Investigation of the Transcriptional Changes Underlying Functional Defects in the Mammary Glands of Prolactin Receptor Knockout Mice

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ABSTRACT

Knockout (KO) mice have been created that carry null mutations of genes encoding molecules essential for prolactin (PRL) release, PRL, the receptor for prolactin (PRLR), and various members of the receptor's signaling pathway. This allowed an *in vivo* genetic analysis of the role of PRL in target organ function. In PRLKO and PRLRKO mice, mammary ductal side branching was absent, terminal end bud (TEB)-like structures persisted at the ductal termini well into maturity, and no alveolar buds formed along the ductal tree. Transplants of recombined mammary glands formed from stromal and epithelial elements with and without PRLR showed normal development, while supplementation of progesterone levels in PRLKO animals restored ductal side branching. During pregnancy, PRLR heterozygous animals initially showed normal ductal and alveolar development. However, alveolar development stalled during late pregnancy, preventing successful lactation. This defect could be rescued by the loss of a single allele of the suppressor of cytokine signaling (SOCS) 1 gene. Transplants of recombined glands containing PRLRKO epithelium and wild-type (WT) stroma formed alveolar buds during pregnancy but showed no lobuloalveolar development. Recombinations of WT epithelium and PRLRKO stroma showed normal development, demonstrating that a direct action of the lactogenic hormones is confined to the epithelium, to promote lobuloalveolar development. Transcript profiling of epithelial transplants expressing or not expressing PRLR was used during early pregnancy to investigate the transcriptional response to lactogens underlying this defect. Such profiling has identified a number of genes with well-characterized roles in mammary development, in addition to a number of novel transcripts.

I. Background

The mouse mammary gland develops in four discrete stages: 1) *in utero,* where a rudimentary ductal structure is first produced; 2) during puberty, when

ducts elongate and bifurcate to fill the mammary fat pad; 3) during each estrus cycle, where in a strain-dependent manner, the density of ductal side branches and alveolar buds increases with each cycle; and 4) during pregnancy, where the alveolar buds that formed on the ductal tree give rise to large, lobuloalveolar structures capable of milk production. Following weaning and each estrus, the gland undergoes involution, losing most of the epithelial component gained during the preceding event. In humans, the gland involutes further, with declining ovarian function in later life.

A number of hormonal factors controlling these developmental stages have been described. Embryonic mammary epithelium appears to develop independently of ovarian and pituitary influence, although responsive to hormonal stimuli (Ceriani, 1970). Hormonal replacement in hypophysectomized, ovariectomized, and adrenalectomized mice showed that development of the mammary ducts (resembling pubertal development) was produced by a combination of estrogen and growth hormone, while further alveolar development (resembling pregnancy) required additional progesterone and prolactin (PRL) (Nandi, 1958). These hormonal combinations were shown to produce similar results in serum-free, *in vitro* culture of whole mammary glands, although mammary development did not achieve the extent seen in normal animals (Ichinose and Nandi, 1964; Vonderhaar, 1998).

In rodents, lobuloalveolar development during pregnancy initially depends upon increased PRL production by the pituitary, maintained by the medial preoptic area of the brain in response to cervical stimulation during copulation (Jakubowski and Terkel, 1986). Development becomes independent of the pituitary from midgestation (Collip *et al.*, 1933), due to lactogen production by the placenta's trophoblast cells (Thordarson and Talamantes, 1987). These lactogenic hormones may act indirectly via the modulation of endocrine organs capable of producing mammotrophic factors, such as the ovary, where lactogenic hormones provide trophic support of the corpora luteum, maintaining estrogen and progesterone production (Galosy and Talamantes, 1995), or the liver, where PRL increases output of insulin-like growth factor-1 (IGF-1) (Wennbo *et al.*, 1997). PRL also may direct mammary development via interaction with prolactin receptors (PRLRs) in the mammary gland, which are expressed by the epithelium and the stroma. The mammary PRLR signals via the Jak2 kinase, to activate the Stat5a, mitogen-activated protein (MAP) kinase, and other signaling pathways.

Knockout (KO) mice now allow these hormonal pathways to be dissected by hormone replacement and tissue recombination in intact animals (Hennighausen and Robinson, 1998). These techniques have shown, for example, that a mammary stromal estrogen receptor (ER) is essential for ductal development (Korach *et al.*, 1996; Cunha *et al.*, 1997) and that a mammary epithelial progesterone receptor is required for alveolar development (Lyndon *et al.*, 1995; Humphreys *et al.*, 1997; Brisken *et al.*, 1998). Genetic analysis of PRL action has been

undertaken through creation of KO mice carrying null mutations of genes that act at multiple points in the PRL pathway. For example, genes have been knocked out that encode regulators of PRL secretion (Wynick *et al.*, 1998), PRL (Horseman *et al.*, 1997; Vomachka *et al.*, 2000), PRLR (Ormandy *et al.*, 1997b; Brisken *et al.*, 1999), and PRL signaling pathway members such as Stat5a (Liu *et al.*, 1997). These models have provided new insight into PRL's action in the mammary gland, which is the subject of this review.

II. Mammary Development *in Utero:* Formation of the Ductal Rudiment

At birth, $PRLR^{-/-}$ animals, both male and female, possessed a rudiment of mammary ductal architecture identical to wild-type (WT) animals (Ormandy *et al.*, 1997a). In males, however, the nipples of $PRLR^{-/-}$ animals were destroyed as normal following testosterone production by the fetal testis, leaving a rudimentary ductal system embedded in the mammary fat pad in two thirds of both $PRLR^{-/-}$ and WT males. Both male and female ductal systems underwent normal allometric growth prior to puberty. These observations show that PRL plays no essential role during mammary organogenesis.

III. Development During Puberty and with Each Estrous Cycle: Ductal Branching and Alveolar Bud Formation

A. FAILURE OF DUCTAL SIDE BRANCHING

At the onset of puberty, terminal end buds (TEBs) formed in females of both PRLR genotypes and ductal elongation and bifurcation commenced. Examination of the mammary gland at sexual maturity (Figure 1A and B) showed that the major ducts appear at the same density in mammary glands of all genotypes but that ductal side branching failed in the $PRLR^{-/-}$ animals (Figure 1B). Ductal side-branch density increased with age in WT animals but the complexity achieved by 14 weeks in $PRLR^{-/-}$ females remained virtually unchanged for the life of the animal. A similar effect was seen in the PRLKO ($PRL^{-/-}$) (Horseman *et al.*, 1997), Stat5a KO (Teglund *et al.*, 1998), and the galanin KO (Wynick *et al.*, 1998). Thus, a distinction needs to be drawn between the processes that cause bifurcation and those that cause side branching.

B. PERSISTENCE OF TEB-LIKE STRUCTURES IN $PRLR^{-/-}$ ANIMALS

By 14 weeks of age, the TEBs of the major ducts and side branches in $PRLR^{+/+}$ animals had differentiated to alveolar buds (Figure 1A and C). However, in $PRLR^{-/-}$ animals, a TEB-like structure persisted at the termini of most ducts (Figure 1B and D). In 20-week-old animals, these TEB-like structures

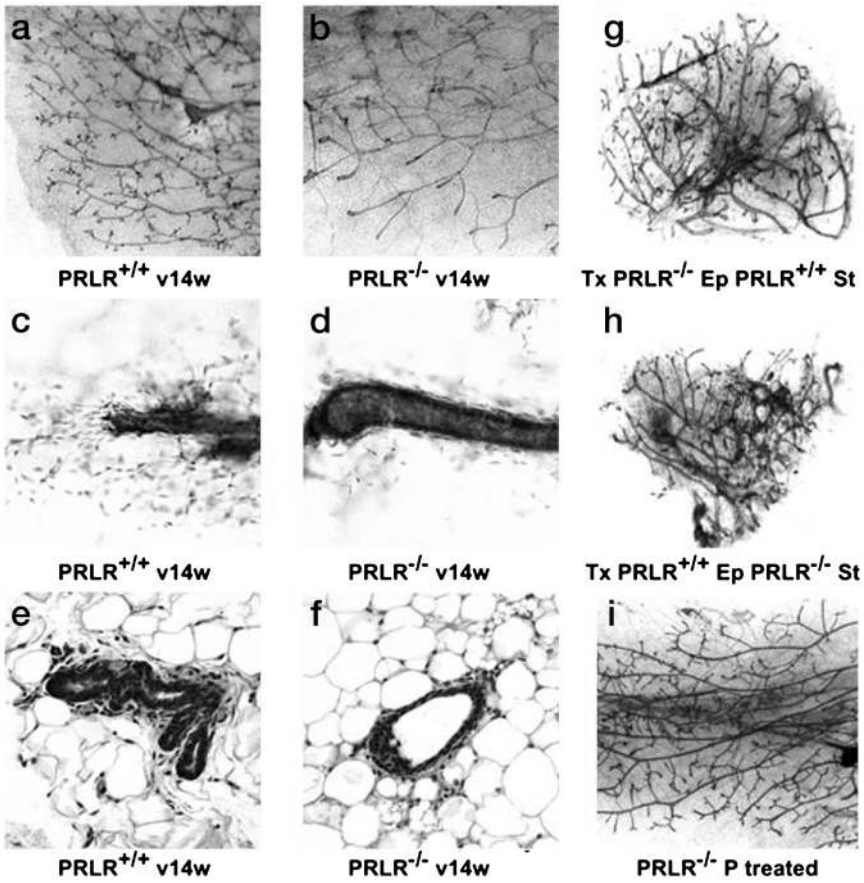


FIG. 1. Prolactin (PRL) indirectly influences mammary development during and following puberty. Development at age 14 weeks of the fourth inguinal gland from virgin animals (A–F). Whole mounts at low magnification (A,B) show failed ductal side branching in PRL receptor (PRLR) knockout (KO) animals ($PRLR^{-/-}$), compared to wild type (WT) ($PRLR^{+/+}$). Failure of alveolar bud differentiation in $PRLR^{-/-}$ animals is revealed by examination of the terminal ductal structures, visualized by high-power whole mounts (C,D) and hematoxylin/eosin (H&E) histology (E,F). Recombination of stroma and epithelium of differing PRLR genotypes, combined with transplantation (Tx) to a $PRLR^{+/+}$ endocrine environment (G,H), reveals the side branching and alveolar bud defects to be indirect effects of PRLR loss. Supplementation for 18 days with a 25-mg progesterone pellet rescues the side-branching defect but not the failure of alveolar bud differentiation (I).

were present at most ductal termini, despite having ceased ductal elongation at the edge of the mammary fat pad, and still could be seen at the ends of some minor ducts at 32 weeks of age. With increasing age, most of the major ducts lost the TEB-like structures and the ducts ended without an apparent terminal

structure. Microdissection of these structures allowed viewing of whole-mount preparations at 200 × original magnification, in addition to hematoxylin/eosin (H&E)-stained sections. The persistent TEB-like structures (Figure 1D and F) showed no resemblance to the alveolar buds seen at the ductal termini of $PRLR^{+/+}$ animals (Figure 1C and E). Comparison with $PRLR^{-/-}$ TEBs observed at 8 weeks of age (which displayed normal histology, data not shown) showed that although the typical direct contact between apical and fat cells was maintained, the TEB-like structures were much smaller, with fewer apical cell layers having no distinct cap cell layer (Figure 1F). These histological observations reflect their dormant behavior and indicate that the persistent TEB-like structures were not typical TEBs. Alveolar buds were never seen in $PRLR^{-/-}$ animals. An identical defect was seen in $PRL^{-/-}$ animals (Horseman *et al.*, 1997).

The persistence of TEB-like structures in $PRLR^{-/-}$ mammary glands is intriguing. As the animals age, most of these structures become simple duct ends lacking a distinctive morphology. These aberrant structures probably result from the failure of TEBs to differentiate into alveolar buds and may represent an intermediate structure in which mitogenesis and ductal elongation have been suspended but differentiation to form an alveolar bud has not occurred.

C. OVARIAN PRLRs ARE REQUIRED FOR NORMAL PUBERTAL DEVELOPMENT OF THE MAMMARY GLANDS

Patterning of the mammary epithelium is influenced by its stroma, which is strongly inductive (Sakakura *et al.*, 1976). This suggests that the failure of ductal side branching may be exerted by the mammary stroma. Although initial investigation failed to detect PRLR in the mammary stroma of the mouse or rat (Meister *et al.*, 1992; Ouhtit *et al.*, 1993a,b; Shirota *et al.*, 1995), immunohistochemistry has found low levels in human breast cancer stroma using an antirat PRLR monoclonal (Reynolds *et al.*, 1997) and through *in situ* hybridization (Mertani *et al.*, 1998). Recently, the stroma of both rat (Camarillo *et al.*, 2001) and mouse (Hovey *et al.*, 2001) mammary gland has been shown to express PRLR. To determine whether PRL acts directly on the mammary epithelial or stromal cells, or indirectly via PRLRs outside the mammary gland, to influence ductal side branching and alveolar bud formation, we transplanted recombined mammary glands formed from epithelium and stroma of both PRLR genotypes, of 129SV background, into $RAG1^{-/-}$ recipients on the C57BL/6 genetic background. Mice homozygous for the inactivated RAG1 allele are immunocompromised and therefore able to accept allografts (Mombaerts *et al.*, 1992). Their mammary glands show typical C57BL/6 morphology of low side branching and little alveolar bud formation. Recombined glands consisted of a fourth mammary

fat pad from a 4-week-old animal (that had been cleared of the undeveloped epithelial rudiment) into which was placed a 1-mm^3 portion of mammary gland from a mature animal. The transplant was placed on the muscle wall, under the skin between the fourth and second/third mammary fat pad, via a small, midline incision. Ten weeks after surgery, the transplanted epithelium had filled the fat pad. The transplanted mammary glands, as well as an endogenous gland, were analyzed by whole-mount histology. Whole-mount analysis showed no differences in ductal side branching between any of the various combinations of epithelium and stroma (Figure 1G and H). This indicates that a mammary PRLR, whether in the epithelium or stroma, is not required for normal ductal side branching to occur. Thus, the side-branching phenotype seen in $PRL^{-/-}$ and $PRLR^{-/-}$ animals is due to PRL action outside the mammary gland.

All transplants reproduced the highly side-branched ductal pattern of the 129Sv mouse, in contrast to the endogenous glands that showed the typical absence of side branching and alveolar bud formation seen in the C57BL/6 strain. When C57BL/6 stroma is recombined with 129Sv epithelium, the C57Bl/6 pattern is reproduced. This indicates that factors within the stroma that are absent from C57BL/6 but present in 129Sv are responsible for the strain-dependent differences in ductal side branching between 129Sv and C57BL/6 (Naylor and Ormandy, 2002). Like the C57BL/6 endogenous gland, none of the transplants produced alveolar buds, suggesting that factors absent from virgin C57BL/6 but present in virgin 129Sv are required for alveolar bud formation. Pregnancy provides this factor in C57BL/6 animals (see below).

D. ROLE OF PROGESTERONE IN SIDE BRANCHING

Transplanted progesterone receptor KO mammary glands show an absence of ductal side branching (Brisken *et al.*, 1998). Progesterone levels are reduced in $PRLR^{-/-}$ animals (Clement-Lacroix *et al.*, 1999), suggesting that progesterone is the mediator and that the ovary is the site of PRL action that controls ductal side branching. Treating 6-week-old $PRL^{-/-}$ females with progesterone pellets (25 mg/21-day release) for 19 days resulted in the formation of ductal side branches but not alveolar buds (Vomachka *et al.*, 2000). PRL supplementation by pituitary implantation resulted in normal development, as both side branches and alveolar buds formed (Vomachka *et al.*, 2000). In $PRLR^{-/-}$ mice, progesterone supplementation also rescued ductal side branching but not alveolar bud formation (Figure 1I). These experiments demonstrate that reduced progesterone levels cause the side-branching deficit in $PRL^{-/-}$ and $PRLR^{-/-}$mice but not the failed formation of alveolar buds. Transplanted progesterone receptor KO mice glands do not show extensive side branches during pregnancy (Brisken *et al.*, 1998).

IV. Development During Pregnancy

A. FAILED FIRST LACTATION IN PRLR$^{+/-}$ ANIMALS

Heterozygous animals on the 129 SvPas/Ola or 129Sv/C57BL/6 background mated at 6 weeks of age are unable to lactate at their first pregnancy. Aging to 20 weeks prior to mating reduces the severity of the lactational deficit (Ormandy *et al.*, 1997b). The subsequent pregnancy results in lactational capacity sufficient for pup survival and normal growth rate but with a developmental lag of approximately 3 days due to slow onset of growth (Ormandy *et al.*, 1997a). There is some variation in this phenotype, however, as some PRLR$^{+/-}$ animals can lactate at the first pregnancy, while others remain incapable of lactation following the second. This heterogeneity was not seen in genetically identical F1 animals. Back cross to the C57BL/6 background greatly increases the severity of the lactational deficit, with some PRLR$^{+/-}$ animals incapable of lactation, despite multiple pregnancies (Gallego *et al.*, 2001). These observations suggest the presence of a factor enhancing lactation that is absent from the C57BL/6 background.

Analysis of mammary development showed that, prior to day 15 of pregnancy, ductal elongation, branching, and the number of lobules formed were similar between PRLR$^{+/-}$ and PRLR$^{+/+}$ in response to the hormonal environment of pregnancy (data not shown). From day 15, development of PRLR$^{+/-}$ glands stalled and greater development of the lobuloalveoli became increasingly apparent in WT animals. At 1 day postpartum, PRLR$^{+/-}$ animals that were incapable of lactation showed lobules mainly at stages 2 and 3, with a few stage 4 lobules at the periphery of the fat pad (Figure 2B). In comparison, PRLR$^{+/+}$ animals showed a fat pad densely packed with stage 4 lobules (Figure 2A). Heterozygous animals exhibiting partial lactation showed many more stage 4 lobules than did animals unable to lactate but far fewer than seen in PRLR$^{+/+}$ animals (data not shown).

To determine whether the lactogenic defect in PRLR$^{+/-}$ mice was epithelial specific, we used epithelial explants from PRLR$^{+/-}$ or PRLR$^{+/+}$ mice transplanted into the cleared mammary fat pads of Rag1$^{-/-}$ recipients. Recombining WT stroma with PRLR$^{+/-}$ epithelium failed to rescue lobuloalveolar development during pregnancy, providing direct evidence that the defect lies in the epithelium (Figure 2C and D).

Microdissection of lobules from animals 1 day postpartum demonstrated that the lobules from PRLR$^{+/-}$ animals showed the formation of multiple alveoli that had failed to engorge with milk postpartum (Figure 2E and F). H&E-stained serial sections (Figure 2G and H) confirmed that the alveoli were not engorged with milk and showed that although alveoli diameters in PRLR$^{+/-}$ animals were smaller, they contained a similar number of epithelial cells as WT animals. Thus,

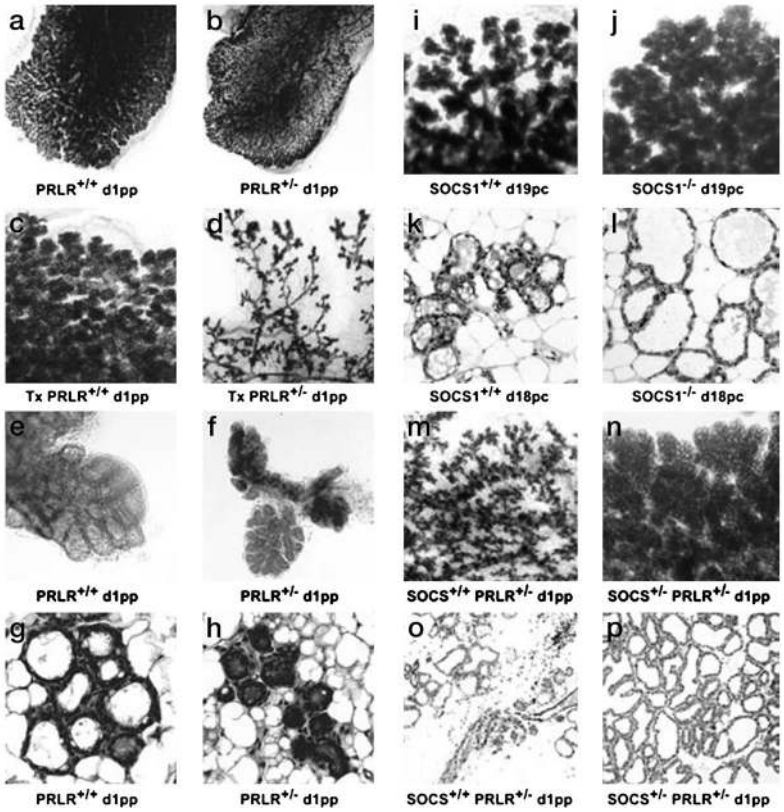


FIG. 2. Mammary development during pregnancy in PRLR$^{+/-}$ animals. Development during pregnancy in PRLR$^{+/-}$ animals (A–H). Low-power magnification of whole mounts reveals failed lobuloalveolar development in PRLR$^{+/-}$ animals, which fail to lactate following their initial pregnancy (A,B). Tx demonstrates that this effect is cell autonomous (C,D). High-power whole mounts (E,F) and H&E histology (G,H) show that although the basic lobuloalveolar architecture has formed, terminal differentiation has failed and lactogenesis has not occurred. Loss of suppressor of cytokine signaling 1 (SOCS1) rescues the PRLR$^{+/-}$ defect (I–P). KO of SOCS1 results in precocious lobuloalveolar development, seen by whole-mount (I,J) and H&E histology (K,L) late in pregnancy. Haploinsufficiency of SOCS1 is without detectable effect on development (not shown) but fully rescues the failure of lobuloalveolar development seen in PRLR$^{+/-}$ mammary glands, seen by whole-mount (M,N) or H&E histology (O,P).

these PRLR$^{+/-}$ lobules showed similar architecture to WT lobules but were not expanded by milk secretion, suggesting a failure of the final stage of functional differentiation as the cause of failed lactation. Interestingly, the alveoli at the periphery of the fat pad were most developed (Figure 2B). This is a consistent finding in both the fourth and second/third glands, suggesting that initiation of

lactation proceeds in a wave from the periphery towards the nipple in $PRLR^{+/-}$ animals. No evidence for this was found in WT animals but it may occur too quickly to be detected.

B. SOCS1 HAPLOINSUFFICIENCY RESCUES THE $PRLR^{+/-}$ DEFECT

Although the intracellular signaling pathways activated by PRL are relatively well understood, the mechanisms by which signaling is attenuated are only now being defined. Negative regulation is likely to involve protein tyrosine phosphatases as well as specific inhibitory molecules such as the suppressor of cytokine signaling (SOCS) proteins. The SOCS family of proteins acts in a classical negative-feedback loop to regulate signal transduction by a variety of cytokines (Yoshimura, 1998; Krebs and Hilton, 2000). The eight members (SOCS1–7 and CIS) of this family are characterized structurally by a C-terminal SOCS box, a central src homology 2 (SH2) domain, and an N-terminal region of variable length and limited homology (Hilton *et al.*, 1998). Functionally, SOCS proteins interact with cytokine receptors and/or Jak kinases, thereby inhibiting activation of kinases and signal transducer and activator of transcription (STAT) proteins (Yoshimura, 1998; Krebs and Hilton, 2000).

SOCS1 (also termed JAB or SSI-1) (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997) is induced in response to a broad range of cytokines and interacts with the kinase domain of Jak proteins. SOCS1-deficient mice die from a complex neonatal disease prior to weaning, involving fatty degeneration of the liver, macrophage infiltration of several organs, and multiple hematopoietic defects (Naka *et al.*, 1998; Starr *et al.*, 1998). This multiorgan disease can be prevented by neonatal treatment with neutralizing anti-interferon gamma (IFN) antibodies. It is absent in mice lacking both SOCS1 and IFN genes, indicating that SOCS1 is a key modulator of IFN effects (Alexander *et al.*, 1999; Marine *et al.*, 1999). Thus, additional disruption of the IFN gene allows the effects of SOCS1 gene deficiency to be studied in adult mice.

Since targeted deletion of the IFN gene rescues $SOCS1^{-/-}$ mice from death at 2 weeks of age (Alexander *et al.*, 1999; Marine *et al.*, 1999), these double KO mice could be used to study the effect of SOCS1 deficiency on mammopoiesis by comparing them with mice lacking IFN alone. $SOCS1^{-/-}/IFN^{-/-}$ mice were crossed to generate females for developmental analysis, while $SOCS^{+/+}/IFN^{-/-}$ mice were bred to generate control $IFN^{-/-}$ females. Loss of IFN had no discernible effect on mammary development, as these mice appeared identical to WT mice at all stages.

SOCS1 deficiency led to increased development of the lobuloalveoli during pregnancy, revealed by whole-mount analysis and histological sectioning. A markedly higher density of lobuloalveoli in mammary glands from $SOCS1^{-/-}/IFN^{-/-}$ mice was apparent from day 16 of pregnancy (Figure 2I and J). By day

18 of pregnancy, the SOCS1$^{-/-}$/IFN$^{-/-}$ lobulalveoli displayed dilated lumens, suggesting precocious lactation (Figure 2K and L). Milk protein levels were elevated from day 16 of pregnancy through to day 1 of lactation in SOCS1$^{-/-}$/IFN$^{-/-}$ mammary glands relative to those from control mice, with the maximal difference occurring at day 18 of pregnancy, confirming precocious lactation (Lindeman *et al.*, 2001).

Stat5 was elevated in SOCS1$^{-/-}$/IFN$^{-/-}$ mice and higher levels of phosphorylated Stat5 were found in mammary glands at day 1 of lactation relative to controls. However, there was no apparent difference during pregnancy. Furthermore, there was no change in Stat5 DNA-binding activity during pregnancy. Interestingly, substantially less MAP kinase activity (phospho-ERK1 and phospho-ERK2) was found in SOCS1$^{-/-}$/IFN$^{-/-}$ mammary glands at day 18 of pregnancy and day 1 of lactation, relative to control mammary tissue. The level of total ERK1/2 remained the same, indicating that MAP kinase activity was reduced. It is not known whether SOCS1 directly influences MAP kinase activity but the diminished levels most likely reflect the differentiated state of the epithelium (Lindeman *et al.*, 2001).

To examine whether a reduction in the level of SOCS1 might rescue signal transduction along the PRL pathway, we generated females that were heterozygous for both PRLR and SOCS1 and compared these to either SOCS1$^{+/-}$, PRLR$^{+/-}$, or WT littermates. We found that six of six double-heterozygous females were capable of lactation after their first pregnancy, whereas four of six PRLR$^{+/-}$ females exhibited reduced lactation. Whole-mount and histological analysis of glands from the rescued mice revealed normal morphology of the lobuloalveolar structures in PRLR$^{+/-}$/SOCS1$^{+/-}$ mice at day 2 postpartum but dramatically reduced development in four PRLR$^{+/-}$ females (Figure 2M–P). The rescue of lobuloalveolar development also was achieved in PRLR$^{+/-}$/SOCS1$^{+/-}$ mice on a different SOCS1 (129Sv) background. Expression of whey acidic protein (WAP) and casein milk protein genes in PRLR$^{+/-}$/SOCS1$^{+/-}$ mammary glands was restored to the level seen in WT glands, in contrast to the lower levels evident in PRLR$^{+/-}$ mice (Lindeman *et al.*, 2001).

C. THE PRLR IS REQUIRED IN THE MAMMARY EPITHELIUM BUT NOT THE MAMMARY STROMA FOR LOBULOALVEOLAR DEVELOPMENT

PRLR$^{-/-}$ females are infertile, preventing an analysis of pregnancy on mammary development in these animals. To circumvent this problem, we made recombined mammary glands from epithelial and stromal elements of both PRLR$^{-/-}$ and PRLR$^{+/+}$ genotypes (from 129Sv background) prior to transfer of the recombined tissue to the abdominal wall of RAG1$^{-/-}$ recipients of the C57Bl/6 background. The engrafted animals were mated 8 weeks after surgery

and the transplanted and endogenous glands analyzed 1 day postpartum. Glands formed from WT epithelium and stroma (Figure 3A) showed development identical to endogenous glands. Glands formed from $PRLR^{-/-}$ epithelium and

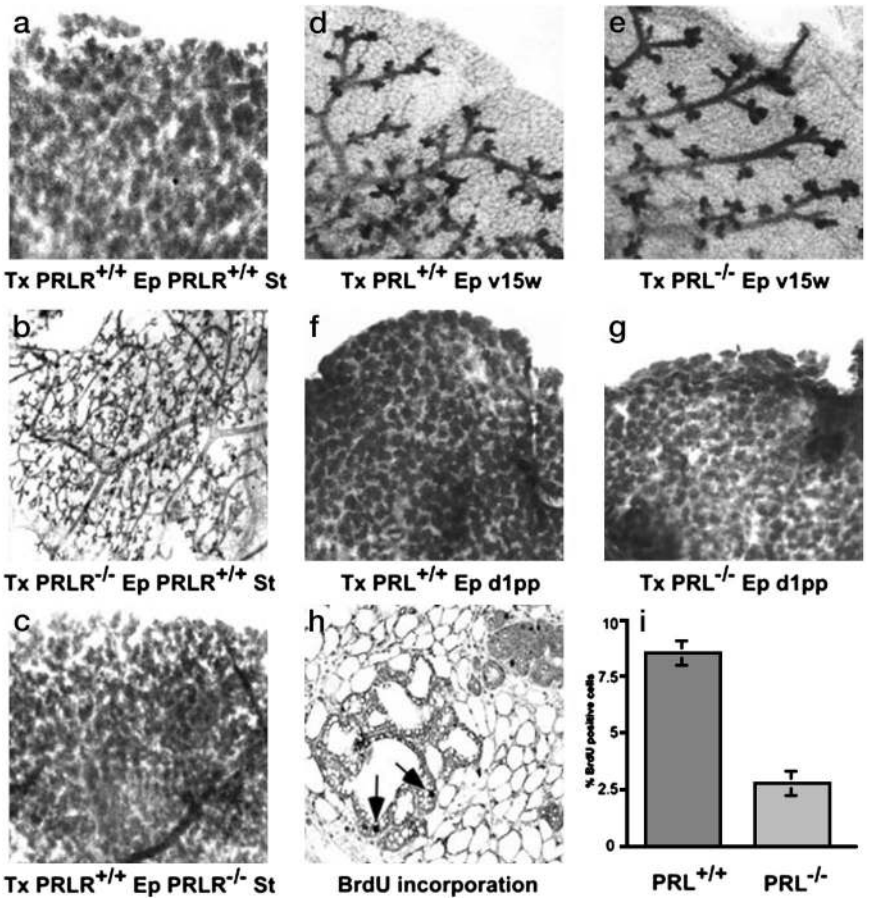


FIG. 3. PRL acts exclusively via the epithelium to direct lobuloalveolar development during pregnancy. Whole mounts of mammary glands formed by tissue recombination and Tx to $Rag1^{-/-}$ hosts, analyzed 1 day postpartum (A–C). Loss of PRLR in the epithelium results in failed lobuloalveolar development (B) but loss from the stroma is without effect (C), compared to WT recombinations (A). Loss of PRL from the mammary epithelium, but not the endocrine system, was produced by epithelial transplant from the $PRL^{-/-}$ mouse to the $Rag1^{-/-}$ host. This had no effect on ductal or alveolar bud development during puberty (D,E) or on lobuloalveolar development during pregnancy (F,G) but results in reduced cell proliferation 1 day postpartum (H,I). No increase in apoptosis was seen in these glands (not shown), suggesting a role for mammary-produced PRL during the onset of lactation. BrdU, bromodeoxyuridine.

WT stroma (Figure 3B) showed no alveolar development but did form side branches and alveolar buds. Identical results for $PRLR^{-/-}$ epithelium were obtained using the cleared fat pad technique (Brisken *et al.*, 1999), where no beta-casein expression was seen in $PRLR^{-/-}$ glands. Glands formed from $PRLR^{-/-}$ stroma and $PRLR^{+/+}$ epithelium developed normally (Figure 3C), demonstrating that stromal PRLR is not required for normal development.

These results demonstrate that an epithelial PRLR is not required for alveolar bud formation during pregnancy, in contrast to the virgin state, where $PRLR^{-/-}$ epithelium cannot form alveolar buds. The formation of alveolar buds in $PRLR^{-/-}$ epithelium at pregnancy, or in $PRL^{-/-}$ glands in response to a pituitary transplant, where none form during the virgin estrous cycle, indicates that PRL induces a systemic factor other than progesterone that is permissive for this event. This represents a second indirect effect of PRL on mammary development.

One of the most-striking features of these experiments is the close similarity between the mammary glands of PRLRKO and progesterone receptor knockout (PRKO) mice. Both models display failed ductal side branching, persistent TEB-like structures, and alveolar bud dysgenesis in virgin glands. The similarity diverges during pregnancy. Although both show lobuloalveolar development stalled at the alveolar bud stage, PRKO glands do not side branch. Both hormones are essential for development of the alveoli at pregnancy and, clearly, PRL and progesterone cooperate to promote alveolar bud formation from the ductal epithelium postpuberty. Previous investigation has shown that these hormones interact. In mouse mammary cells (Edery *et al.*, 1985) and human breast cancer cells (Ormandy *et al.*, 1997c), PRL and progesterone upregulate each other's receptors, providing a mechanism for their synergistic interaction during alveolar formation. The nature of the interaction changes in late pregnancy, as progesterone holds PRLR levels in check (Djiane and Durand, 1977), preventing lactation before parturition. This suggests a modulation of the interaction between these hormones with changing mammary epithelial cell phenotype (Vonderhaar, 1987; Vonderhaar and Biswas, 1987). PR and Stat5a also interact, to redirect transcriptional activity (Richer *et al.*, 1998).

Progesterone treatment of $PRLR^{-/-}$ females following mating fully restores the deficits of preimplantation embryo development and implantation but cannot fully sustain fetal growth past midterm. Thus, only 20% of implantations survive long enough to be delivered by Caesarian section. These animals, when successfully fostered, are normal, indicating a maternal or placental defect (Binart *et al.*, 2000). In $PRL^{-/-}$ animals, progesterone supplementation can fully restore fertility. The mammary glands from pregnancies maintained to term by progesterone show normal development in $PRL^{-/-}$ animals (Vomachka *et al.*, 2000) and failed lobuloalveolar development in $PRLR^{-/-}$ animals (Binart *et al.*, 2000). This indicates that the action of placental lactogen (PL), which can act in $PRL^{-/-}$

but not $PRLR^{-/-}$ animals, is able to fully compensate for PRL, for mammary development and possibly for maintenance of the placenta. PL cannot act in the absence of PRLR. The simplest explanation is that PRLR is the PL receptor or is an essential component of the PL receptor (e.g., as a heterodimer with the growth hormone (GH) receptor) (Herman *et al.*, 2000). If PL acts via the GH receptor homodimer, it must be conditional on PRLR activation.

D. MAMMARY PRL PRODUCTION IS NOT REQUIRED FOR NORMAL DEVELOPMENT BUT INFLUENCES PROLIFERATION

PRL is synthesized primarily in the anterior pituitary. Studies utilizing bromocriptine, which inhibits pituitary PRL synthesis, or pituitary isographs, which secrete large amounts of PRL, have established that endocrine PRL is largely responsible for PRL's reported physiological functions (Freeman *et al.*, 2000). However, PRL is also synthesized in several extrapituitary sites, including mammary epithelial cells (Lkhider *et al.*, 1996; Escalada *et al.*, 1997; Iwasaka *et al.*, 2000), raising the possibility that in addition to PRL's demonstrated direct and indirect endocrine roles, it may regulate mammopoiesis via an autocrine or paracrine mechanism. We addressed this question by comparing the development of transplanted mammary epithelium with and without a null mutation of the PRL gene, using the endpoints of morphology/histology, cell proliferation, and cell apoptosis.

Deletion of the PRL gene from the epithelium, stroma, or both did not alter ductal side branching (Figure 3D and E) or histology (not shown) in virgin mature animals. The amount of cell proliferation assessed by bromodeoxyuridine (BrdU) staining in these glands did not differ significantly (percentage of epithelial cells positive for BrdU, $PRL^{+/+}$ epithelium = 1.17 + 0.09, $PRL^{-/-}$ epithelium = 1.47 + 0.47; $P = 0.56$). These data demonstrate that mammary PRL does not regulate mammary gland development in virgin animals and plays no detectable role in epithelial cell proliferation at this stage.

During pregnancy, normal lobuloalveolar development was observed in whole mounts of mammary glands carrying a null mutation of the PRL gene (Figure 3F and G). H&E-stained sections at day 1 postpartum showed the presence of colostrum and oil droplets, indicating normal epithelial secretory function (data not shown). Cell proliferation was assessed by measuring BrdU incorporation in transplanted mammary glands on day 1 postpartum (Figure 3H and I). Both epithelial and stromal cells were scored for BrdU staining; however, the number of proliferating stromal cells was too few to analyze. In mammary glands formed using $PRL^{+/+}$ epithelium, the percentage of proliferating epithelial cells was 7.94 ± 0.20, compared to 2.82 ± 0.08 in $PRL^{-/-}$ epithelium-derived glands. This represents a 2.8-fold ($P < 0.0001$) decrease in epithelial cell proliferation in mammary glands unable to produce PRL, suggesting an autocrine

or paracrine mechanism for PRL during mammopoiesis. Apoptosis also was assessed in these transplants using the terminal-deoxy UTP nick end labeling (TUNEL) assay. As expected for this stage of development, rates of apoptosis were very low (i.e., three to five cells per section) in the epithelium of both genotypes. This prevents accurate measurement of the frequency of apoptotic cells but shows that no dramatic increase in apoptosis in $PRL^{-/-}$ glands has occurred.

Perplexingly, no difference in morphology was seen, despite the large difference in proliferation rates that apparently is not to be balanced by increased apoptosis. It is possible that, at this late stage of development, the mammary gland shifts from reliance on endocrine PRL to local PRL influence as part of the changes in regulatory control accompanying the shift from proliferation to milk production. Thus, the effect of an altered proliferation rate may not have had time to exert an effect on morphological endpoints. This is consistent with the role of pituitary PRL during lactation. Although hypophysectomy or treatment with a dopamine agonist will stop lactation, the level of pituitary PRL secretion falls as lactation proceeds, without diminution in milk supply (Tyson *et al.*, 1972). Local PRL may play a role in maintaining lactation during falling pituitary PRL secretion.

Several studies to treat breast cancer using inhibitors of pituitary PRL secretion have either been unsuccessful or have produced inconsistent findings (Vonderhaar, 1999). It has been hypothesized that these studies failed because these compounds do not inhibit extrapituitary synthesis or secretion (Vonderhaar, 1999). Our data provide evidence in support of a role for mammary-produced PRL during late pregnancy but did not detect a difference in virgin glands. The number of proliferating epithelial cells is low outside pregnancy, making any effect of local PRL difficult to detect, so a subtle role for PRL outside pregnancy cannot be excluded. If such a subtle effect occurs, it may be significant across a life span.

V. Model of PRL Action in the Mammary Gland

Figure 4 summarizes PRL's hormonal actions. PRL acts indirectly to control ductal side branching via an action in the ovary to control progesterone secretion. PRL also acts indirectly via an unknown factor (X) to regulate alveolar bud formation in virgin animals. It acts directly on the mammary epithelium to drive lobuloalveolar development during pregnancy. At this point, alveolar buds form on $PRLR^{-/-}$ ducts. Mammary-produced PRL may influence mammary epithelial proliferation from a stage in late pregnancy. During involution, PRL has a cell-survival action that prevents the second stage of involution. In the transgenic models of mammary cancer examined to date, the absence of PRL or Stat5a reduces the rate of tumor formation.

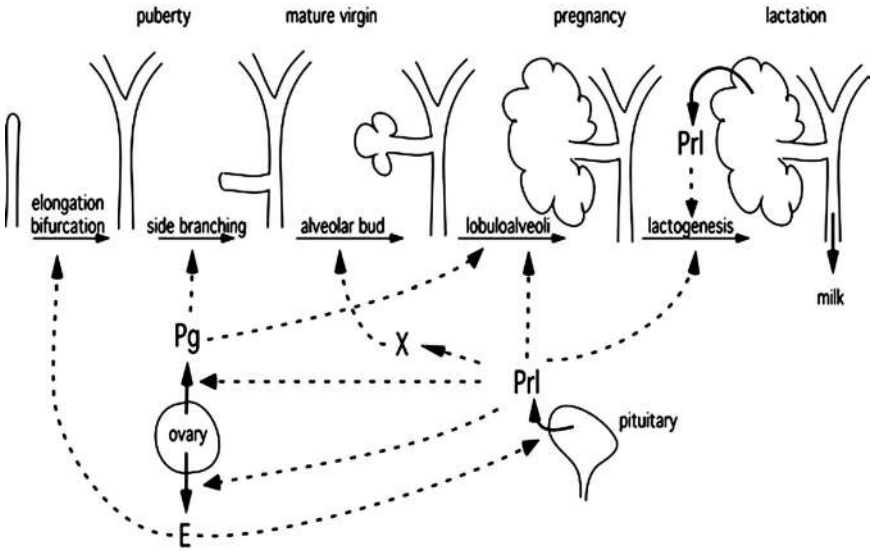


FIG. 4. Model of PRL action on the mammary gland. The stages of mammary ductal and lobuloalveolar development are shown schematically, with causative endocrine states displayed at the top of the diagram. The mechanisms by which PRL influences these events are indicated below. Hormone secretion is represented by solid arrows and regulatory influence by broken arrows. E, estrogen; Pg, progesterone.

VI. Mammary Transcriptional Response to PRL

Having defined and described the morphological and functional defects produced in the mammary gland by the loss of PRLR signaling, we turned our attention to understanding the altered transcriptional events that underlie these defects. To discover the genes that PRL regulates during lobuloalveolar development, we utilized high-density oligonucleotide arrays (Affymetrix MGU74A GeneChips) to profile the transcriptional differences between $PRLR^{+/+}$ and $PRLR^{-/-}$ epithelial transplants during early pregnancy. Days 2, 4, and 6 of pregnancy were chosen to minimize the effect of the difference in epithelial content between $PRLR^{+/+}$ glands, which develop normally, and $PRLR^{-/-}$ glands, in which epithelial development stalls following differentiation to alveolar buds. This approach also allows detection of the early transcriptional response to PRL. We also profiled glands without epithelial transplants, to determine which genes showed an epithelial-specific pattern of expression. Glands from four to six animals were pooled to reduce nonspecific changes in gene expression due to interanimal variation and thus amplify consistent changes in gene expression due to PRLR loss. Data were analyzed using MicroArray Suite 4.0 (MAS 4.0 Affymetrix) and sorted using Excel (Microsoft). Fold

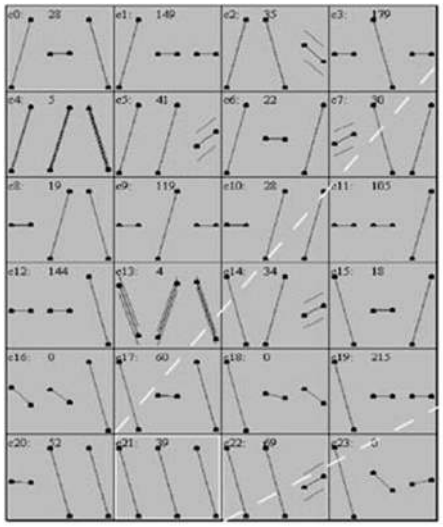
changes calculated by MAS 4.0 for a number of genes were confirmed by quantitative polymerase chain reaction (PCR) using the LightCycler (Roche).

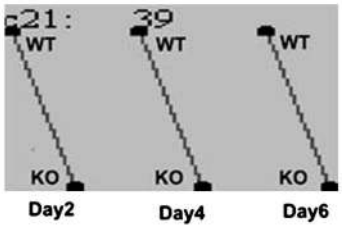
Self-organizing maps were constructed using GeneCluster (Whitehead Institute) to investigate genes that have similar patterns of transcript expression across the experiments. Genes were filtered for those that changed the most and were placed into 24 clusters representing similar patterns of change at each of the 3 days (Figure 5A). The most-interesting cluster generated from this analysis was cluster 21, containing 39 transcripts that decrease in the $PRLR^{-/-}$ epithelial transplants, compared to $PRLR^{+/+}$ transplants at each of the 3 days. Many of these genes are known to be important for mammary gland development or are expressed in the mammary gland during pregnancy, indicating that the self-organizing maps were able to identify a functionally distinct set of genes.

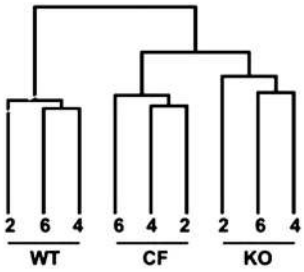
Hierarchical clustering using Cluster and TreeView (Stanford) was employed to investigate the relationship between the types of mammary glands, based on their transcript profiles (Figure 5B). It revealed that $PRLR^{+/+}$ epithelial transplants are distinct from $PRLR^{-/-}$ epithelial transplants and fat pads cleared of epithelium, the latter two groups being more similar and forming a separate branch. In both the $PRLR^{+/+}$ and $PRLR^{-/-}$ epithelial transplants, day 6 of pregnancy was more closely related to day 4 than day 2 of pregnancy. This may represent the peak in cell proliferation in the mammary gland during early pregnancy that occurs at day 4 (Traurig, 1967), in response to a rise in progesterone and PRL levels (McCormack and Greenwald, 1974). This indicates that $PRLR^{-/-}$ epithelium does respond, at least in part, to the hormonal changes of early pregnancy, as also seen in the failure of development after formation of alveolar buds in the transplanted $PRLR^{-/-}$ glands, which are not seen in virgin animals.

The genes identified by MAS 4.0 and the GeneCluster program were sorted into groups, depending on their Gene Ontology as annotated in NetAffx (Affymetrix) (Figure 5C). This abbreviated list (to be published in full elsewhere) does not include cDNAs of unknown function or genes associated with expressed sequence tags (ESTs) and, although not discussed here, they are the focus of ongoing investigation. From extensive literature searching, we have found that many of the genes we have identified as decreasing in $PRLR^{-/-}$ transplants by GeneCluster and MAS 4.0 are upregulated during pregnancy, show predominantly epithelial expression, and some have been shown to be important for mammary gland development.

Four milk protein genes (casein alpha, casein beta, casein kappa, and WDNM1) were decreasing in the $PRLR^{-/-}$ epithelium at days 2, 4, and 6 of pregnancy. WDNM1 and β-casein are expressed during early pregnancy and increase during alveolar proliferation (Robinson *et al.*, 1995). The decrease in these markers of epithelial differentiation in the $PRLR^{-/-}$ transcript profiles confirms that our model is able to detect epithelial transcripts important for







GenBank	Name	Symbol	Annotation	Day 2	Day 4	Day 6
X15662	keratin complex 2, basic, gene 8	Krt2-8	cytoskeleton	-2.9	-4.5	-3.4
M36120	keratin complex 1, acidic, gene 19	Krt1-19	cytoskeleton	-3.3	-3.3	-3
M13805	keratin complex 1, acidic, gene 17	Krt1-17	cytoskeleton	-1.6	-2.3	-2.4
M22832	keratin complex 1, acidic, gene 18	Krt1-18	cytoskeleton	-3	-3	-3.4
U43298	laminin, beta 3	Lamb3	cell adhesion	-3.1	-3.6	-2.4
U32107	procollagen, type VII, alpha 1	Col7a1	cell adhesion	-2.5	-1.9	-4.2
AJ131395	procollagen, type XIV, alpha 1	Col14a1	cell adhesion	-2.5	-2.6	-1.3
X55123	GATA binding protein 3	Gata3	transcription factor	-3	-3.5	-3.2
X94694	transcription factor AP-2, gamma	Tcfap2c	transcription factor	-4.8	-6.4	-7.8
AF095905	claudin 3	Cldn3	tight junction	-3.6	-3.1	-2.8
AF087825	claudin 7	Cldn7	tight junction	-3.7	-6.6	-5.3
M81445	connexin 26	Cx26	gap junction	-15.7	-17.7	-10.9
AF019048	tumor necrosis factor, member 11	Tnfsf11	ligand	-4.7	-3.1	-3.5
L41352	amphiregulin	Amph	ligand	-6.7	-4.7	-3.5
M89797	wingless-related MMTV integration site 4	Wnt4	ligand	-2.8	-3.7	-4.1

FIG. 5. Transcript profiling of PRLR$^{+/+}$ and PRLR$^{-/-}$ epithelial transplants during early pregnancy. Mammary epithelial transplants from PRLR$^{+/+}$ and PRLR$^{-/-}$ animals were made to cleared Rag1$^{-/-}$ mammary fat pads and allowed to develop for 6 weeks prior to timed mating and collection of the transplants at days 2, 4, and 6 of pregnancy. Fat pads without epithelial transplants also were collected at these times, to allow epithelial-specific genes to be identified. RNA was extracted, then pooled prior to analyses of gene expression using the Affymetrix mouse U74A chip and MAS 4.0 software. Results are presented as a self-organizing map analysis performed using the Gene Cluster program from the Whitehead Institute and hierarchical clustering using Cluster and Map Viewer from Stanford University. Cluster 21 contained genes decreasing at all time points, some members of which are shown below, including their functional annotation and fold change in expression given by MAS 4.0.

lobuloalveolar development. β-casein is recognized as a classical PRL-regulated gene in both *in vivo* and *in vitro* models. Its appearance in our list of genes confirms that our model can identify PRL-regulated genes involved in mammary epithelial differentiation.

Keratins are traditional markers of the epithelium. However, their regulation is specific to tissue type and state of differentiation of the epithelial cell. Their major role is maintaining the structure of epithelial cells. Keratins have been implicated in cell signaling, stress response, and regulation of other cellular proteins (Coulombe and Omary, 2002). Our study implicates keratins 8, 17, 18, and 19 as PRL-regulated genes required for lobuloalveolar development in the mammary gland. Two pieces of data show that the decrease in keratin expression between $PRLR^{-/-}$ and $PRLR^{+/+}$ epithelial transplants is not due to differences in the epithelial content of the glands. First, keratins 5 and 14, markers of myoepithelial cells, did not decrease (Neville and Daniel, 1987). Second, the proportion of expressed genes designated as epithelial specific by the stroma-only profiles remained unchanged between days 2 and 6 of pregnancy (i.e., at $\approx 15\%$ of all expressed genes at days 2, 4, and 6 in both KO and WT profiles). Interestingly, keratin 18 protein levels are increased during lactation in the human breast, during which keratin 18 and 8 appear as intracellular aggregates rather than as components of the filamentous network seen in the nulliparous state (Michalczyk *et al.*, 2001). While keratins remain useful markers of epithelial cells, their use to correct for variable epithelial content is called into question by this result.

Mammary gland development is influenced not only by systemic hormones such as PRL but also by cell microenvironment. One component of this environment is the extracellular matrix (ECM), which harbors factors that are known to regulate tissue-specific gene expression (Howlett and Bissell, 1993). We have identified a number of ECM components involved in cell adhesion as important for lobuloalveolar development, including two members of the collagen family and laminin.

A number of transcription factors were discovered to be important for PRL-stimulated development of lobuloalveolar cells. These are key molecules in the transcription response to PRL, as they act as turning points in the transcription cascade by activating the transcription of further genes.

GATA binding protein 3 (GATA3) belongs to a family of transcription factors that bind to DNA through a highly conserved zinc finger domain. GATA3 KO mouse embryos show kidney development failure early in embryogenesis. Their embryonic lethality is attributed to noradenaline deficiency and cardiac failure (Lim *et al.*, 2000). GATA-3, as well as keratin 19 transcript levels, were elevated in ER-positive breast cancer cells lines, when compared to ER-negative breast cancer cell lines. An association was found between ER and GATA-3 expression in hormone-responsive breast cancers. However, estradiol did not

induce GATA-3 expression in MCF-7 cells, suggesting a role for GATA-3 in establishing the hormone-responsive phenotype in breast cancer (Hoch *et al.*, 1999). The level of ER expression is closely correlated with the level of PRLR expression (Ormandy *et al.*, 1997c).

Similarly, the transcription factor activator protein-2 gamma (AP-2γ) was identified as ER factor 1 (ERF-1) in ER-positive breast cancer cell lines (deConinck *et al.*, 1995). Its expression is limited to ER-positive cancer cell lines and is upregulated in breast cancers (Turner *et al.*, 1998). Gel-shift assays suggest that this molecule plays a critical role in regulating ER gene transcription (McPherson *et al.*, 1997). AP-2γ KO mice are embryonic lethal due to failure of trophectodermal cell proliferation (Werling and Schorle, 2002).

Claudins are recently discovered integral membrane proteins that are major structural components of tight junction strands. Tight junctions form between epithelial cells to block transport of solutes to neighboring cells and to minimize diffusion of molecules to maintain cellular polarity. Claudin-3 is expressed mainly in the lung and liver, while claudin-7 is primarily found in the lung and kidney (Morita *et al.*, 1999). In order to prevent diffusion of molecules across the mammary epithelium during lactation, tight junction closure is increased. This is mediated by progesterone withdrawal following parturition and requires PRLR activation (Nguyen *et al.*, 2001). Our experiments indicate that PRL not only plays a role in the closure of tight junctions during pregnancy but also may influence the formation of these junctions by regulating transcription of their components.

Connexin-26 is a member of a large family of proteins that form similar junctions between epithelial cells, gap junctions that allow exchange of small ions and metabolites. Connexin-26 mRNA and protein expression are upregulated significantly during pregnancy and remain elevated during lactation (Tu *et al.*, 1998). Furthermore, connexin-26 expression is confined to the alveolar epithelium, specifically localized to where adjacent alveolar cells are in contact (Locke *et al.*, 2000). A functional binding site for the AP-2 transcription factors has been identified in the connexin-26 promoter (Tu *et al.*, 2001), indicating that our transcript-profiling experiment may have found at least one transcription factor cascade following PRL binding to its receptor on mammary epithelial cells.

A number of extracellular ligands also were identified in our screen as transcribed following PRL action on the mammary epithelium during pregnancy. These ligands generally are important during the cell-cell communication necessary for differentiation.

Wnt4 is a member of the Wnt family of secreted glycoproteins implicated in cell-cell signaling. Wnt4 has been shown to act downstream of progesterone to induce ductal side branching during pregnancy (Brisken *et al.*, 2000). Overexpression of Wnt4 in the mammary gland by retroviral delivery resulted in

increased ductal side branching and alveolar-like structures in virgin animals, similar to those seen at day 10 of pregnancy in normal animals (Bradbury *et al.*, 1995). This study and our transcript profiles suggest that Wnt4 may play an additional role in the PRL-stimulated development of lobuloalveolar cells.

Amphiregulin is a member of the epidermal growth factor (EGF) family that all bind to the EGF receptor. Amphiregulin can restore ductal proliferation in mammary glands of ovariectomized mice; overexpression of this ligand induces hyperplastic ducts and lobules (Kenney *et al.*, 1996). KO studies have shown that amphiregulin is essential for ductal morphogenesis, suggesting a role in epithelial cell migration or adhesion. During pregnancy, alveoli appear small, dense, and immature in amphiregulin-deficient mammary glands, a phenotype aggravated by the loss of other EGFR ligands, EGF and transforming growth factor alpha (TGFα) (Luetteke *et al.*, 1999).

Calcitonin, a peptide hormone produced in the thyroid, is known to inhibit osteoclast-mediated bone resorption. Expression of calcitonin mRNA and peptide is induced during mid- to late pregnancy in the rat mammary gland, decreasing at parturition. Calcitonin receptor mRNA is induced during pregnancy, suggesting a paracrine role for this ligand in the mammary gland (Tverberg *et al.*, 2000).

Tumor necrosis factor (TNF) (ligand) superfamily member 11 (Tnfsf11) — also known as receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) and osteoprotegrin ligand (OPGL) — was found to decrease in the $PRLR^{-/-}$ epithelium at all three timepoints. The mammary glands of the $RANKL^{-/-}$ mouse show a phenotype similar to $PRLR^{-/-}$ mammary glands — the mice are unable to lactate because lobuloalveolar cells failed to form during pregnancy. PRL was able to induce RANKL expression in the mammary gland, independent of progesterone and estrogen (Fata *et al.*, 2000). A recent discovery has placed RANKL at the head of a signaling cascade resulting in lobuloalveolar proliferation in the mammary gland. A mouse expressing an inactivated form of the alpha subunit of IkB kinase (IKKα) had a mammary gland defect in lobuloalveolar development (Cao *et al.*, 2001). IkB kinase also is known to activate the transcription factor NF-κB. Another KO mouse with a defect in lobuloalveolar development is that of cyclin D1 (Fantl *et al.*, 1999), a molecule that requires activation of NF-κB for its induction. Overexpression of cyclin D1 in the IKKα-inactivated mouse restored lobuloalveolar development, confirming that cyclin D1 is a molecule acting downstream of IKKα. As RANKL was able to induce NF-κB activation in WT and not IKKα-inactivated mammary epithelial cells, it would seem that RANKL initiates the signaling cascade that results in cyclin D1-induced lobuloalveolar cell development during pregnancy (Cao *et al.*, 2001). Our study has shown that PRL modulates RANKL expression during early pregnancy, suggesting that PRL is the master regulator of the signaling events necessary for lobuloalveolar development.

Thus, PRL acts to induce the transcription of a number of genes in the mammary epithelium that are essential for the complex interactions necessary for lobuloalveolar development and subsequent milk production and secretion. These transcriptional actions are summarized in Figure 6. PRL acts to induce transcription of genes that encode milk proteins at the final stage of differentiation. It also induces transcription of genes important for intracellular structure (keratins), extracellular structure (laminins, collagens), cell permeability (claudins, connexins), cell-cell communication (Rankl, amphiregulin, Wnt4), and the

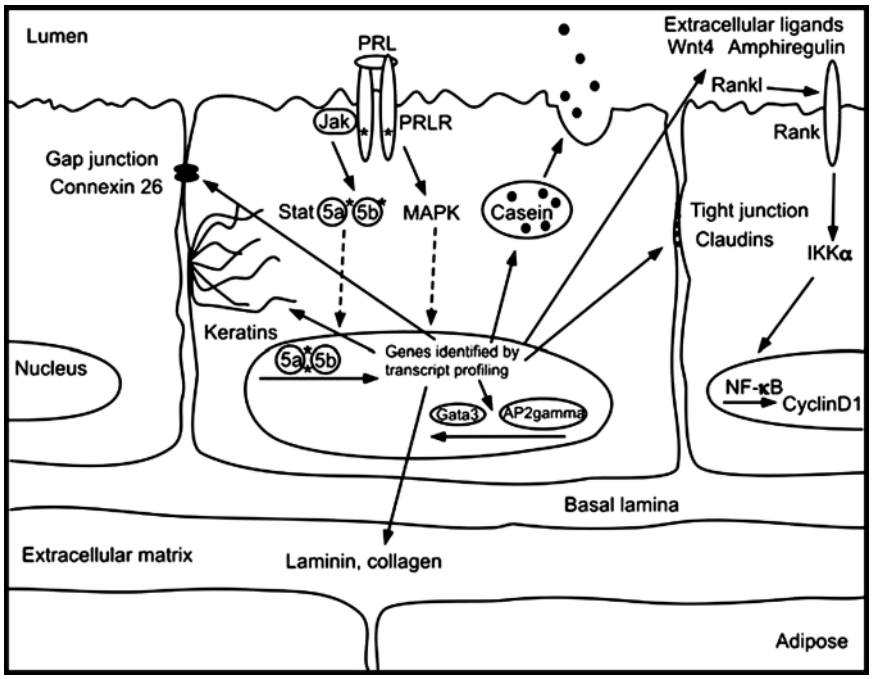


FIG. 6. The transcriptional response to PRL. PRL acts on the mammary epithelium by binding to its receptor, activating a number of signaling cascades, including the Jak/Stat pathway and the mitogen-activated protein (MAP) kinase pathway. This results in the transcription of genes necessary for epithelial differentiation and formation of lobuloalveolar cells in the mammary gland. Transcript profiles of mammary glands capable of producing lobuloalveolar cells ($PRLR^{+/+}$ epithelial transplants), compared to profiles of mammary glands unable to produce lobuloalveolar cells ($PRLR^{-/-}$ epithelial transplants), identified a number of genes within the mammary epithelium whose function is known. These genes include those important for cell structure (keratins) and components of the extracellular matrix (laminin and collagen) as well as components of junctions necessary for cell permeability (connexin-26, claudin-3 and -7). A number of transcription factors were identified that act to transcribe further genes necessary for differentiation (activator protein (AP)-2 gamma, GATA-3). These genes may include extracellular ligands such as those identified by our screen (Wnt4, amphiregulin, Rankl) that act on neighboring cells to stimulate their differentiation.

continuation of differentiation (transcription factors). Thus, our transcript-profiling experiments have confirmed the morphological phenotype of the PRLR$^{-/-}$ mouse and have shown that PRL is necessary for lobuloalveolar development in the mammary gland by allowing transcription of genes essential for a number of structures, signals, and transcription factors necessary for cell differentiation.

VII. Conclusion

The combination of epithelial/stromal recombination with transcript profiling has provided an opportunity to uncover the transcriptional program underlying the formation of lobuloalveoli in the mammary gland in response to pregnancy. Some of these genes (Figure 6; to be published elsewhere in detail) have well-established roles in mammary development, demonstrating the success of this approach and the likely importance of the novel genes that we currently are analyzing. Which of these effects are direct and which are mediated via the modulation of transcription factor activity remain to be elucidated, as does the exact temporal sequence of events. The growing understanding of the development of various cell lineages within the mammary gland will be central to fully understanding the global changes in gene expression that we now can observe. We look forward to future advances that will allow the separation of these cell types.

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