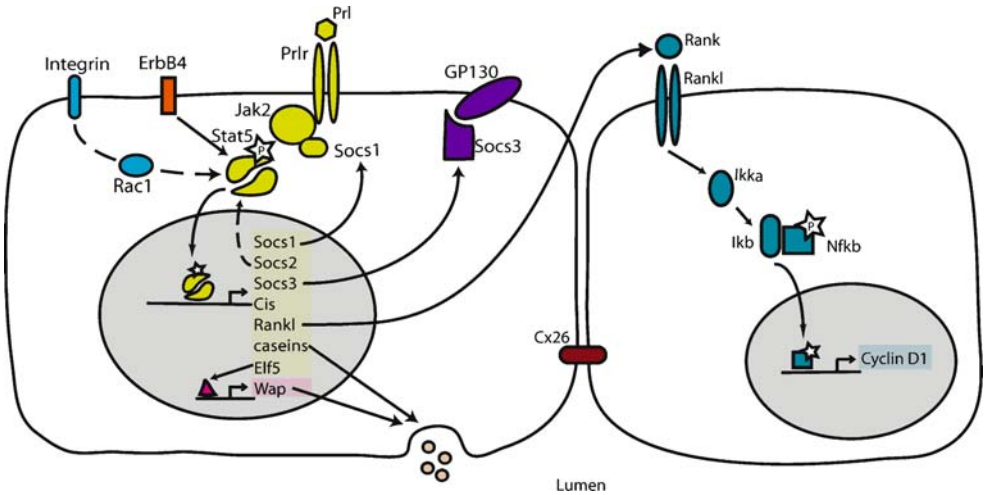

Figure 2 Regulation of Prl Signaling. Prl signalling occurs predominantly via the Jak2/Stat5 signaling cascade. Stat5 is activated by the Prlr associated Jak2, which enables Stat5 translocation into the nucleus, resulting in the transcription of a variety of genes involved in the formation of the lobuloalveolar compartment during pregnancy (*highlighted in green*). ErbB4 and β1-integrin act to enhance Stat5 activation, with ErbB4 acting to directly phosphorylate Stat5. The exact mechanisms by which β1-integrin and Socs1 modulate Stat5 activity are currently unknown, however β1-integrin may act via the Rho GPTase Rac1. Prl signaling also results in increased Socs1 and Socs2 levels, which act in a negative feedback loop to attenuate Stat5 activation. Socs1 does this by directly binding the kinase Jak2, preventing its activation of Stat5. Socs3 is also a target of Prl signalling, which negatively regulates Prl signalling via binding to the receptor GP130, which subsequently leads to epithelial cell apoptosis. Prl induced activation of Stat5 also leads to the induction of Rankl, Elf5 and α- and β-caseins. Rankl acts in a paracrine fashion by stimulating an NfκB signalling cascade in neighbouring cells via its receptor Rankl. Elf5, an Ets transcription factor, binds the promoter of the milk protein gene Wap inducing its transcription, so that it can be secreted into the alveolar lumen with other milk constituents.

of proteins, Socs1, Socs2, Socs3 and Cis, function as negative regulators of the Prl signaling cascade. Expression of all four of these genes can be induced by Prl in mammary epithelial cells [119–121]. Socs1 acts to negatively regulate Prl signaling via a classical negative feedback loop. Prl induces the expression of Socs1, which then in turn binds Jak2, antagonising its association with Stat5 [122]. Socs2 also attenuates Prl signaling via the modulation of Stat5 activation [119]. In contrast, Socs3 acts via binding to the GP130 receptor, which then leads to the increased Stat3 expression [123] and subsequent mammary epithelial cell apoptosis. Studies using mouse knockout mouse models and genetic complementation have defined the roles for the Socs genes in regulating Prl signaling in the mammary gland.

Mice with a null mutation for Socs1 exhibited augmented alveolar development and milk production during pregnancy [124]. This enhanced development was due to increased Prl signaling as demonstrated by increased Stat5 phosphorylation at 1dpp [124]. As further proof of its role in regulating Prl signaling, Socs1 heterozygosity rescued the lactational defect of the $Prlr^{+/-}$ mouse [124]. Similarly, homozygous deletion of both Socs2 alleles in $Prlr^{+/-}$ females restored lactation [119]. Deletion of Socs3 in the mouse mammary epithelium during lactogenesis II (using a Cre recombinase under promotional control of the Wap promoter), resulted in alveolar destruction during lactation and accelerated apoptosis 12 hours after pup removal [125]. This increase in alveolar programmed cell death was so dramatic, that involution was almost fully complete after only 3 days in mice lacking Socs3 in the mammary epithelium [125], a process in wildtype mice that takes approximately 6–8 days [126]. Socs3 expression in the mammary gland is induced by Prl [103] although independently of Stat5 [127]. Socs3 expression is regulated by Prl in both epithelial and stromal cells [127], demonstrating that Prl actions influence multiple cell populations within the mammary gland.

Unlike the Socs1 and Socs3 knockout mice, a null mutation for Cis had no effect on mammary gland development or function [128]. However over-expression of Cis under the control of the β-actin promoter resulted in reduced Stat5 activation and impaired terminal differentiation of the mammary epithelium during pregnancy, which rendered these mice incapable of lactation [129]. This revealed that ectopic Cis expression acts as a negative regulator of mammary development and the Prl signaling pathway. It is feasible that in the absence of Cis expression, another Socs gene is able to compensate for the function of Cis, and hence the absence of a mammary phenotype in the Cis knockout mouse. Further investigation is required to determine the exact role of Cis in regulating Prl signaling. These experiments demonstrate the importance of the members of the Socs family of proteins play in the control of Prl signaling in the mammary epithelium.

Uncovering the Prl-induced Genomic Regulatory Network in the Mammary Gland

At their simplest level genomic regulatory networks illustrate the regulatory interactions that occur within a cell, linking transcription factors to their target genes. However genomic regulatory networks exemplify the complexity of transcriptional regulation that occurs within a cell to allow it to respond to various stimuli. Transcription factors act to induce the expression of target genes by binding to a specific sequence or motif within the promoter regions of their target genes. However transcriptional control is often exerted by multi-protein complexes that bind a cluster of binding sites, collectively known as a regulatory motif, within the promoter region of their target genes. The multi-protein complexes and the regulatory motifs they bind are the fundamentals of genomic regulatory networks.

The recent advent in microarray technology has allowed the identification of many transcriptional targets of the Prl signaling pathway, and the beginnings of a Prl induced genomic regulatory network to be elucidated. This has been achieved by transcript profiling of different models of altered Prl signaling, such as the Prl and Prlr null and heterozygous mice [41, 103], the Gal-knockout mouse [34] and mice treated with a S179D Prl [41]. Targets identified via the examination of these models include Rankl, Connexin 26, Amphiregulin, Srebf1, Gata3 [41, 103], Igf2 [130] and Elf5 [119]. We have chosen to discuss these genes here as many have since been shown via knockout and genetic complementation experiments to play key roles in mammary development and the Prl pathway.

Receptor activator of NFκB ligand (RankL) expression was decreased $Prlr^{-/-}$ mammary transplants [103], and increased in response to Prl stimulation of primary mammary epithelial cell cultures [131], which suggested that RankL is a direct target of Prl signaling. RankL binds to its receptor Rank on the surface of mammary epithelial cells and induces a signaling cascade involving NFκB (reviewed in [132]). Recently, RankL was shown to induce mammary epithelial cell proliferation via the helix-loop-helix protein Id2 [133] whose activation allows cell cycle progression. Thus mammary alveolar morphogenesis in RankL-null mice was impaired. This was attributed due to reduced alveolar proliferation during alveolar morphogenesis, concomitantly with increased alveolar apoptosis due to impaired Akt signaling [134]. These studies indicated a pro-proliferative and anti-apoptotic role for RankL signaling in the mammary epithelium. Indeed a recent study showed that forced mammary specific over-expression of the receptor Rank, in the presence of RankL, increased alveolar proliferation [135]. In addition to Prl, progesterone is also able to regulate the expression of RankL in mammary

epithelial cells [136]. Progesterone exerts its effects via the progesterone receptor, of which there are two isoforms—PgR-A and PgR-B [137]. Defective alveolar morphogenesis in PgR-B knock-out mice was associated with reduced paracrine signaling via RankL, indicating that PgR-B and not PgR-A is responsible for the activation of RankL signaling [136]. The fact that RankL responds to stimulation by both Prl and progesterone indicates that the RankL/Rank pathway is a point of cross-talk between these two hormones. However the specific nature of RankL regulation by Prl and progesterone and whether they cooperate with one another or act at different stages of mammary gland development to induce RankL expression is not yet known. Further evidence for cross-talk between these pathways is the observation that Prlr expression levels increased in MCF7 and T47D cells after progestin treatment [138]. Also, the observation that PgR expression was increased in Prlr-null glands and Prlr expression decreased in PgR-null glands [139], further supports a synergistic relationship of these two receptors. Furthermore, steroid receptor positive cells predominantly dissociate with proliferating cells in the mammary gland, a characteristic of the mammary gland, which is required for ductal and alveolar morphogenesis [140, 141]. Thus the steroid receptor positive cells represent a sensor population, which appear to mediate the expansion of steroid receptor negative luminal cell progenitors [142]. It would therefore be interesting to determine the expression patterns of Prlr within these populations, to further characterise the role Prl plays in regulating these cellular lineages. The development of a highly specific anti mouse Prlr antibody would be a great advantage to further understand this facet of mammary gland development.

The insulin growth factor binding protein, Igf2, was identified as a target of Prl signaling by a transcript profiling experiment that compared the global transcriptome of $Prlr^{-/-}$ mammary glands with that of $CyclinD1^{-/-}$ mammary glands [130]. Due to its decreased expression in $Prlr^{-/-}$ mammary glands and unchanged expression in CyclinD1 mammary glands, Igf2 was deemed to act downstream of the Prlr, but upstream of CyclinD1. Stimulation of primary mammary epithelial cells with Prl also resulted in increased Igf2 expression [130], further indicating that Igf2 is a target of Prl. Using retroviral infection, Igf2 was re-expressed in primary mammary epithelial cells from $Prlr^{-/-}$ mice, and these cells subsequently transplanted into the cleared mammary fat pads of wildtype hosts. After mating, the glands generated from these cells developed small alveolar structures on the ductal tree, however the cells of these alveoli did not terminally differentiate [130], indicating that Igf2 was not a sole mediator of Prl-signaling in the mammary gland. Furthermore, Igf2 knockout mammary epithelial cells were able to form a functional mammary gland upon transplantation, albeit at a slower rate than wildtype cells [130]. These results suggest that other factors can compensate for the loss of Igf2 in the mammary epithelium. Over-expression of Igf2 in the mammary gland resulted in delayed involution due to reduced alveolar cell apoptosis [143]. Delayed apoptosis in these mammary glands was associated with sustained activation of Akt [143], suggesting a survival role for Igf2 in the alveolar epithelium.

Amphiregulin expression was also down-regulated in Prlr-null mammary epithelium in transcript profiling experiments [103]. The amphiregulin gene encodes the ligand for the Egfr receptor tyrosine kinase [144]. Amphiregulin expression in the mammary gland is increased during puberty in response to estrogen [145], and is expressed on the basal surface of mammary epithelial cells. To permit localisation of amphiregulin to its stromally located receptor, amphiregulin is proteolytically cleaved from the epithelial cell surface by Adam17 [146]. Amphiregulin then acts as a paracrine mediator of estrogen induced mammary epithelial cell proliferation and terminal end bud formation [145]. This was demonstrated in mice with targeted deletion of amphiregulin in the mammary gland, which displayed defective ductal morphogenesis [145]. Although amphiregulin has been demonstrated to be an Egfr ligand, it is not known whether Egfr is involved in the paracrine signaling by amphiregulin induced by estrogen. Furthermore, the regulation of amphiregulin by Prl in mammary epithelial cells has not been investigated any further than the initial transcript profiling experiments performed in our laboratory. If amphiregulin expression is indeed regulated by Prl during pregnancy, the ability of this factor to act on neighbouring stromal cells indicates that Prl signaling not only impacts on the mammary epithelial cell population, but on adjacent cell populations of the mammary gland.

Connexin 26 encodes a gap junction protein, whose expression is up-regulated in the mammary gland during pregnancy [147]. Connexin 26 complexes with connexin 30 at the adjacent edge of neighbouring mammary epithelial cells to form gap junctions to allow cell–cell communication (reviewed in [148]). Connexin 26 expression was decreased in $Prlr^{-/-}$ mammary transplants [103], suggesting that it is a target of Prl signaling in the mammary gland. Connexin 26 expression was undetectable in Stat5-null mammary glands [149], indicating that connexin 26 is likely a direct target of Stat5, however this still remains to be proven experimentally. Defective alveolar differentiation and lactation was observed when connexin 26 was deleted in the mammary gland using Cre recombinase driven by the mouse mammary tumor virus promoter (MMTV) [150]. Increased alveolar cell apoptosis was also observed in these glands during pregnancy, indicating that connexin 26 is required for mammary epithelial cell survival [150]. Conversely, when connexin 26 was deleted after the

mammary differentiation program had initiated using the Wap promoter, alveolar differentiation proceeded normally [150], which indicated that connexin 26 does not play an essential role during secretory activation and lactation.

The transcription factor Srebf1 was consistently down-regulated in transcript profiling of three models of altered Prl action resulting in failed secretory activation, suggesting that Srebf1 is a target of Prl signaling [41]. Srebf1/Srebp1 is at the origin of a genomic regulatory network involved in the synthesis of cholesterol and fatty acids, major components of milk [151]. In the normal mouse mammary gland, Srebf1 expression greatly increases during secretory activation, a time when Prlr-signaling is necessary for milk synthesis and secretion [152]. Whether Srebf1 is a direct target of Prlr-mediated gene transcription in the mammary gland has not yet been demonstrated, however these experiments suggest that Srebf1 plays a critical role in Prl/Prlr-mediated secretory activation.

Through the identification of these targets of Prl, the Prl-induced transcriptional networks are beginning to be uncovered. However, while many of the genes discussed here have been hypothesised to be direct transcriptional targets of the Prl signaling mediator Stat5, this has been proven experimentally for only few of these genes. Our expanding data libraries of microarrays of altered Prl action, combined with more comprehensive in vitro and in vivo evidence, will permit the elucidation of a more complete understanding of the transcriptional network directly induced by Prl. The identification of new targets of Prl has uncovered mechanisms of cross-talk between different hormones, mechanisms by which Prl affects multiple cell types, and also how Prl induced targets play key roles in specifying mammary cell fate. Such functions confirm the enormity of the role played by Prl in the mammary gland. This network will almost certainly become more complex when examined within the context of the recent emerging evidence of a mammary stem cell hierarchy.

Gata3—A Transcription Factor Potentially Regulated by Prolactin, that Specifies Mammary Epithelial Cell Fate

Gata3 was demonstrated to be significantly down-regulated in the $Prlr^{-/-}$ mammary transplants at days 2, 4 and 6 of pregnancy [103], indicating that it may be a target of Prlr-induced transcription. Transcript profiling experiments performed independently of these studies, demonstrated that Gata3 expression was increased in the terminal end buds of the pubertal mammary gland compared to the adjacent stroma [153]. More recently, Gata3 was demonstrated to specify ductal and alveolar mammary cell fate [142, 154], implying that Prl may act to specify cell fate in the mammary gland by regulating the levels of transcription factors other than just Stat5.

Gata3 regulates mammary cell fate at multiple time points throughout mammary gland development. Deletion of Gata3 in mammary primordial cells via the use of the cytokeratin 14 promoter to drive Cre recombinase, resulted in the absence of the mammary placode in these mice [16]. MMTV-Cre mediated knockout of Gata3 in the virgin ductal epithelium resulted in defective ductal elongation and invasion, and aberrant terminal end bud formation during puberty [16, 154]. Furthermore, deletion of Gata3 in the alveolar epithelium during lactogenesis II using the Wap promoter to drive Cre, resulted in reduced Stat5 phosphorylation and reduced expression of multiple luminal cell markers [16]. These studies indicated a crucial role for Gata3 in embryonic mammary development, mammary ductal morphogenesis and in the maintenance of the differentiated state of luminal mammary cells during pregnancy.

Visvader and colleagues noted an expansion of the luminal progenitor cell population ($CD29^{lo}CD24^{+}CD61^{+}$) and a subsequent decline in the differentiated luminal cell population ($CD29^{lo}CD24^{+}CD61^{-}$), in both virgin and pregnant mice in which Gata3 had been deleted using MMTV-Cre [142]. Retro-viral re-expression of Gata3 in freshly isolated mammary stem cells ($CD29^{hi}CD24^{+}$), resulted in their differentiation to mature luminal cells and the expression of milk proteins Wap and β-casein in the absence of lactogenic stimuli [16]. Similarly, Werb and colleagues noted that loss of Gata3 (using MMTV-Cre) resulted in an accumulation of a population of luminal epithelial cells, which lacked the expression of multiple differentiation markers. These studies clearly identify a role for Gata3 as determinant of mammary luminal cell fate. Gata3 was additionally shown to directly regulate the expression of the transcription factor Foxa1 [154], a transcriptional co-activator of estrogen receptor signalling [155]. Whether Prl signaling is directly involved in the expression of Gata3 remains to be determined. However, the role of Gata3 in maintaining the differentiation of the alveolar epithelium combined with its decreased expression in Prlr-null mammary transplants, suggests a role for Gata3 in Prl-mediated alveolar morphogenesis, and the specification of mammary cell fate.

Elf5—A Critical Mediator of Prl Signaling

Expression of the epithelial-specific Ets transcription factor Elf5 was increased following Prl and/or galanin treatment

of mammary gland explant cultures [29] and Scp2 cells, which differentiate in response Prl treatment and produce β-Casein [111]. Conversely, Elf5 expression was decreased in $Prlr^{-/-}$ mammary transplants at days 2, 4 and 6 of pregnancy [119], suggesting that Elf5 is indeed a target of Prl signaling. Like Gata3, Elf5 has also previously been identified as playing a role in cell lineage determination, specifically in the extraembryonic ectoderm [156], indicating that Elf5 could also play a role in the specification of mammary cell fate. Indeed, FACS sorting of MECs derived from Elf5-null and Elf5-overexpressing mammary epithelium illustrated that Elf5 drives the specification of differentiated alveolar cells from CD61+luminal cell progenitors [157]. Therefore we hypothesise that Prl may drive the specification of alveolar cell fate via Elf5.

Elf5 is a member of the Ets transcription factor family, and is predominantly expressed in secretory epithelium of organs such as the mammary gland, prostate and salivary gland [158, 159]. Members of the Ets transcription factor family are involved in the differentiation of multiple tissues [160]. Mice lacking Elf5 are unable to survive due to a placentation defect that occurs during early embryogenesis [156, 161]. However mice lacking just one Elf5 allele are viable, and displayed defective alveolar morphogenesis, which phenocopied that of the $Prlr^{+/-}$ mouse. Mammary transplantation of $Elf5^{+/-}$ epithelium demonstrated that these effects were mammary epithelial cell autonomous [161], thus Elf5 is essential for alveolar morphogenesis.

Elf5 expression was down-regulated in the $Prlr^{+/-}$ mammary glands, but Prlr expression was unaltered in the $Elf5^{+/-}$ gland, which suggested that Elf5 acts downstream of the Prlr. Decreased milk protein gene expression and lobuloalveolar development observed in both the $Prlr^{+/-}$ [25] and $Elf5^{+/-}$ mammary glands [161], suggested that Elf5 acts downstream of the Prlr to modulate alveolar morphogenesis and secretory activation. It has been hypothesised that Elf5 is a direct target of Stat5 [76], however this has not yet been shown experimentally. The molecular mechanisms downstream of the Prlr, which lead to Elf5 expression requires further investigation. Recently, we demonstrated that retro-viral re-expression of Elf5 in isolated $Prlr^{-/-}$ mammary epithelial cells rescued failed alveolar morphogenesis as a result of loss of Prlr-mediated signaling [119]. These data demonstrate that Elf5 is a bona fide target of Prlr signaling, and is at the origin of a genomic regulatory network responsible for co-ordinating all of the mechanisms that lead to a differentiated alveolar compartment. The role of Elf5 in the differentiation of the alveolar epithelium during pregnancy, as well as its well-defined role as a cell lineage determinant in the embryonic ectoderm, suggests that the Prl target Elf5 may regulate mammary development via the specification of alveolar cell fate.

Conclusion

The pituitary hormone Prl is responsible for the regulation of a complex transcriptional regulatory network in the mammary gland, which controls both epithelial cell proliferation and differentiation. Further dissection of the Prl-induced transcriptional network will allow a distinction to be drawn between the role played by Prl in mammary epithelial cell proliferation and the role it plays in mammary epithelial cell differentiation. Prl must now also be considered within the context of the emerging members of the mammary cell hierarchy, as many of its target genes play a critical role the differentiation of the alveolar epithelium, and may achieve this via specification of mammary cell fate.

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