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Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene

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Prolactin (PRL) has been implicated in numerous physiological and developmental processes. The mouse PRL gene was disrupted by homologous recombination. The mutation caused infertility in female mice, but did not prevent female mice from manifesting spontaneous maternal behaviors. PRL-deficient males were fertile and produced offspring with normal Mendelian gender and genotype ratios when they were mated with heterozygous females. Mammary glands of mutant female mice developed a normal ductal tree, but the ducts failed to develop lobular decorations, which is a characteristic of the normal virgin adult mammary gland. The potential effect of PRL gene disruption on antigen-independent primary hematopoiesis was assessed. The results of this analysis indicated that myelopoiesis and primary lymphopoiesis were unaltered in the mutant mice. Consistent with these observations in PRL mutant mice, PRL failed to correct the bone marrow B cell deficiency of Snell dwarf mice. These results argue that PRL does not play any indispensable role in primary lymphocyte development and homeostasis, or in myeloid differentiation. The PRL$^{-/-}$ mouse model provides a new research tool with which to resolve a variety of questions regarding the involvement of both endocrine and paracrine sources of PRL in reproduction, lactogenesis, tumorigenesis and immunoregulation.

Keywords: B cell/granulocyte/macrophage/mammary gland/T cell

Introduction

Prolactin (PRL) is closely associated with the stimulation of lactogenesis in mammals, but its secretion in non-mammalian species, and the multiple effects caused by administration of exogenous PRL (Nicoll and Bern, 1972) have led to suggestions that PRL may play important roles in systems other than the mammary gland. Recent discoveries have reinforced the notion that PRL may be fundamental to the development of the reproductive (Cooke, 1995) and hematopoietic (Hooghe-Peters and Hooghe, 1995; Koojiman *et al.*, 1996) systems.

The fact that the PRL receptor is expressed in cells in the testis, prostate gland, seminal vesicles and ovary (Nicoll and Bern, 1972; Cooke, 1995) is consistent with a role for the hormone in reproduction. Similarly, the PRL receptor has been detected on up to 95% of bone marrow cells and thymocytes (Dardenne *et al.*, 1991; O'Neal *et al.*, 1991; Gagnerault *et al.*, 1993; Touraine *et al.*, 1994), suggesting a potential role in hematopoiesis. The PRL receptor is a member of the type I cytokine receptor superfamily, which includes numerous hematopoietic growth factor receptors (Kelly *et al.*, 1993; Horseman and Yu-Lee, 1994). However, despite reports that PRL can potentiate effector function of lymphoid and myeloid cells during secondary immune responses (Gala, 1991; Matera *et al.*, 1991; Kelley *et al.*, 1992; Sabharwal *et al.*, 1992; Murphy *et al.*, 1993; Hooghe-Peters and Hooghe, 1995; Warwick *et al.*, 1995; Koojiman *et al.*, 1996), its involvement in the primary, antigen-independent development and homeostasis of these populations is not resolved.

Our current understanding of the physiology and pathobiology of PRL is based on administration of exogenous hormones to animals or cell cultures, and ablation of pituitary function by surgery, drugs or spontaneous genetic mutations that suppress pituitary PRL secretion. PRL is synthesized in many tissues other than the pituitary gland (Ben-Jonathan *et al.*, 1996). Pituitary ablation cannot remove potentially important local sources of PRL in some tissues. Consequently, many long-standing controversies regarding the role of PRL in key developmental and physiological processes have remained unresolved.

To circumvent various limitations in our understanding of PRL actions, a strain of mice with a targeted disruption of the PRL gene was generated and analyzed. The results of these analyses demonstrate that PRL is required for normal reproduction and mammary gland development in adult females; but PRL is not required for somatic growth, male reproduction, spontaneous maternal behavior, or primary differentiation or homeostatic maintenance of the blood cells.

Results

Generation of PRL-deficient mice

The PRL gene was mutated by a targeted insertion of a neomycin resistance gene (PGK–*neo*) into the region encoding the second α helix of the PRL protein (Figure 1A). Targeting was confirmed by genomic Southern blotting using enzyme cuts that were both 5′ (*Bpm*I) and 3′ (*Hin*dIII) of the targeting site (Figure 1B). PCR

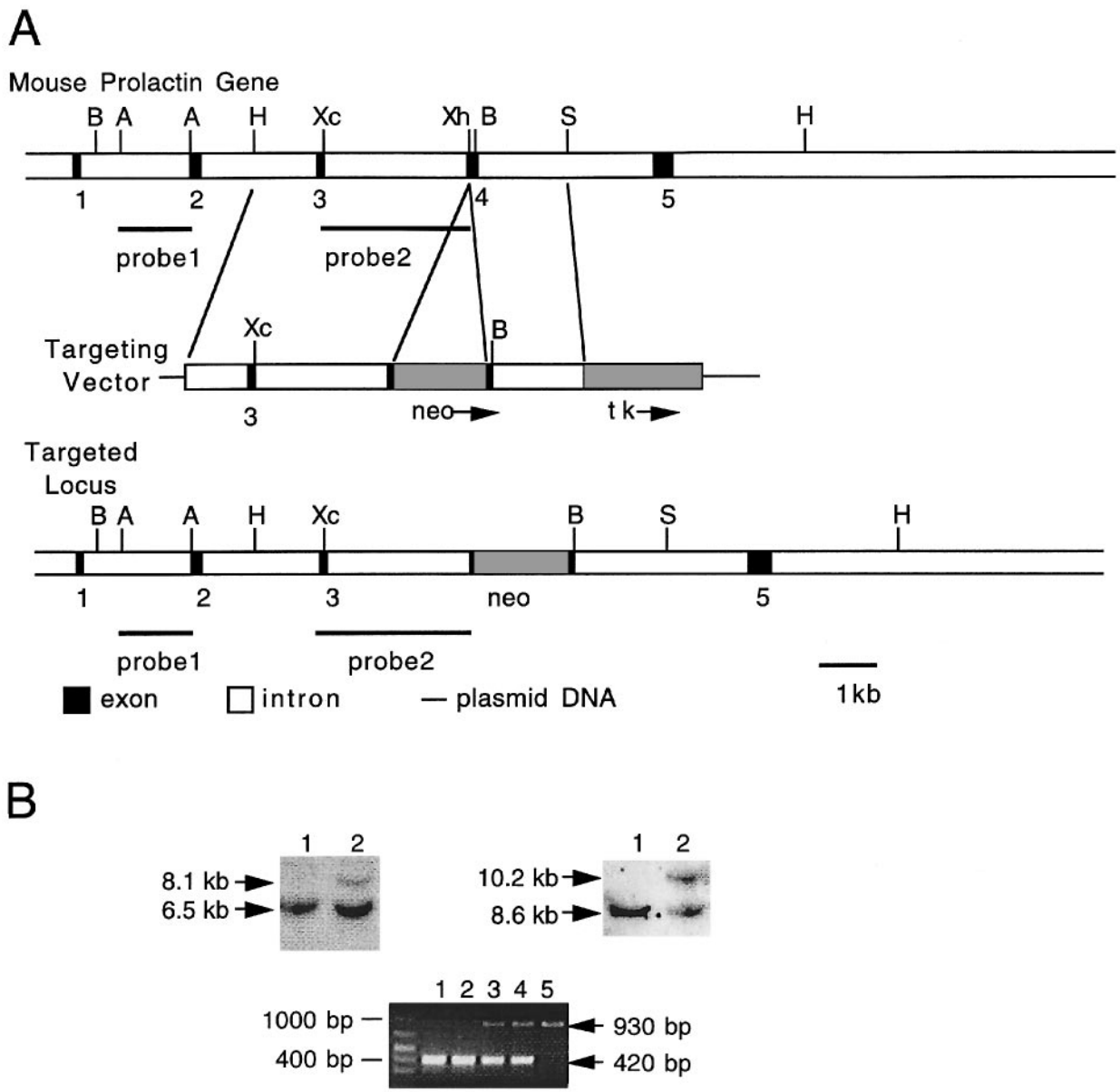

Fig. 1. Targeted disruption of the mouse PRL gene. (**A**) The organization of the wild-type mouse PRL gene is shown in relation to the targeting vector. The relative positions of the exons and introns are depicted along with restriction enzyme sites that were used for preparing the vector, probes and diagnostic fragments. Restriction enzyme sites are labeled as follows: A, *Alw*N1, B, *Bpm*I, H, *Hin*dIII, S, *Sac*I, Xc, *Xcm*I, Xh, *Xho*I. The targeted locus is described in the third line with two diagnostic probes. (**B**) Genomic Southern blotting of wild-type (lanes 1) and targeted (lanes 2) DNA following digestion with either *Bpm*I (left panel) or *Hin*dIII (right panel) and probing with outside probe 1 or probe 2 (see A), respectively. The sizes of the hybridizing bands are labeled at the right margin of the gels. The map of the wild-type locus predicts bands of 6.5 and 8.6 kb with *Bpm*I and *Hin*dIII, respectively, and the map of the targeted locus predicts bands of 8.1 and 10.2 kb with *Bpm*I and *Hin*dIII, respectively. A probe for the neomycin cassette detected only the upper (8.1 and 10.2 kb) bands in each blot (data not shown). Genotyping of five mice by PCR is depicted in the bottom panel. Primers for genotyping consisted of 5′ oligos directed to intron C and to the *neo* gene, and a 3′ oligo directed to exon 4. These primers produced a 420 bp product from the wild-type allele and a 930 bp product from the targeted allele under the conditions used for amplification. The size markers are labeled in the left-most lane. Lanes 1 and 2 were DNA from wild-type, lanes 3 and 4 were heterozygous, and lane 5 was DNA from a homozygous mutant mouse.

amplification of genomic DNA yielded the predicted 420 and 930 bp fragments from the normal and targeted gene (Figure 1B). Pituitary mRNA was assayed by reverse transcriptase–PCR to confirm the loss of the PRL gene product at the mRNA level. Amplification of the 5′ portion of the PRL mRNA (encoded by exons 1–3) yielded an expected 276 bp product in +/+, +/– and –/– mice. Primers for the 3′ portion of the mRNA (encoded by exons 4 and 5) yielded the expected 312 bp product in +/+ and +/– mice, and no product in the –/– mice. These results are consistent with the prediction that the mice should express a 3′-truncated PRL transcript as a consequence of the PGK–*neo* insertion.

Western blotting of pituitary gland extracts was used to assess the status of PRL gene expression at the protein level (Figure 2). Purified mouse PRL migrated as a 25 kDa monomer band with a larger (50 kDa) band that presumably represents a PRL dimer. A band identical to 25 kDa PRL was present in extracts from male and female +/+ mice. Heterozygous mice synthesized predominantly the 25 kDa

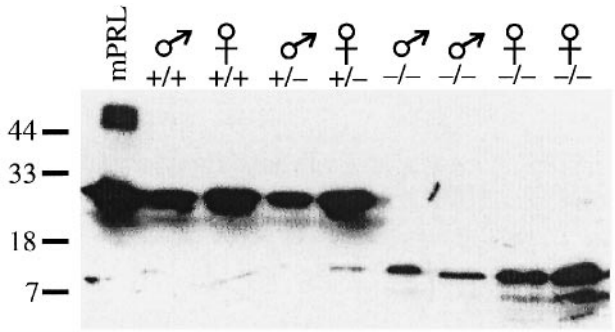

Fig. 2. Western blot analysis of pituitary proteins. Pituitary extracts (10 μg) were immunoblotted as described in Materials and methods. Molecular weight markers are depicted at the left margin. The first lane is control mouse PRL (3 μg). The sex and genotypes of mice from which each extract was prepared are labeled at the top of each lane. Both wild-type and heterozygous mouse pituitaries contained similar amounts of the full-length immunoreactive PRL. Homozygous mutant mice produced only the predicted truncation product at a molecular weight of ~11 kDa.

Table I. PRL bioactivity in mouse pituitary extracts

Genotype	[PRL] (ng/μg protein) ± SD and (*n*)[a]
Male +/+	2.8 ± 0.4 (4)
Male +/–	3.9 ± 0.7 (4)
Male –/–	<0.001 (4)
Female +/+	28.3 ± 1.7 (3)
Female +/–	28.6 ± 5.9 (5)
Female –/–	<0.001 (3)

An Nb2 cell bioassay was performed on lysates of pituitary glands of individual mice. Each lysate was assayed in duplicate. The minimum detectable level of PRL bioactivity in the assay was 1 pg.
[a]*n* = number of individual animals in each group.

product; and female heterozygotes, which synthesized higher PRL levels than males, also had a detectable amount of immunoreactive protein which migrated at 11 kDa. Homozygous male and female mice synthesized exclusively the 11 kDa immunoreactive PRL product. The PGK–*neo* cassette insertion truncated the pre-PRL polypeptide at serine 117 in the second α-helix and added a 12-amino-acid extension (sequence: PRL_{117}–TDPPGCRNSIS–stop) before the first stop codon in PGK–*neo*. This results in a 129-amino-acid precursor that would be cleaved to 100 residues by removing the signal peptide. The calculated molecular weight of the polypeptide predicted from the sequence of the targeted gene was 11 306 Da, which corresponds to the 11 kDa immunoreactive band in the $PRL^{-/-}$ mice. Under the conditions of these Western blots, where 10 μg of pituitary extracts were loaded, small amounts of immunoreactive protein migrated faster than the main bands. These smaller products may represent proteolytically cleaved PRL.

Bioassay of pituitary PRL

Pituitary extracts from homozygous wild-type and heterozygous mice had indistinguishable levels of PRL bioactivity (Table I), The average level of PRL in male mice pituitaries was ~12% of that in female mice pituitaries. PRL bioactivity was completely undetectable in pituitaries from both male and female –/– mice.

Table II. Mating records for heterozygous pairs

+/– (M)×+/– (F)	+/+	+/–	–/–
Male	77 (33.6%)	109 (47.6%)	43 (18.8%)
Female	42 (21.4%)	112 (57.2%)	42 (21.4%)
Total	119 (28%)	221 (52%)	85 (20%)

Each cell of the table records the number and frequency of offspring with the genotypes identified at the top of the columns.

Table III. Mating records for homozygous-null male×heterozygous female matings

–/– (M)×+/– (F)	+/–	–/–
Male	60 (50.4%)	59 (49.6%)
Female	67 (51.9%)	62 (48.1%)
Total	127 (51%)	121 (49%)

Each cell of the table records the total number and frequency (in parentheses) of male or female offspring with the genotypes identified at the top of the columns. Gender and genotype distributions did not vary significantly from the Mendelian 1:1 ratios (χ^2 test).

Fertility, maternal behavior and somatic growth of $PRL^{-/-}$ mice

Heterozygous crosses yielded the expected ratios of genotypes and genders in the offspring (Table II). There was no measurable difference in fetal survival of –/– mice and their littermates. Heterozygous females produced normal litter sizes and had no problems with nursing their offspring.

Homozygous $PRL^{-/-}$ female mice were completely infertile. Female $PRL^{-/-}$ mice were mated with male mice of known fertility and no litters were produced following more than 15 matings. Each female mated repeatedly at irregular intervals, without entering a state of pseudopregnancy. Estrous cycles of females were assessed by vaginal smear cytology (Champlin, 1973) in six $PRL^{-/-}$ mice. The females all underwent cycles that displayed all of the phases, but the patterns were very irregular. Unlike normal female mice, which have only a single proestrus and a single estrus day in each cycle, the $PRL^{-/-}$ mice had cycles with multiple days of proestus and/or multiple days of estrus. Individual females did not establish any consistent pattern of cycling; subsequent cycles could be either longer or shorter than the normal 4–5 day cycle, with no predictable cycle length for any individual.

Nulliparous female mice (8 weeks old) were tested for maternal behaviors (pup retrieval and crouching) toward foster pups. Four of six +/+ females, five of seven +/– females and six of seven –/– females retrieved 2-day-old foster pups and crouched over them in a nursing position within 30 min after placement of the pups in the home cage. Thus, PRL deficiency does not cause any profound defect in the spontaneous maternal behavior exhibited by laboratory mice.

In contrast to the reproductive abnormalities of the female –/– mice, males with the disrupted PRL gene were fully fertile. Matings between –/– male and +/– female mice produced normal litter sizes and normal 1:1 Mendelian gender and genotype ratios (Table III).

Somatic growth of the mice was not significantly

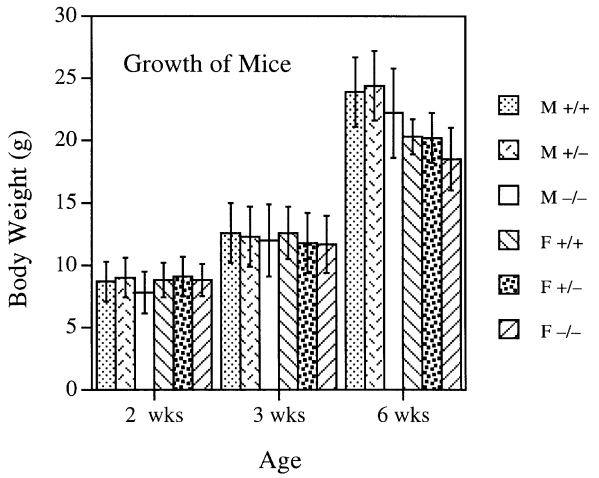

Fig. 3. Growth curves for normal and PRL-deficient mice. The mean body weight (± standard deviation) is depicted for each group. The number of mice in each group was at least 17.

affected by disruption of the PRL gene. Figure 3 shows growth curves for both male and female mice through the first 6 weeks. The –/– mice grew normally, increasing to approximately triple their weight between 2 and 6 weeks of age. There was no significant difference between the body weights of control and targeted mice at any of the ages. In addition to the growth curves done on the young mice, –/– mice were examined at 6 months old, at which age the males averaged 38.9 g and the females averaged 28.6 g. Therefore, PRL deficiency had no discernible effect on growth of the mice at any age.

Gross pathology and histopathology at necropsy

No macroscopic lesion was found in either male or female –/– mice, nor was there any difference at the gross level between control and $PRL^{-/-}$ mice. Of the wide array of tissues examined (see Materials and methods), the only histological abnormality in the 6-week-old –/– mice was a subtle effect in the pituitary glands, where there was a decrease in the volume of the acidophilic cells, consistent with deficient PRL biosynthesis and storage. As there was no bioactive PRL detected in the pituitary glands of –/– mice, pituitary gland function has not yet been studied in any more detail. Further analysis of the pituitary glands will require development of specific N-terminal and C-terminal antibodies that can differentially detect the wild-type and the targeted PRL polypeptides. Histological examination of the mammary glands at 6 weeks of age was equivocal, showing only some modest signs of hyperplasia and neutrophil infiltration in the –/– female mice.

Abnormal mammary gland development in adult virgin $PRL^{-/-}$ mice

Mammary gland development includes the formation of a branched ductal system that is decorated with terminal and lateral lobules in virgin adult mice (Figure 4A). The growth and differentiation of the mammary gland is under the control of several hormones whose precise roles are still unclear. In $PRL^{-/-}$ mice, terminal end buds form during puberty and the ductal tree grows normally (Figure 4B). However, in adult $PRL^{-/-}$ mice, the mammary gland ductal system grows into an extended branching network

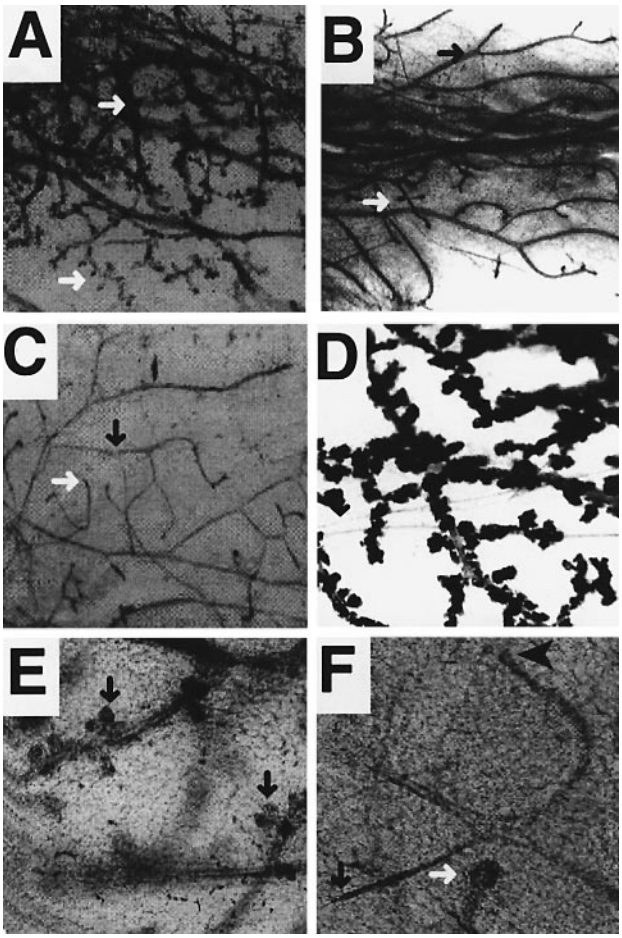

Fig. 4. Mammary gland development is defective in adult PRL-deficient mice. Whole mounts of abdominal mammary glands were stained with safronin O (A, B, E and F) or iron hematoxylin (C and D). (**A**) Mammary gland of 5-month-old normal ($PRL^{+/-}$) mouse showing the extensive decoration of the ductal tree by lobulations (white arrows). (**B**) Pubescent (6-week-old) $PRL^{-/-}$ mouse mammary gland. The branching ducts (black arrow) and terminal end buds (white arrow) are indistinguishable in normal and PRL-deficient mouse mammary glands at this age. (**C**) Mammary gland from 5-month-old $PRL^{-/-}$ littermate of the mouse in (A). Note the nakedness of all of the ducts (black arrow) and termination of some ducts by end buds (white arrow). (**D**) Lobuloalveolar development of midpregnant $PRL^{+/-}$ mouse mammary gland Development of the glandular system is more extensive during pregnancy that in the virgin adult (A). (**E**) Magnification of normal ($PRL^{+/-}$) virgin adult (5 month) mammary gland showing the development of lobulations associated with the ductal system (black arrows). (**F**) Magnification of $PRL^{-/-}$ virgin adult (5 month) mammary ductal system showing termination of ducts as tapered tubes (black arrow), blunt-ending tubes (arrowhead) or terminal end buds (white arrow).

that is completely devoid of either terminal or lateral lobulation (Figure 4C). In normal mice, the differentiation of the ductal system results in a compact glandular structure which undergoes full lobuloalveolar development during pregnancy (Figure 4D). In $PRL^{-/-}$ mice, the mammary ducts ended as blunt tubes, or extended tapered tubes, without any lateral or terminal decorations (Figure 4E and F). Despite the lack of lobulation, there was no compensatory increase in the number of branches formed during the development of the mammary gland.

Effects of PRL on lymphopoiesis

In view of speculation that PRL is a lymphopoietic hormone, B and T cell development was assessed in

PRL$^{-/-}$ mice. The data in Figure 5A and Table IV indicate that the frequency of CD45R$^+$sIgM$^-$ and surface IgM$^+$ (sIgM$^+$) cells is comparable in the knockout mice and their +/– littermates. In order to assess the potential of PRL to affect development in a minor subpopulation of developing B lineage cells, the expression of CD43, CD45R, HSA and IgM was used to resolve bone marrow B lineage cells into CD45R$^+$CD43$^+$HSA$^-$ pro-B cells (Fraction A), CD45R$^+$CD43$^+$HSA$^+$ progenitors (Fraction B + C), CD45R$^+$CD43$^-$HSA$^+$ pre-B cells (Fraction D) and CD45R$^+$sIgM$^+$ B cells (Fraction E and F). The data in Figure 6 demonstrate that the frequency of cells in these various B lineage fractions is comparable between the PRL$^{-/-}$ mice and their PRL$^{+/-}$ littermates. As bone marrow cellularity in PRL$^{-/-}$ mice and their PRL$^{+/-}$ littermates was nearly identical (Table IV), there was no significant difference in the absolute number of B lineage cells in the mice.

The analysis of PRL effects on B cell development in Snell dwarf (*dw/dw*) mice supports the above findings. As previously reported and confirmed in Table V, the frequency of CD45R$^+$ B lineage cells in the bone marrow of these mice is significantly depressed when compared with levels present in their +/? littermates (Murphy *et al.*, 1992; Montecino-Rodriguez *et al.*, 1996, 1997). The data in the table indicate that while treatment of *dw/dw* mice with 100 μg of ovine PRL/day for 2 weeks resulted in an increase in the number of bone marrow cells, the frequency of CD45R$^+$sIgM$^-$ and sIgM$^+$ B lineage cells remained depressed. However, as reported previously (Montecino-Rodriguez *et al.*, 1996), B cell defects in the bone marrow of *dw/dw* mice can be corrected by thyroxine.

The potential role of PRL in primary T cell development was determined by comparing the number and frequency of CD4- and/or CD8-expressing thymocytes in PRL$^{-/-}$ mice and their PRL$^{+/-}$ littermates. As shown in Figure 5B and Table VI, the frequencies of CD4$^-$CD8$^-$ double negative, CD4$^+$CD8$^+$ double positive and single positive CD4 and CD8 thymocytes were comparable in PRL$^{-/-}$ mice and their PRL$^{+/-}$ littermates. However, although not statistically significant, the average number of cells in the thymus of the PRL$^{+/-}$ mice was lower than in the PRL$^{-/-}$ mice.

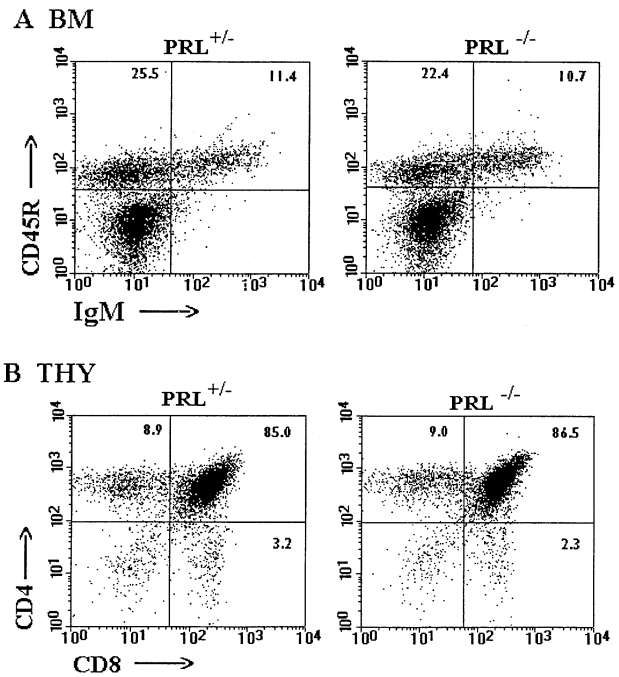

Fig. 5. Representative FACScan profiles of (**A**) B lineage cells in the bone marrow of PRL$^{+/-}$ and PRL$^{-/-}$ mice and (**B**) of CD4- and CD8-expressing cells in the thymus of PRL$^{+/-}$ and PRL$^{-/-}$ mice.

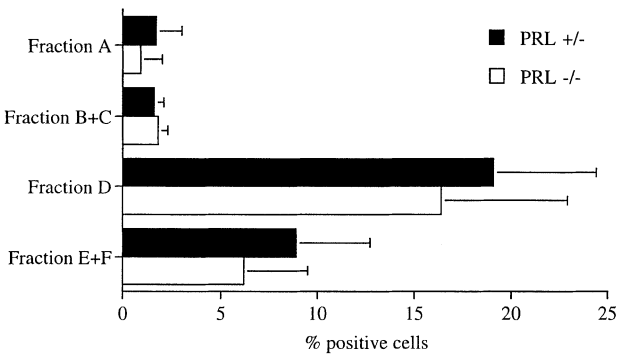

Fig. 6. Frequency of B lineage cells in fractions A–F in PRL$^{-/-}$ (open bars; $n = 12$) and PRL$^{+/-}$ (filled bars; $n = 6$) mice.

Table V. Prolactin treatment does not stimulate bone marrow B lymphopoiesis in dwarf mice

Treatment (n)[a]	Cells $\times 10^{-6}$	Percentage CD45R$^+$, sIgM$^-$	Percentage IgM$^+$
Saline (8) *dw/dw*	7.1 ± 2.3	5.2 ± 4.6	7.4 ± 5.1
PRL (10) *dw/dw*	9.3 ± 1.9[b]	5.5 ± 3.8	4.2 ± 1.8
GH (4) *dw/dw*	17.2 ± 8.4[c]	7.3 ± 1.1	4.6 ± 0.7
GH + PRL (4) *dw/dw*	15.6 ± 5.9[c]	8.5 ± 4.2	4.6 ± 0.6
Thyroxine (5) *dw/dw*	10.0 ± 2.8	22.9 ± 4.1[d]	8.9 ± 1.2

All values are given as mean ± SD.
[a]Number in parenthesis represents the number of mice in each group.
[b]Value significantly different from saline-treated group ($P < 0.025$).
[c]Value significantly different from saline-treated group ($P < 0.005$).
[d]Value significantly different from saline-treated group ($P < 0.0025$).

Table IV. Bone marrow B lymphopoiesis and myelopoiesis in prolactin knockout mice

Genotype	Cells $\times 10^{-6}$	CD45R$^+$IgM$^-$	IgM$^+$	CD11b (Mac–1)	CFU-GM/5$\times 10^4$
+/–[a]	18.7 ± 6.7[c]	22.4 ± 5.2	8.9 ± 5.3	51.7 ± 5.3	147.5 ± 38.0[d]
–/–[b]	13.9 ± 6.8	19.1 ± 6.8	6.2 ± 3.3	57.3 ± 11.3	162.0 ± 17.0[d]

All values are given as mean ± SD.
[a]$n = 6$.
[b]$n = 12$.
[c]Cell counts based on analysis of two femurs and two tibia per mouse.
[d]Assays run on four mice in each group.

Table VI. Thymus cellularity and frequency of thymocyte subpopulations in PRL knockout mice

Genotype	Cells $\times 10^{-6}$	Percentage of cells expressing:			
		$CD4^- 8^-$	$CD4^+ 8^+$	$CD4^+$	$CD8^+$
+/–[a]	82.7 ± 16.8	2.0 ± 0.4	81.5 ± 4.0	13.1 ± 3.0	3.5 ± 1.7
–/–[b]	129.7 ± 41.8	1.6 ± 0.3	83.0 ± 3.1	12.5 ± 2.3	2.9 ± 0.8

All values are given as mean ± SD.
[a]$n = 5$.
[b]$n = 9$.

Table VII. Frequency of B and T cells in the spleen (SPL) and lymph nodes (LN)

Organ	Genotype (n)[a]	Cells $\times 10^{-6}$	Percentage of cells expressing:			
			IgM^+	$CD4^+$	$CD8^+$	CD11b (Mac–1)
SPL	+/– (6)	143.3 ± 30.7	39.7 ± 8.1	29.8 ± 1.9	14.7 ± 3.1	7.6 ± 3.1
SPL	–/– (12)	93.5 ± 39.5	31.6 ± 7.7	30.7 ± 4.6	16.5 ± 3.5	6.8 ± 2.8
LN	+/– (4)	1.2 ± 0.2	3.9 ± 1.2	63.5 ± 1.3	30.5 ± 1.3	9.3 ± 2.4
LN	–/– (4)	1.4 ± 0.3	3.8 ± 2.1	63.2 ± 5.5	30.2 ± 4.7	8.6 ± 1.2

All values are given as mean ± SD. Lymph node cellularity is given per single lymph node and the phenotypic analysis was carried out on cells pooled from two axillary and two inguinal lymph nodes.
[a]The number of mice in each analysis is given in parentheses.

Cellularity of secondary lymphoid tissues in $PRL^{-/-}$ mice

The data in Table VII demonstrate that spleen and lymph node cellularity and the frequency of IgM-, CD4-, CD8- and CD11b (Mac-1)-expressing cells in $PRL^{-/-}$ mice and their $PRL^{+/-}$ littermates were comparable. The mean number of $sIgM^+$ cells was lower in the spleen of $PRL^{-/-}$ mice than in their $PRL^{+/-}$ littermates, although not significantly so.

Myelopoiesis in $PRL^{-/-}$ mice

The expression of the PRL receptor on the majority of hematopoietic cells also raised the possibility that PRL was required for normal development of myeloid cells. Therefore, the frequency of granulocyte–macrophage progenitors responsive to GM-CSF and of CD11b (Mac-1)-expressing cells were enumerated in the $PRL^{-/-}$ mice. The data in Table IV demonstrate that no differences in the frequency of CFU-GM and CD11b $(Mac\text{-}1)^+$ cells between the $PRL^{-/-}$ mice and their littermates were observed. The hematocrits (51.1 ± 1.5 in $PRL^{-/-}$ and 53.0 ± 0.14 in $PRL^{+/-}$ mice) were similar as well.

Discussion

While PRL has been demonstrated to be required during lactogenesis in the pregnant mammal, its importance in reproductive function in the non-pregnant female has been poorly understood. In addition, there has been speculation that PRL might play a key role in the development of the hematopoietic system. One difficulty in addressing these issues has been the lack of an appropriate animal model for assessing PRL function. There have been three approaches to studying the effects of PRL deficiency in animals: surgical hypophysectomy (Bates *et al.*, 1962), pharmacological inhibition of PRL secretion (Nagy *et al.*, 1983) and analysis of pituitary dwarf models (Murphy *et al.*, 1992). None of these approaches has been completely satisfactory, for two reasons. First pituitary ablation does not inactivate potentially important PRL synthesis in extrapituitary tissues and, secondly, many of the changes in these animals might be due to other hormone or drug effects rather than PRL deficiency, *per se*. To circumvent some of these limitations, we chose to produce a mouse model with an isolated PRL deficiency. The aim of the studies reported herein was to assess the effects of PRL deficiency during postnatal development, with particular emphasis on mammary gland development, reproductive capacity, somatic growth and hematopoiesis.

PRL deficiency in these mice was produced by using homologous recombination in embryonic stem cells to interrupt the coding region of the PRL gene with a neomycin-selectable marker. Disrupted PRL synthesis was confirmed by biochemical and biological assays. The mutant mice produced a truncated peptide that reacted with anti-mouse PRL, which was consistent with disruption of the PRL gene by insertion of *neo* downstream of the bases that encode serine 117. Putative 16K PRL, which has anti-angiogenic properties, is produced by proteolytic cleavage downstream of the third α-helix (residue 174) (Ferrara *et al.*, 1991). Because the biological properties and receptor for 16K PRL are not well known, it is impossible to infer whether the truncated product in $PRL^{-/-}$ mice might have any activity similar to that proposed for 16K PRL. As the N-terminally truncated fragement is immunoreactive, Western blotting, rather than serum radioimmunoassay, was used to show the deficiency of the protein.

Heterozygous mice produced the full-length PRL protein and bioactive PRL at levels that were identical to homozygous wild-type mice. Therefore, the loss of one PRL locus was compensated for in the mice so that the overall level of PRL synthesis was not reduced. Consistent with this, the heterozygous mice had no difficulties in bearing normal sized litters and nursing their offspring. In contrast, mice heterozygous for a PRL receptor mutation

had problems with lactation and nurturing of their first litters because the mammary glands did not reach a full lactational state of differentiation (Ormandy *et al.*, 1997). This suggests that there is no feedback system to upregulate the PRL receptor in compensation for the loss of one allele, whereas normal levels of PRL can be synthesized from a single allele in the heterozygous mice.

Mice homozygous for the PRL gene mutation were born in normal Mendelian proportions. Similarly, disruption of the PRL receptor gene was also not embryonically lethal (Ormandy *et al.*, 1997). Despite these similarities, the characteristics of the PRL ligand and receptor knockout mouse strains were not identical. Half of the male receptor –/– mice were infertile or had reduced fertility, whereas the ligand-disrupted male mice were normally fertile. It is possible that reduced fertility in male $PRL^{-/-}$ mice might be revealed if they were challenged with multiple females or were placed in competition with non-mutant males, but these experiments have not been done. PRL receptors are widely distributed in fetal tissues (Freemark *et al.*, 1995). During the perinatal period, PRL bioactivity may be supplied from the maternal circulation and in the milk (Soares *et al.*, 1991; Kacsóh *et al.*, 1993). Thus, the $PRL^{-/-}$ mice are probably not absolutely PRL deficient until some time during the nursing period, when maternal proteins are no longer passed to the offspring via the milk. Some differences between PRL ligand- and receptor-mutant mice may be attributable to the supply of maternal PRL to the offspring. It is also important to consider that PRL is probably hypersecreted in receptor-mutant mice, which may cause non-specific effects during development.

The female $PRL^{-/-}$ mice had irregular estrous cycles and did not become pregnant when mated to stud males. Although PRL is required for maintenance of the corpus luteum in mice, it is not obvious why the estrous cycles of $PRL^{-/-}$ mice should be altered, since there is no functional luteal phase in the estrous cycle of mice, as is present in humans and many other mammals. There were no obvious defects in the ovaries or other genital structures of the $PRL^{-/-}$ mice when examined histologically. PRL receptor knockout mice fail to become pregnant owing to multiple pre-implantation reproductive defects, which result in poor embryo survival and progression (Ormandy *et al.*, 1997). Similarily, preliminary observations (data not shown) indicate that $PRL^{-/-}$ mice ovulate normal numbers of eggs which can be fertilized but do not progress to implantation. Future experiments will be necessary to address whether the altered cyclicity in $PRL^{-/-}$ mice is caused peripherally (i.e. an ovarian defect) or centrally (i.e. hypothalamic), and whether the fertility defects are similar to those in receptor-null mice. It will be possible to use the $PRL^{-/-}$ mice to determine whether the fertility defects are caused by direct PRL actions on the oviduct and uterus or are secondary to changes in other organs.

Spontaneous maternal behaviors were expressed in $PRL^{-/-}$ mice as well as normal heterozygous and wild-type controls. Unlike some other species, where prior experience is required for the expression of maternal behaviors, naive virgin laboratory mice display a complex suite of maternal behaviors (Noirot, 1969). PRL appears not to be required to induce receptivity of naive female mice to foster pups. Disruption of the *fos*B gene resulted in profoundly defective pup retrieval and crouching behaviors (Brown *et al.*, 1996); and preliminary data indicated that PRL levels were normal in the *fos*B mutant mice. Our results affirm definitively that PRL is not required in order for mice to manifest maternal behaviors. It remains possible that PRL plays some role in maternal behavior in other species, such as rats, where maternal behaviors are dependent on prior experience (Bridges *et al.*, 1985, 1990).

Postnatal growth of $PRL^{-/-}$ mice was unaltered. Mice grew normally during the first 6 weeks, and the males and females were of normal size at 6 months of age. These results indicate that the PRL mutation did not have any effects on GH or IGF-1 secretion even though GH and PRL are closely related molecules and they are synthesized by cells that can be interconverted (Ben-Jonathan *et al.*, 1996).

Mammary gland development was altered by PRL deficiency in the virgin adult mice. The ductal system in –/– females grew into an extensively branched, extended network of ducts, with no evidence of terminal or lateral lobular decorations. In contrast, normal mice produced compact mammary gland networks with extensive lobulation. It has been suggested that GH and IGF-1 may be important for both end bud development in early puberty, and differentiation of 'alveolar structures' in the adult (Feldman *et al.*, 1993; Kleinberg, 1997), which we refer to as 'lobulations' in this paper. Based on our results we can conclude that GH and IGF-1, along with estrogens, may be sufficient to stimulate pubertal growth of terminal end buds. However, PRL is required to drive the subsequent growth and differentiation of the lobular strucures of the adult mouse mammary gland. The terminal end buds of early puberty, which may be stimulated by GH rather than PRL, appear to be physiologically and developmentally different from the lobulations of the adult mouse mammary gland. The end buds of puberty are associated with growth of an extended ductal tree, whereas the lobulations in the adult mammary gland are associated with growth and differentiation of the alveolar structures, which lead to a compact system of glandular tissue.

The disruption of the genes for Stat5a (Liu *et al.*, 1997) and Stat5b (Udy *et al.*, 1997), as well as the PRL receptor (Ormandy *et al.*, 1997) and ligand (our results) offer some insight into the potential complexity of intracellular signaling mechanisms for PRL. Stat5 was identified as a PRL-stimulated transcription factor in the mammary gland of sheep (Wakao *et al.*, 1994), and has been associated with mediating PRL actions in a variety of cell types (Gouilleux *et al.*, 1995). Whereas both the PRL ligand- and receptor-null mice showed major defects in fertility and mammary gland development in the females, the Stat5a and Stat5b null mice were each fertile and their mammary glands underwent nearly complete development. Stat5a-deficient mice did not lactate, although the mammary glands developed to a full lobulo alveolar state and synthesized milk proteins (Liu *et al.*, 1997). This suggests that Stat5a is essential for the full manifestation of lactation, but not for development of the mammary gland or synthesis of milk proteins. Stat5b-deficient mice were fertile and lactated, although the pregnancies of Stat5b-null mice often aborted, and the mice did not produce enough milk to feed their pups (Udy *et al.*, 1997). In contrast, the PRL receptor (Ormandy *et al.*, 1997) and

PRL ligand (our studies) are absolutely necessary for fertility of females, and for pre-lactogenic mammary gland development. It is possible that Stat5a and b, or other Stat proteins, have many redundant functions in female reproduction and mammary gland development. If so, disruption of multiple Stat genes would be necessary to cause the reproductive defects seen in PRL-deficient mice. Alternatively, other PRL-activated signal transducers (Horseman *et al.*, 1997) may mediate PRL actions in the female reproductive tract or during some of the pre-lactational stages of mammary gland development. The availability of mice with disruptions in various genes that have been implicated in PRL action will greatly facilitate studies on the physiological basis of PRL signal transduction.

Maturation of breast tissue in the post-pubertal adult, or during pregnancy, has been associated with a marked reduction in breast cancer risk in both rodents (Welsch and Nagasawa, 1977) and humans (MacMahon *et al.*, 1973). It has not previously been possible to determine the potential role of PRL in the maturation of breast tissue in the adult because of the inadequacies of the available experimental models. PRL-deficient mice allow us to conclude that PRL is necessary for mammary gland tissue to progress past a pubertal stage of ductal development to an adult state. This indicates that PRL has an important role during the post-pubertal maturation of the breast, which may contribute to the refractoriness of breast cells to carcinogenesis. The PRL-null mice will provide an *in vivo* model in which to examine the relationships between mammary gland development, signal transduction and breast cancers.

A major goal of this study was to determine whether PRL is an obligate hematopoietic hormone in the mouse. No major deficiency in the production of lymphoid or myeloid cells was observed in the $PRL^{-/-}$ mice, strongly suggesting that PRL is not critical for primary, antigen-independent development of lymphoid and myeloid cells. The frequency and absolute number of B lineage cells in the bone marrow of PRL-deficient mice did not differ from values in their normal littermates, and these results were complemented by independent studies performed on *dw/dw* mice. This strain, which is deficient in growth hormone, PRL and thyroid hormone production, has a well-documented deficiency in the generation of B lineage cells in the bone marrow (Murphy *et al.*, 1992; Montecino-Rodriguez *et al.*, 1996). While administration of thyroid hormone can restore B lymphopoiesis to normal in this strain (Montecino-Rodriguez *et al.*, 1996), the potential role of PRL has not been reported. Treatment of *dw/dw* mice with PRL alone, or in combination with growth hormone (data not shown), had no effect on the frequency of bone marrow B lineage cells. These results suggest that PRL is not an important regulator of primary B lymphopoiesis.

Although PRL was not required for B cell development, the number of cells in the bone marrow of PRL-treated dwarf mice was higher than in saline-treated mice. Since PRL has been reported to potentiate the proliferative response of granulocytic and erythroid cells to lineage-specific hematopoietic growth factors (Nagy and Berczi, 1989; Bellone *et al.*, 1995), the increased bone marrow cellularity may be a consequence of that effect. Nevertheless, while these results suggest the potential of PRL to enhance growth in particular hematopoietic lineages, the presence of a normal hematocrit and normal numbers of myeloid and lymphoid cells in the bone marrow of $PRL^{-/-}$ mice indicates that PRL is not required for normal, steady-state hematopoiesis.

Thymopoiesis also appeared normal in the $PRL^{-/-}$ mice, as the frequency of the thymocyte populations expressing CD4 and/or CD8 was comparable with that in their normal littermates. This observation further suggests that PRL is not an obligate thymopoietic factor. Although not statistically significant, the mean number of cells in the thymus of $PRL^{-/-}$ mice was higher than in their littermates. This observation, together with a report that PRL treatments depress thymocyte cellularity in Snell dwarf mice (Murphy *et al.*, 1993), raises the possibility that PRL may have an inhibitory effect on cell production in the thymus. However, this conclusion must remain tentative, because an inhibitory effect of PRL on the dwarf mouse thymus was not observed in this (data not shown) or another laboratory (Villanua *et al.*, 1992), and PRL has no effect on thymic cellularity in normal mice (Murphy *et al.*, 1995).

Our results also demonstrate that there is a normal distribution of B and T cells in secondary lymphoid organs of $PRL^{-/-}$ mice. This finding does not necessarily imply that PRL has no effect on the secondary responses involving these populations. In fact, there are numerous reports indicating that PRL may modulate the secondary immune response (Gala, 1991; Kelley *et al.*, 1992; Hooghe-Peters and Hooghe, 1995; Murphy *et al.*, 1995). Additional studies using the $PRL^{-/-}$ mice, as well as the recently described PRL-receptor knockout (Ormandy *et al.*, 1997), should be of value in delineating the role of PRL in secondary immune responses.

In summary, the data in this report demonstrate that PRL is essential for female reproduction and the post-pubertal differentiation of the mammary gland. The mechanisms involved in PRL actions in the female reproductive tract and in the early differentiation of the mammary gland are not known. In contrast to its essential roles in the female systems, PRL does not appear to be required for normal development of the hematopoietic system. Nevertheless, the expression of the PRL receptor on the majority of developing blood cells provides for the possibility that PRL can have subtle effects on these populations, and it is important to stress that PRL may play a role in modulating secondary immune responses. Future studies will be necessary to assess reproductive defects, secondary immune responses and the neoplastic potential of the mammary gland of $PRL^{-/-}$ mice.

Materials and methods

Generation of PRL mutant mice

A positive–negative selection targeting vector was constructed using the MJK-KO plasmid (Li *et al.*, 1996) kindly provided by Steven Potter (Department of Pediatrics, University of Cincinnati). A region encompassing 5.2 kb of the mouse PRL gene was cloned into MJK-KO such that the PGK–neomycin (*neo*) selectable marker was inserted into the fourth exon at a unique *Xho*I site, and the thymidine kinase (tk) negative selection cassette was inserted at a *Sac*I site in intron D (Figure 1). The *neo* insertion bisects the PRL gene in the region encoding the second α helix so as to eliminate both sites 1 and 2 of the receptor-binding regions.

Fifty million 129/Sv D3 embryonic stem (ES) cells were electroporated in the presence of 5 nM of *Not*I linearized targeting vector, and were plated on mitomycin C-treated feeder cells. After 24 h G418 was added at 500 μg/ml and the concentration of G418 was reduced to 20 μg/ml after

24 h. Gancyclovir (2 nM) was added 3 days after electroporation to select against tk^+ cells. Selection of neo^r/$ganc^r$ cells was completed in 10–14 days. Individual clones were expanded in 24-well culture plates and then screened by PCR. Of 119 clones selected, 28 were confirmed to be targeted by PCR and Southern blotting (Figure 1). Blastocyst injections and mating of chimeric mice was carried out as described (Shull *et al.*, 1992). Gene targeting and blastocyst injections were performed in the Gene-Targeted Mouse Core Facility of the University of Cincinnati. Heterozygous F1 pups were identified by PCR and sibling matings were carried out to produce homozygous PRL-deficient mice.

Mouse husbandry and behavior testing

$PRL^{-/-}$ mice were maintained in barrier facilities within AAALAC-accredited animal quarters under Institutional Animal Care and Use Committee-approved protocols. Snell dwarf ($DW/JPit1^{dw}$) mice (*dw/dw*) and their +/+ and +/– littermates (referred to as +/?) were obtained from the Jackson Laboratories, Bar Harbor, ME. Mice were administered saline, ovine PRL (100 µg/day, lot AFP-10677C; National Hormone and Pituitary Program), or DL-thyroxine (T4; 2 µg/day; Sigma, St Louis, MO) by a single subcutaneous injection daily for 2 weeks.

Retrieval of foster pups and crouching in a nursing posture were assayed to determine whether $PRL^{-/-}$ mice displayed spontaneous maternal behaviors. Three newborn foster pups (2–3 days of age) were placed in the home cage of the nulliparous test females (8 weeks of age) at positions distant from the female and from each other. The mice were observed continuously for 30 min. A positive maternal behavior test was defined as retrieval of all of the foster pups, followed by the female assuming a nursing posture, crouched over the pups (Bridges *et al.*, 1985, 1990)

PRL bioassay

Pituitary PRL content was determined by the Nb2 lymphoma cell bioassay (Gout *et al.*, 1980) as modified by Kascóh (1997). Briefly, Nb2 cells were growth-arrested by removal of FBS for 24 h. Cells plated in 96-well tissue culture plates were given either PRL standard (NIH sheep PRL-20, 31 IU/mg, gift of the National Hormone and Pituitary Program) or pituitary cell extracts. Individual pituitary glands were suspended in 100 µl PBS and were disrupted by trituration through a micropipette. Cells were lysed by three cycles of freezing and thawing and the lysates were cleared by centrifugation. Protein content was determined by the micro BCA assay according to the manufacturer's protocol (Pierce Chemical Co., Rockford, IL). Cells were incubated with standards and test samples for 48 h and cell proliferation was quantified by MTT (3-[4,5-dimethyl-thiazol–2-*yl*]–2,5-diphenyltetrazolium) dye conversion. The minimum detectable level of PRL in this assay was 1 pg/well.

Western blotting

Pituitary extracts (10 µg protein/lane) or purified mouse PRL (3 µg, gift of F.Talamantes and L.Ogren, University of California, Santa Cruz) were separated through a 15% (monomer) sodium dodecyl sulfate–polyacrylamide gel. Proteins were electroblotted to nitrocellulose membrane and the transferred proteins were stained with Ponceau S to confirm equivalent loading and transfers. The membrane was blocked by incubation in 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20 and 5% non-fat dried milk. Anti-mouse PRL (gift of F.Talamantes and L.Ogren) was added in the same buffer and incubated with the transferred proteins for 1 h. The membrane was washed twice and incubated for 1 h with goat anti-rabbit IgG–peroxidase conjugate (Sigma Chemical Co., St Louis). After washing three times, the immunoreactive bands were visualized by chemiluminescence using Enhanced Nu-Glo reagent (Alpha Diagnostics, San Antonio) according to the manufacturer's protocol.

Histopathology and morphological analyses

Mice were examined at 6 weeks of age for gross and histological pathologies. The mice were anesthetized and fixed by whole-body formalin perfusion. Organs were examined grossly at necropsy, and were then removed and weighed. The following organs were harvested for examination: eye, conjunctiva, harderian gland, salivary gland, masseter muscle, mandibular lymph node, heart, aorta, renal artery, lung, trachea, thymus, kidney, liver, spleen, stomach, duodenum, jejunum, ileum, ceacum, colon, pancreas, adrenal gland, skin, femur, quadriceps muscle, cranium, cerebrum, cerebellum, mammary gland, vagina, uterus, ovary, testis, epididymis, seminal vesicle, vas deferens, prostate, bulbourethral gland and pituitary gland. Samples of tissues were embedded in paraffin; histological sections were stained with hematoxylin/eosin and examined microscopically by comparing age-matched control and gene-disrupted mouse tissues. For whole-mount analyses, the mammary glands were spread on glass microscope slides and fixed with Carnoy's fixative for at least 30 min. The tissues were washed by incubation in 70% ethanol and defatted with acetone for a minimum of 12 h. They were stained with either iron hematoxylin or safronin O, and photographed at low power under a dissecting microscope or under a 10× objective using an inverted compound microscope. This method was modified from the protocol distributed via the Mammary Gland Biology Internet posting at URL (http://mammary.nih.gov). Morphogenesis of the mammary ducts was compared by counting the number of branches along the primary duct extending along the longest axis of the gland.

Preparation of cell suspensions from hematopoietic organs

Bone marrow cell suspensions were prepared by flushing bones with 3 ml of α-MEM (GIBCO, Grand Island, NY) containing 5% fetal calf serum (Hyclone, Logan, UT). Spleen and thymus cell suspensions were prepared by teasing the organs apart with bent needles or gently pressing them through a fine mesh screen. Cells were counted with a hemacytometer and cell viability, which was always greater than 95%, was determined by eosin dye exclusion. All bone marrow cell counts are expressed as the total number of cells obtained from two femurs and two tibiae.

Immunofluorescence analysis

Expression of particular cell surface determinants was detected by labeling cells with phycoerythrin, fluorescein or biotin-conjugated antibodies to the following cell surface determinants: CD11b (Mac-1; clone M1/70), CD45R (B220; Clone RA3-6B2), CD43 (Clone S7), CD24 (Heat Stable Antigen; clone M1/69). The above antibodies were obtained from Pharmingen, La Jolla, CA. Antibodies to IgM were obtained from Southern Biotechnology, Birmingham, AL. T lineage cells were identified based on labeling with antibodies to CD4 (Clone H129.19) or CD8 (Clone 53-6.7) from Pharmingen. For triple staining, streptavidin conjugated to PerCP (Becton-Dickinson, San Jose, CA) was used.

Cell suspensions were treated with NH_4Cl to lyse erythrocytes before staining, and prior to the addition of one or more of the above antibodies, samples were incubated with an antibody to the FcγII and III receptor (CD16/32; clone 2.4G2 from Pharmingen) to reduce non-specific labeling of cells. All staining protocols were conducted in calcium, magnesium-free phosphate-buffered saline at 4°C. Dead cells were excluded based on their SSC vs. FSC profile or their staining with propidium iodide, added to a final concentration of 0.5 µg/ml per sample. Cell analysis was performed on a Becton-Dickinson FACScan. Gates were set on the basis of staining with either an isotype control antibody conjugated to the same fluorochrome or the secondary reagent alone.

B lineage cells were resolved into various fractions (A–F) based on the technique initially reported by Hardy *et al.* (1991). $CD45R^+CD43^+HSA^-$ fraction A cells were distinguished from $CD45R^+CD43^+HSA^+$ fraction B + C cells on the basis of HSA expression. Fraction D cells were defined as $CD45R^+CD43^-sIgM^-$ cells.

Myeloid colony assay

Bone marrow cells that formed colonies (CFU-GM) in semisolid medium in response to recombinant granulocyte-macrophage colony stimulating factor were enumerated by plating 5×10^4 cells in 35 mm tissue culture dishes containing 1 ml of methylcellulose medium. This contained 40% of a 0.8% methylcellulose solution, 30% fetal calf serum, 30% Iscoves medium, 5×10^{-5} M 2-β-mercaptoethanol and 50 U/ml recombinant GM-CSF (AMGEN, Thousand Oaks, CA). Colonies were counted on day 11.

Statistical analysis

Data were analyzed using a single-tailed Student's *t*-test. Data are presented as mean ± standard deviation.

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