ABSTRACT: At face value there are clear and established roles for prolactin (PRL) in the regulation of mammary gland growth, lactogenesis, and galactopoiesis. These actions of PRL do not occur in isolation; rather, they are finely attuned to and coordinated with many local, reproductive, and metabolic events in the female. Hence, to understand PRL action at the level of the mammary gland is to understand the systemic and local contexts in which it acts and functions. Herein we review the functions of PRL, its receptors, and the pathways leading to the phenotypes it evokes within the mammary glands, including growth and lactation, across a variety of species. At one level, the actions of PRL are mediated by several PRL receptor (PRLR) isoforms, including its long form and various short PRLR variants that are generated by alternative splicing in a species- and tissue-dependent manner. In turn, these PRLR activate a variety of intracellular signaling cascades. We also focus on how PRL coordinates with other endocrine cues to impart its effects on the mammary glands, where the ovarian hormones can independently and substantially modulate PRL action. Many of these effects of PRL are also realized at the local level of the mammary gland, either through the autocrine or paracrine synthesis of a multitude of molecules and transcription factors or through its effects on adjacent supporting tissues, including the mammary vasculature. Taken together, it is clear that PRL directs a variety of mechanisms during growth and function of the mammary gland and is deserving of its classification as the master hormone.

Keywords: angiogenesis, estrogen, lactation, mammary gland, progesterone, prolactin

INTRODUCTION

Prolactin (PRL) was originally identified in the late 1920s and named for its ability to stimulate mammary growth and lactogenesis in several species (Trott et al., 2008). Since then, more than 300 roles for PRL have been identified in a wide range of species including mammals, fish, and birds (Bole-Feyt et al., 1998). Among its various target organs, the mammary gland is one of the most sensitive to PRL. More importantly, PRL exerts multiple effects on this organ, ranging from the stimulation of growth to the initiation of milk synthesis and the maintenance of lactation. To realize these tissue- and stage-specific effects, PRL must collaborate with other reproductive, metabolic, and local cues. The role for PRL in breast cancer is beyond the scope of this review; however, it also likely influences progression of the normal mammary gland to its cancerous state, as discussed elsewhere (Vonderhaar, 1999). Likewise, PRL is part of the larger PRL and GH superfamily that includes various relatives such as placental lactogens and PRL-related proteins that have been reviewed extensively. Herein we focus specifically on some of the pathways and partners that PRL utilizes to realize the diverse phenotypes it incites in the mammary gland. To review these roles for PRL and to identify areas for future research is also a timely tribute to the late Allen “Tuck” Tucker, who made many seminal contributions...
regarding the functions of PRL in livestock and was a mentor, colleague, and friend of many scientists whose work is cited here.

ENDOCRINE PRL

Prolactin is secreted by lactotrophs in the anterior pituitary gland under dopaminergic control from the hypothalamus and exists in numerous molecular forms, primarily because of alternative posttranslational modifications (Freeman et al., 2000). Whereas native PRL is secreted as a protein of approximately 23 kDa, cleavage of the full-length product by cathepsin D results in 16 kDa N-terminal PRL (Clapp et al., 2008). This cleaved PRL still exerts many of the same effects as full-length PRL, including its mitogenic and lactogenic actions on mammary epithelial cells (Corbacho et al., 2002). A large proportion of PRL in the circulation of various species is phosphorylated, a modification that reduces its biological activity and may allow it to function as a PRL antagonist (Freeman et al., 2000). Glycosylated PRL also exists in serum, where its proportional abundance relative to unglycosylated PRL changes during both gestation and lactation (Markoff et al., 1988; Hashim et al., 1990). Surprisingly, the full effects of these modifications on mammary gland function are largely undefined.

Prolactin generally circulates as a free monomeric protein; however, it can also be found in human serum as either a dimer (i.e., big PRL) or complexed with IgG (i.e., big big or macro PRL) that can modify the clearance and bioactivity of PRL (Freeman et al., 2000). Interestingly, because none of these forms is distinguishable by RIA, some diagnoses of hyperprolactinemia in humans are incorrect. Similarly, because measurement of PRL in animals is generally achieved by RIA, the relative abundance of these PRL forms has not been investigated in animals despite their likely presence (Gala and Hart, 1980) and the fact that their concentrations do change during lactation in humans (Kamel et al., 1993). The activity of PRL in the circulation may also be modulated by PRL binding proteins (Trott et al., 2003). We have demonstrated that alternative splicing of the human PRL receptor (hPRLR) gene can exclude the transmembrane region, giving rise to a soluble PRL binding protein (Figure 1; Trott et al., 2003). Whether this mechanism is at play in other species is unclear.

LOCAL PRL SYNTHESIS

The partial failure of hyperprolactinemia treatment in breast cancer patients using long-term dopaminergic suppression of pituitary PRL secretion led to the conclusive demonstration that PRL could also be synthesized by the mammary gland, breast cancers, and breast cancer cell lines (Clevenger et al., 1995). This paradigm-shifting discovery raises important questions for the role of local PRL synthesis during normal mammary function. In rats, expression of PRL mRNA in the mammary glands is increased during lactation (Iwasaka et al., 2000). Along these lines, female mice that were neonatally estrogenized underwent precocious lactogenesis coincident with abundant local expression of PRL mRNA (Hovey et al., 2005). Local overexpression of PRL in the mammary glands of transgenic mice induced a similar phenotype (Manhès et al., 2006). Surprisingly, little has been done to relate any role for local PRL synthesis in dairy animals to functional states such as lactation.

PRL RECEPTORS

PRL Receptor Structure and Function

The PRL receptor (PRLR) is a transmembrane protein comprising an extracellular domain (ECD), a transmembrane domain, and an intracellular domain
(ICD; Figure 1). The ECD binds PRL in a 2:1 stoichiometry of receptor:ligand and activates signaling by the ICD (Goffin et al., 2005; van Aghoven et al., 2010). Generally speaking, the most abundant PRLR is the long isoform (PRLR-LF), whereas other intermediate (PRLR-IF) and short forms (PRLR-SF) also exist, so named for the length of their ICD. The PRLR gene of mammals generally consists of at least 11 exons, with the PRLR-LF generated by alternative splicing from exons 1 through 10 and PRLR-SF generally resulting from alternative splicing to exons 11, 12, or 13. An exception appears to be in ruminants, where exon 9 splices to a splice donor site at 40 to 41 bp upstream of exon 10, producing premature in-frame stop codons just 5′ of exon 10 (Trott et al., 2011). Interestingly, birds and the leoparded gecko have duplication of the ECD region, yielding a gene of 14 exons that contains 2 ligand-binding units (Xing et al., 2011). The PRLR-IF appears unique to humans and rats, where the human PRLR-IF results from splicing out a segment within exon 10 (Kline et al., 1999), whereas in rats it results from a deletion in exon 10 of the rat PRLR (rPRLR) gene that is found only in Nb2 lymphoma cells (Ali et al., 1992).

Short forms of the PRLR have been identified in the human (Trott et al., 2003), rat (Boutin et al., 1988), mouse (Davis and Linzer, 1989), sheep and goat (Bignon et al., 1997), pig (Trott et al., 2011), cow (Schuler et al., 1997), and carp (San Martin et al., 2007). The mouse and human are the only species with multiple short isoforms; 3 have been identified in mice [mouse PRLR (mPRLR)-PR1, -PR2, and -PR3; Davis and Linzer, 1989] and 2 have been identified in humans (hPRLR-SF1a and -SF1b; Trott et al., 2003). Numerous other PRL variants have also been identified, often with little or no further functional characterization (Nagano et al., 1995; Kline et al., 1999; Laud et al., 2000; Trott et al., 2003).

Prolactin binds 2 molecules of cell surface PRLR to initiate downstream signaling. The traditional model for PRLR activation involves binding of PRL to the first PRLR at binding site 1 that leads to significant structural changes to PRL binding site 2. This complex recruits a second PRLR, which binds to PRL at binding site 2 (Goffin et al., 2005). The PRLR dimers also form independent of ligand and can subsequently bind PRL (Gadd and Clevenger, 2006; Qazi et al., 2006), first at binding site 1 to yield conformational changes in the PRLR before allowing PRL to bind at site 2 (Binart et al., 2010). Subsequent to ligand binding, tyrosine kinases, such as Janus associated kinase 2 (Jak2), Fyn, and Src, that are associated with the ICD cross-phosphorylate each other and phosphorylate tyrosines in the receptor ICD (Hennighausen et al., 1997; Acosta et al., 2003). Signal transducer and activation of transcription (STAT) proteins are recruited to the ICD of the activated receptors, are phosphorylated by Jak2, dimerize, and are translocated to the nucleus, where they bind gamma-interferon activation sites in gene promoters (Hennighausen et al., 1997). Activated Jak2 and Src family kinases activate various pathways leading to cell division or survival (Yamauchi et al., 1998; Acosta et al., 2003; Aksamitienne et al., 2011), including K+ channels (Van Coppenolle et al., 2004).

The PRLR-LF activates STAT1, 3, 5a, and 5b (DaSilva et al., 1996) but does not activate STAT2, STAT4, or STAT6 (Meraz et al., 1996; Bole-Feytson et al., 1998). Little evidence indicates that PRL activation of STAT1 or STAT3 is of major importance for mammary gland function in vivo. However, both STAT5a and STAT5b are required, either individually or, ideally, together, in almost all physiological responses by the mammary glands to PRL (Teglund et al., 1998). In both STAT5a−/− and STAT5b−/− mice, there is an absence of secondary and tertiary ductal branching in virgin mammary development (Teglund et al., 1998; Santos et al., 2010), as occurs in PRL−/− mice (Horsemaman et al., 1997). Signal transducer and activation of transcription 5a is required for alveolar proliferation and differentiation, whereas STAT5b is unable to compensate for the loss of STAT5a because the activity of STAT5b is also reduced in the mammary glands of STAT5a−/− mice (Liu et al., 1997). Conversely, the presence of STAT5a appears to compensate for the loss of STAT5b during pregnancy-associated mammary gland development and lactation in STAT5b−/− mice (Teglund et al., 1998) while being unable to rescue GH signaling (Udy et al., 1997).

The PRLR-IF appears to have different activities in rats and humans. The rPRLR-IF can activate 2.3 kb of rat β-casein or 4 kb of ovine β-lactoglobulin promoter via the Jak2/STAT5 pathway as effectively as the rPRLR-LF (Ali et al., 1992) and stimulates cell growth (Lebrun et al., 1994; Chang and Clevenger, 1996). By contrast, the hPRLR-IF promotes cell survival, but not cell proliferation (Kline et al., 1999), and is therefore unlikely to transduce a differentiative signal via Jak2/STAT5.

Very few signaling pathways have been identified as being activated downstream of the PRLR-SF. This paucity of data partly reflects the fact that the ICD of the PRLR-SF lacks regions that are essential for signaling by the PRLR-IF and -LF (Figure 1), where both Box 1 and Box 2 in the ICD of the rPRLR are needed for Jak2 activation and PRL-induced proliferation (DaSilva et al., 1994). One of the 3 mouse PRLR-SF isoforms, mPRLR-PR1, contains just Box 1 plus unique C-terminal AA (Figure 1). Surprisingly, this isoform activates mitogen-activated protein kinase in 3T3 fibroblast cells (Das and Vonderhaar, 1995) and conversely causes cell death in both uterine and luteal cell lines (Devi et al., 2009). Overexpression of the mPRLR-PR1 in mice in the ab-
sence of the mPRLR-LF did not activate Jak2 or any STAT proteins in the ovary or decidua, but inhibited the DNA binding activity of Sp1 (Devi et al., 2009). By contrast, overexpression of mPRLR-PR1 in the mammary gland rescues the failure of heterozygous PRLR knock-out (i.e., PRLR<sup>+/−</sup>) mice to lactate after their first pregnancy (Binart et al., 2003). The ability of mPRLR-PR1 to activate proliferation in some systems and cell death in others may reflect tissue-specific roles for PRL. The rPRLR-SF, like mPRLR-PR1, also contains only Box 1 and a short unique C-terminal sequence (Figure 1). The rPRLR-SF weakly activates Jak2 in vitro when increased concentrations of Jak2 are present (Lebrun et al., 1995), but the ICD cannot activate downstream pathways in the presence of native Jak2 (Chang and Clevenger, 1996).

Box 1, Box 2, the region between them, and phosphorylation of the C-terminal tyrosine residue (Y382 in the rPRLR-IF and Y580 in the rPRLR-LF; Figure 1) are required for full activation of Jak2 and STAT5 and transcription from milk protein gene promoters (Bole-Feyssot et al., 1998). These regions exist in PRLR-LF from all species as well as the rPRLR-IF (Figure 1). Contrary to this finding, the ovine PRLR-SF has Box 1 but lacks Box 2 and a C-terminal tyrosine (Figure 1); although it can activate the Jak2/STAT5 pathway in vitro, it cannot activate the β-lactoglobulin promoter (Bignon et al., 1999). Whether this result is specific to ruminant PRLR-SF or is an artifact of the in vitro system is unclear. The hPRLR-SF1a, which has both Box 1 and Box 2 and 2 COOH-terminal tyrosines (Y323 and Y329; Figure 1), weakly activates a 2.4-kb rat β-casein promoter via Jak2/STAT5 (Tan et al., 2005) but not a shorter promoter fragment (Trott et al., 2003) and does not transduce a proliferation signal (Tan et al., 2008). This hPRLR-SF1a has 2 tyrosines in the ICD (Figure 1), which may explain its ability to signal, albeit weakly, to the β-casein promoter.

**Dominant-Negative Functions of PRLR-SF**

The ability of PRL with short or truncated ICD to act as a dominant negative against signaling-competent PRLR-LF is well established. The hPRLR-SF1b, rPRLR-SF, and pPRLR-SF (porcupine PRLR-SF (p)) are all potent dominant negatives against PRLR-LF-induced Jak2/STAT5 activation in vitro (Berlanga et al., 1997; Trott et al., 2003, 2011) and, in the case of the rPRLR-SF, in vivo (Saunier et al., 2003). In the case of the hPRLR-SF1b isoform, it interferes with hPRLR-LF signaling through heterodimerization of the 2 receptors (Tan et al., 2005; Qazi et al., 2006) and via increased degradation of hPRLR-LF mRNA in the presence of hPRLR-SF1b (Tan and Walker, 2010). The efficacy of the hPRLR-SF1b dominant negative activity is so pronounced that a ratio of 10:1 LF:SF1b halves the proliferation rate of T47D cells (Tan and Walker, 2010). The potency of this hPRLR-SF1b isoform lends credence to the theory that an increased ratio of LF:SF1b in breast tumors may modify their growth (Meng et al., 2004). Heterodimerization of the ICD of rPRLR-SF and rPRLR-IF blocks activation of Jak2 and Fyn by the rPRLR-IF (Chang and Clevenger, 1996). Likewise, heterodimerization of the ICD of various rPRLR (rPRLR-SF/IF, rPRLR-IF/LF, and rPRLR-SF/LF) all fail to induce cell proliferation (Chang et al., 1998). The fact that rPRLR-IF/LF heterodimers fail to activate proliferation while homodimers of rPRLR-IF or rPRLR-LF can (O’Neal and Yu-Lee, 1994) further emphasizes the importance of homodimers for PRL-activated signaling.

**Regulation of PRLR Expression in the Mammary Gland**

Expression of PRLR in the mammary glands is regulated by both endocrine and local influences. Within the mammary glands of gilts, PRLR expression is regulated in a cell type-dependent manner; whereas hyperprolactinemia induced PRLR mRNA expression in the stroma, exogenous estrogen (E) increased PRL expression in the epithelium (Trott et al., 2009). During lactogenesis and lactation, the abundance of PRLR in mouse mammary epithelial cells is upregulated by glucocorticoids (Sakai and Banerje, 1979). Meanwhile, PRL downregulates the expression of its own receptor, as occurs in lactating rats (Barash et al., 1983). Along these lines, PRLR gene expression and PRL binding in the mammary glands of lactating sows decreases postpartum and then rebounds at peak lactation (Plaut et al., 1989; Manjarin et al., 2012) in an inverse relationship with the concentration of circulating PRL (Farmer et al., 1998). That said, exogenous PRL did not alter the number of PRLR in the mammary glands of pigs (Farmer et al., 1999). On the other hand, increased milking frequency upregulates PRLR abundance in the mammary glands of goats and cows concomitant with increased milk production (Bernier-Dodier et al., 2010).

**FUNCTIONS OF PRL IN THE MAMMARY GLAND**

**Mammary Growth**

Prolactin indirectly affects ductal side branching and terminal end bud regression within the mammary glands of mice. During the estrous cycle of mice, PRL acts through stromal PRLR to elicit ductal side branching and terminal end bud regression (Brisken et al., 1999). Similarly, the mammary glands of prepubertal gilts respond to the mammogenic effects of PRL (Farmer and Palin, 2005), exogenous PRL promotes the proliferation of both epithelial and stromal cells within the mammary
glands of ovary-intact pubescent female mice (Hovey et al., 2001b). All 4 of the mPRLR isoforms (Figure 1) are differentially expressed in the epithelium-free mammary stroma of mice during this period of growth; whereas the mPRLR-LF is expressed constitutively in the stroma at all stages, expression of all mPRLR-SF isoforms declines during puberty, which is coincident with the onset of ductal branching (Hovey et al., 2001b). Whether reduced expression of these PRLR-SF isoforms in the stroma, and their dominant-negative activities, facilitates increased PRLR-LF activity during branching morphogenesis remains untested. Prolactin is absolutely essential for lobuloalveolar development to occur and proceed during puberty (Neville et al., 2002), whereby E and progesterone (P4) cannot facilitate this process in the absence of PRL (Schams et al., 1984).

**Lactogenesis**

Lactogenesis is a 2-stage differentiative process that occurs prepartum and through parturition. During lactogenesis I, the mammary epithelium becomes polarized, begins transcribing certain milk proteins, and assumes a cytological organization consistent with milk synthesis (Neville et al., 2002). Prolactin assumes a key role during the assumption of this state, in conjunction with cues from the extracellular matrix and mechanical cues (Roskelley et al., 1994; Stiening et al., 2008), the supporting actions of glucocorticoids and insulin (Neville et al., 2002), and the context of decreasing P4 concentrations at parturition (Horseman, 1999). During the same window of time, the mammary epithelium transitions from transporting immunoglobulins into the alveolar lumen to a state of secretory activation, where immunoglobulin receptors at the basal surface of epithelial cells are concurrently downregulated by PRL (Barrington et al., 1997). Not surprisingly, the PRLR is required for full differentiation of the mammary gland epithelium in mice (Ormandy et al., 1997).

Lactogenesis II coincides with the synthesis of all milk components, increased absorption of precursors from the perivascular space, closure of tight junctions, the onset of lactose synthesis, and apically directed secretion of milk fat globules (Neville et al., 2002). The combined length of lactogenesis I and II varies among species. For example, lactogenesis I ranges from 3 mo prepartum in goats to 1 wk prepartum in rats, whereas lactogenesis II starts at the time of parturition in pigs and mice, shortly before parturition in dairy cows, and up to a few days after birth in humans (Neville et al., 2002).

Generally speaking, the basal concentration of PRL increases during late gestation and peaks around parturition, then gradually decreases during lactation (Neville et al., 2002). As revealed by multiple studies, PRL is essential for the onset of milk production in all species. Administering bromocriptine to dairy cows before parturition blocked the periparturient PRL surge and the milking-induced postpartum surges of PRL, reducing milk yield by 45% during the first 10 d of lactation (Akers et al., 1981). Likewise, similar treatment of goats with bromocriptine from d 147 of pregnancy to d 4 of lactation blocked the prepartum PRL surge, delayed lactogenesis, and depressed milk yield for the first week of lactation (Forsyth and Lee, 1993). A similar intervention evoked comparable responses in pigs (Farmer et al., 1998).

**Galactopoiesis**

Galactopoiesis is defined as the maintenance of established lactation, as facilitated by frequent suckling or milk removal or both. The role for PRL during galactopoiesis differs across species. Administering bromocriptine to lactating sows completely abolishes lactation (Farmer et al., 1998), whereas lactating goats and cows treated with bromocriptine demonstrate only a small decrease in milk yield (Smith et al., 1974). One interpretation of these findings is that a reduced concentration of circulating PRL is sufficient to sustain milk production in ruminants and that these concentrations remain during bromocriptine treatment. Accordingly, treating cows with quinagolide, a dopamine agonist that is 200-fold more potent that bromocriptine, significantly reduced milk production at peak lactation (Lacasse et al., 2011). One cannot discount the possibility that differences in the galactopoietic activity of PRL across species may reflect the activation of different intracellular pathways by PRL or, for certain cases, cross-reactivity between the actions of PRL, GH, and their respective receptors.

Conversely, exogenous PRL administered during early or peak lactation does not increase milk production by sows (Farmer et al., 1999), cows (Plaut et al., 1987), or goats (Jacquemet and Prigge, 1991) despite the fact that PRL induced a significant increase in the α-lactalbumin content of milk from cows (Plaut et al., 1987). The large doses of exogenous PRL in these experiments may have inhibited the normal pulsatile release of PRL. Basal concentrations of PRL are not associated with milk yield, as shown by the lack of correlation between milk yield and PRL concentrations in cows of different genetic merit (Sorensen et al., 1998), nor does the magnitude of the milking-induced PRL release correlate with milk yield (Lacasse et al., 2011). Accordingly, a reduced dose of exogenous PRL during the first 3 wk of lactation increased milk production from cows (Wall et al., 2006). The galactopoietic effect of PRL may be more related to the density of PRLR abundance or affinity or both than to the circulating PRL concentrations (Plaut et al., 1989; Farmer et al., 1999).
PRL CONVERGENCE WITH OTHER REGULATORS OF MAMMARY FUNCTION

Estrogen

Exogenous E and PRL synergize to stimulate epithelial proliferation in the mammary glands of hypophysectomized-ovariectomized rats (Stoudemire et al., 1975). By contrast, when we treated ovariectomized mice for 5 d with E and PRL, the rate of epithelial proliferation was intermediate between the effects of either hormone alone (Hovey et al., 2001b). These different outcomes may not reflect only timing or dose of hormone or both, but may also reflect the parallel effects of E on PRL secretion by the pituitary gland. By contrast, when we treated hormone-depleted gilts with various hormone combinations, E plus PRL yielded the greatest epithelial cell proliferation and promoted the morphological progression of type 2 terminal ductal lobular units to the more developed type 3 morphotype (Horigan et al., 2009). It is unclear whether any of these differential responses to E plus PRL in the mammary glands of mice and pigs is species specific. The effects of E on the developing mammary glands can be potentiayed by PRL-modulated E signaling or E receptor (ER) expression or both (Frasor and Gibori, 2003). For instance, PRL increased activation of ER in the mammary glands of mice, leading the author to conclude that PRL potentiated ER-dependent signaling (Muldoon, 1981). Locally synthesized PRL can also modulate ER expression, as shown by an increase in ERα abundance and downstream signaling in response to endogenous human PRL (hPRL), but not exogenous hPRL, in MCF-7 cells (Gutzman et al., 2004). These relationships between PRL and ER may also be manifest in breast cancer, where women in the top quartile of PRL concentrations had a 60% greater risk for developing ER-positive tumors compared with women in the bottom quartile (reviewed by Tworoger and Hankinson, 2008).

Prolactin can also induce E-independent activation of ERα, at least in breast cancer cells (González et al., 2009). Furthermore, the combination of E and PRL enhanced activity of the transcription factor activator protein 1 (AP-1; Gutzman et al., 2005), whereas others found that mRNA expression of the early growth response 3 (EGR3) was induced synergistically by E plus PRL (Rasmussen et al., 2010). Conversely, E can also modulate PRL action, where E induces autocrine PRL in breast cancer cells (Duan et al., 2008) further to the established effects of E on lactotroph proliferation and PRL secretion (Freeman et al., 2000). Estrogens also increase PRLR expression in breast cancer cells (Ormandy et al., 1997) and the mammary glands of gilts (Trott et al., 2009).

Progesterone

Beyond the individual effects of P4 and PRL on the mammary glands, compelling evidence indicates that P4 and PRL synergize to promote mammary growth during the estrous cycle and pregnancy. The serum concentrations of both P4 and PRL in rodents fluctuate similarly during parts of the estrous cycle, whereby P4 concentrations begin to increase during proestrus, peak at estrus, and then decrease during diestrus 1 and 2 (Ben-Jonathan et al., 2008). Similarly, PRL concentrations increase during proestrus in response to E-induced secretion (Ben-Jonathan et al., 2008). Prolactin declines slightly during estrus, then dramatically during diestrus 1 and 2 (Ben-Jonathan et al., 2008). Surges in PRL occur twice daily for the first 10 to 11 d of pregnancy in rodents (Ben-Jonathan et al., 2008), whereas serum P4 concentrations increase steadily postcoitus and plateau around d 16 of pregnancy (Virgo and Bellward, 1974). These parallel changes in P4 and PRL concentrations present the potential for these hormones to collaboratively promote branching morphogenesis and alveolar development. Accordingly, mice lacking P4 receptor undergo normal mammary gland (MG) development postnatally (Lydon et al., 1995) but fail to undergo ductal side branching during recurrent estrous cycles and pregnancy-associated lobuloalveolar development (Ismail et al., 2003). Similarly, PRL and PRLR knockout mice fail to develop side-branching and lobuloalveolar structures during pregnancy (Horsemman et al., 1997; Ormandy et al., 1997).

Given these relationships, we examined the coregulation and convergence of P4 and PRL signaling in the mammary glands of nulliparous female mice. Parallel temporal changes in P4 receptor and PRLR mRNA expression across the course of development supported the potential for crosstalk between the effects of these 2 hormones. Specifically, both PR and PRLR are expressed in the mammary glands of mice by 3 wk of age and reach the greatest abundance in mature nulliparous and early-pregnant females (Hovey et al., 2001b). Expression of both receptors declined during pregnancy, diverging only during lactation when P4 receptors became undetectable (Hovey et al., 2001b). In the same study we also showed that P4 receptors and PRLR colocalize to a subpopulation of epithelial cells, indicating that both hormones act on the same target cells (Hovey et al., 2001b). Likewise, P4 receptors and PRLR were coordinately expressed in the mammary glands of mice lacking C/EBP-β (Seagroves et al., 2000), whereas deletion of P4 receptors reduced PRLR expression (Grimm et al., 2002). This coregulation and coexpression of P4 receptors and PRLR supports the notion that a subset of cells responds to the combination of P4 and PRL in the mammary gland.
Several models support a synergistic effect of P₄ and PRL on mammary epithelial proliferation, at least in rodents. Rat mammary epithelium increased cell number 3- to 4-fold during 7 d of treatment with P₄ and PRL, coincident with increased P₄ receptor expression (Edery et al., 1984). In the same way, proliferation was increased in the mammary epithelium of hypophysectomized and ovariectomized adult rats treated with P₄ and PRL (Stoudemire et al., 1975). Likewise, we found that P₄ plus PRL increased epithelial and stromal cell proliferation in 9-wk-old ovariectomized female mice by 400- and 17-fold, respectively (Hovey et al., 2001b). Conversely, the mammary epithelium of nulliparous female pigs did not respond to the combination of P₄ and PRL-activated STAT5 in the promoter region of the β-lactalbumin and β-casein genes (Doppler et al., 2001). For instance, the glucocorticoid receptor and PRLR signaling in vitro. Treatment of T47D cells with P₄ and PRL synergistically activated the distal enhancer of the long-terminal repeat within the mouse mammary tumor virus (MMTV) promoter (Haraguchi et al., 1992; Morabito et al., 2008) via recruitment of a mammary-specific transcription factor and activation of a nearby STAT5 site (Morabito et al., 2008). Importantly, this response to P₄ and PRL occurs independent of P₄ receptor binding domains in the proximal promoter (Morabito et al., 2008). This synergistic response to P₄ and PRL occurred downstream of Jak2, c-Src, and Fyn (Morabito et al., 2008). These data collectively demonstrate that P₄ and PRL converge, at least in part, at the level of intracellular signaling within mammary epithelial cells. In keeping with this notion, the concentrations of insulin receptor substrate (IRS)-1 and IRS-2 are greatest in the mammary epithelium of ovariectomized mice treated with P₄ and PRL (Hovey et al., 2003). A similar situation exists for induction of receptor activator of nuclear factor kappa B ligand (RANKL) mRNA transcription, whereby P₄ or PRL alone can induce its transcription, whereas both hormones were required for epithelial cell transcription of RANKL mRNA in PRL-deficient mice (Srivastava et al., 2003).

**Glucocorticoids**

Glucocorticoids fulfill a critical role during lactogenesis (Casey and Plaut, 2007), where they synergize with PRL to activate the transcription of several milk protein genes (Doppler et al., 2001). For instance, the glucocorticoid receptor-corticoid complex acts with PRL-activated STAT5 in the promoter region of the whey acidic protein and β-casein genes (Doppler et al., 2001). Beyond the effects of PRL and glucocorticoids on milk protein gene expression, both hormones also regulate tight junction formation in cultured mammary epithelial cells, where glucocorticoids potentiate the actions of PRL (Stelwagen et al., 1999). This response occurs after the induction of lactogenesis II by the rapid withdrawal of P₄ (Nguyen et al., 2001). Meanwhile, loss of glucocorticoid receptor in parallel with decreased PRL stimulation leads to postlactational involution of the mammary epithelium (Bertucci et al., 2010).

**Insulin**

Insulin is essential for a successful lactation given that milk secretion is decreased in streptozotocin-treated rats (Lau et al., 1993). Conversely, increased circulating insulin in hyperinsulinemic or euglycemic cows increased milk protein production (Gruniari et al., 1997). Along with PRL and glucocorticoids, insulin is essential for the transcription of milk protein genes such as α-lactalbumin and β-casein (Rosen et al., 1999). Recent evidence indicates that insulin alone increases the transcription of E74-like factor 5 (ELF5) and STAT5, which then furthers the ability of PRL to phosphorylate STAT5, thereby leading to increased milk protein transcription (Menzies et al., 2010). Furthermore, insulin and PRL synergize to regulate the posttranscriptional processing of milk protein mRNA that leads to their increased stability and translational output (Rhoads and Grudzien-Nogalska, 2007).

**LOCAL EFFECTORS OF PRL ACTION IN THE MAMMARY GLANDS**

Prolactin was historically viewed as acting directly on target cells to activate events including mitosis and lactogenesis. Clearly there are also additional local mediators of PRL action on target tissues. Of these, an interesting situation exists with the IGF. Stroma-derived IGF-I is an established local effector of GH action in the mammary gland. Given the homology between GH and PRL, and the similar homologous relationship between IGF-I and IGF-II, we posited that IGF-II may have coevolved as a local regulator of PRL action in the mammary glands. Indeed, our results and those of others point to a role for IGF-II during PRL-induced branching morphogenesis and alveolar development (Brisken et al., 2002; Hovey et al., 2003).

Several other candidate autocrine and paracrine regulators of alveolar development are also positively regulated by PRL, as revealed by transcript profiling of PRLR−/− mammary epithelium. These include wingless-type MMTV integration site family member 4 (Wnt4), the epidermal growth factor receptor ligand amphiregulin, and RANKL (Oakes et al., 2006). Not surprisingly, all of these molecules have also been implicated during
P₄-stimulated alveologenesis, further emphasizing the potential interplay between PRL and P₄. One additional candidate effector of PRL action in the lactating mammary gland is serotonin, a monoamine synthesized from tryptophan by tryptophan hydroxylase (Brunton et al., 2011). Both serotonin and tryptophan hydroxylase are also present in the murine and bovine mammary glands, where they apparently provide autocrine-paracrine homeostatic feedback to oppose PRL-stimulated mammary development and milk secretion (Matsuda et al., 2004). Indeed, treatment of mammary epithelial cells with serotonin suppresses PRL-induced expression of milk protein genes and causes involution of mammary tissue, whereas blockade of serotonin receptors in mammary epithelial cells increases milk protein gene expression (Matsuda et al., 2004). Serotonin also accumulates within the lumen of mammary alveoli, where it increases the permeability of tight junctions between epithelial cells, disrupting the transepithelial gradients necessary for milk secretion (Stull et al., 2007). Hence, PRL relies on autocrine and paracrine factors not only during pregnancy but also during lactation.

**REGULATION OF VASCULAR FUNCTION IN THE MAMMARY GLAND**

The mammary epithelium has an essential requirement for vascular support, where these 2 tissues grow and function intimately together. Capillary density dramatically changes in the mouse mammary gland during pregnancy, lactation, and involution, with the greatest density occurring at the end of pregnancy (Pepper et al., 2000). The intralobular capillaries become highly ordered by d 18 of gestation, appearing in basket-like structures of approximately 70 to 80 μm (Yasugi et al., 1989). In early lactation the basket-like appearance remains and expands, likely as the result of glandular growth (Andres and Djonov, 2010).

New blood vessel development occurs in the form of angiogenesis. Sprouting angiogenesis occurs as the result of several steps, including sprouting, branching, proliferation, differentiation, and remodeling (Adams and Alitalo, 2007), whereas intussusceptive angiogenesis results in the increased density of a capillary network, such as a plexus, through a process of splitting vessels via insertion of tissue pillars (Adams and Alitalo, 2007). All of these blood vessels develop from the preexisting vasculature or, to a lesser extent, from undifferentiated precursors (Andres and Djonov, 2010). Delivery of nutrients from capillaries to the perivascular space for use by the differentiated mammary epithelium is regulated by multiple mechanisms, including blood flow, vasomotor activity, permeability, and new blood vessel growth (Clapp et al., 2008). Vascular permeability changes markedly during the transition period, whereby the rate at which circulating ferritin moves into the perivascular space increases through pregnancy and peaks at midlactation, then starts decreasing during involution back to the concentrations found in nulliparous females (Matsumoto et al., 1994).

**VASCULAR Endothelial Growth Factor**

Vascular endothelial growth factor A (VEGF) is a key regulator of endothelial cell proliferation, vasodilation, and vascular permeability (Ferrara and Davis-Smyth, 1997). The many VEGF isoforms differ in their affinity for heparin and exert their effects through 2 vascular endothelial growth factor receptors (VEGFR): VEGFR-1 and VEGFR-2 (Ribeiro et al., 2007). The VEGFR-1 (also known as Flt-1) binds VEGF with the greatest affinity and is necessary for maintenance of the endothelium, whereas VEGFR-2 (also known as KDR or flk-1) binds VEGF with less affinity but is responsible for exerting all of the biological effects of VEGF, including proliferation and permeability (Ferrara and Davis-Smyth, 1997; Robinson and Stringer, 2001).

Abundance of VEGF mRNA in the mammary glands of pregnant rodents undergoes stage-specific changes. Expression increases during pregnancy, is greatest during lactation, and decreases upon involution (Pepper et al., 2000). These changes are associated with altered abundance of different VEGF transcripts, where VEGF₁₈₈ expression is increased in prepubescent females, then declines to low concentrations in pregnancy, becomes undetectable in lactation, and then increases again during involution. On the other hand, expression of the VEGF₁₂₀ and VEGF₁₆₄ transcripts during midpregnancy and lactation increases more than 2-fold (Hovey et al., 2001a). In the mammary gland of virgin animals, VEGF is localized in the endothelium and ductal epithelial cells, whereas during late pregnancy VEGF is found in the endothelium and ductal and alveolar epithelium (Islam et al., 2010). Two of the targets of VEGF in the mammary gland of mice, VEGFR-1 and VEGFR-2, decline through pregnancy but increase during early lactation and decline to low concentrations again around weaning (Hovey et al., 2001a). Not surprisingly, inactivation of the VEGF gene in the mouse mammary epithelium impaired blood vessel function and angiogenesis, thus decreasing subsequent milk production (Rossiter et al., 2007).

**A Case for PRL-Regulated Vascular Function in the Mammary Gland**

The timing of changes in serum PRL concentrations positions PRL as a likely regulator of vascular function in the mammary gland. Indeed, PRL induced angiogen-
esis from the quiescent vasculature in the chicken cho-
rioallantoic membrane assay (Struman et al., 1999). One
possible mechanism behind this PRL-induced angiogenesis is PRL-induced VEGF expression (Hovey et al.,
2001a; Corbacho et al., 2002). Along these lines, both mouse mammary epithelial cells and rat lymphoma cells increase their transcription of VEGF_{164} and VEGF_{120} in response to PRL (Goldhar et al., 2005).

The expression of PRLR by endothelial cells is variable. Bovine pulmonary artery endothelial cells expressed mRNA for the PRLR-LF (Merkle et al., 2000), whereas bovine brain capillary endothelial cell membranes did not have PRL binding sites (Clapp and Weiner, 1992) and rat retina capillary endothelial cells did not express PRLR mRNA (Clapp and Weiner, 1992; Ochoa et al., 2001). Bovine umbilical vein and thoracic aorta endothelial cells expressed PRLR, with the PRLR-SF predominating (Ricken et al., 2007). The fact that VEGF can be produced by mammary epithelial cells and that at least some endothelial cells have PRLR indicates that VEGF and PRL may coregulate angiogenesis and permeability in a paracrine manner (Corbacho et al., 2002; Goldhar et al., 2005).

Cleaved PRL is a potent inhibitor of angiogenesis that inhibits VEGF-stimulated proliferation in human and bovine endothelial cells (Corbacho et al., 2002). The 16-kDa cleaved PRL inhibited angiogenesis in the growing capillaries whereas it did not affect the quiescent capillaries during the late stage of development (Struman et al., 1999). The 16-kDa PRL also stimulated endothelial apoptosis, supporting its potential role during regression of the mammary gland vasculature (D’Angelo et al., 1999; Dimmeler and Zeiher, 2000). Production of nitric oxide, a potent vasodilator, was increased by the N-terminal PRL product in rat pulmonary fibroblasts and lung alveolar type II cells through stimulation of the inducible isoform of nitric oxide synthase (Corbacho et al., 2000). Given that PRL upregulates VEGF production, that PRLR are found on some endothelial cells, and that cleaved PRL is antiangiogenic, we suggest that a convincing case exists for the control of angiogenesis and vascular function in the mammary gland by PRL.

**SUMMARY AND CONCLUSIONS**

From the preceding overview it is clear that PRL truly is a multifaceted potentiator of mammary growth and function, probably more so than any other hormone. In many ways, these roles are not surprising given that the mammary glands must grow and synthesize milk in a tissue- and species-specific manner in close coordination with various stages of female reproduction. Hence, PRL clearly does deserve the title of master hormone when it comes to its effects on the mammary gland. Although many of the effects of PRL may seem less than novel relative to emerging new discoveries at the molecular level, the consideration of these new findings in the context of their regulation by PRL will likely explain a great deal as we proceed through this era of functional genomics.

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