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Socs2 and Elf5 Mediate Prolactin-Induced Mammary Gland Development

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The proliferative phase of mammary alveolar morphogenesis is initiated during early pregnancy by rising levels of serum prolactin and progesterone, establishing a program of gene expression that is ultimately responsible for the development of the lobuloalveoli and the onset of lactation. To explore this largely unknown genetic program, we constructed transcript profiles derived from transplanted mammary glands formed by recombination of prolactin receptor (Prlr) knockout or wild-type mammary epithelium with wild-type mammary stroma. Comparison with profiles derived from prolactin-treated Scp2 mammary epithelial cells produced a small set of commonly prolactin-regulated genes that included the negative regulator of cytokine signaling, Socs2 (suppressor of cytokine signaling 2), and the ets transcription factor, E74-like factor 5 (Elf5). Homozygous null mutation of Socs2 rescued the failure of lactation and reduction of mammary signal transducer and activator of transcription 5 phosphorylation that characterizes Prlr heterozygous mice, demonstrating that mammary Socs2 is a key regulator of the prolactin-signaling pathway. Reexpression of Elf5 in Prlr nullizygous mammary epithelium restored lobuloalveolar development and milk production, demonstrating that Elf5 is a transcription factor capable of substituting for prolactin signaling. Thus, Socs2 and Elf5 are key members of the set of prolactin-regulated genes that mediate prolactin-driven mammary development. (*Molecular Endocrinology* 20: 1177–1187, 2006)

MAMMARY GLAND development differs from the development of most other organs as it proceeds in adults in response to endocrine changes associated with the timing of reproductive events. The hormonal changes of puberty induce the ductal morphogenesis phase of development, when the mammary rudiment developed *in utero* forms terminal end buds that elongate and bifurcate to fill the mammary fat pad with a branched ductal network. Ductal density then increases due to secondary and tertiary side branching in response to each estrous or menstrual cycle. The hormonal changes of pregnancy cause the gland to enter the alveolar morphogenesis phase, characterized by an initial proliferation phase, during which the alveolar architecture is established, followed by the onset of lactation comprised by phases of secretory initiation during the later part of pregnancy and secretory activation after parturition. At weaning the gland commences involution, with the loss of most of the epithelial component gained during the preceding lactation (1–3).

Mouse knockout models have revealed both the requirement for these hormones for mammary development and their complex interactions, which involve receptors located in the ovary and pituitary, in addition to the mammary epithelium and stroma. Ductal morphogenesis is initiated by rising estrogen via a complex mechanism (4–6). Progesterone is required for ductal side branching and alveolar bud formation after puberty and the formation of lobuloalveolar structures during pregnancy (7). Loss of prolactin (8), or the prolactin receptor (Prlr) (9, 10), also prevents side branching after puberty, but indirectly via failure of progesterone secretion from the corpora lutea (11, 12). During pregnancy, complete loss of Prlr stalls development at an early point in the alevolar proliferation phase, after the formation of alveolar buds (9). In contrast, loss of just a single Prlr allele has no effect during the early alveolar proliferation phase, and development proceeds past the formation of alveolar buds to produce lobuloalveolar structures with normal architecture. These structures fail to fully differentiate late in the proliferative phase or early in the secretory initiation phase, however, and lactation fails during secretory

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Abbreviations: Elf5, E74-like factor 5; Expi, extracellular proteinase inhibitor; H&E, hematoxylin and eosin; IRES, internal ribosome entry site; MEC, mammary epithelial cell; Plet-1, placenta-expressed transcript 1; Prlr, prolactin receptor; Socs2, suppressor of cytokine signaling 2; Stat, signal transducer and activator of transcription.

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activation (9). The effects of prolactin on lobuloalveolar development are exerted via the mammary epithelium and not the stroma (9, 13). A hierarchy of action is apparent from these and other studies. Estrogen is essential from the earliest stage of ductal development, and progesterone is required for subsequent side branching. Prolactin and progesterone are necessary for the proliferative phase of alveolar development. A further increase in prolactin-generated signaling is required for functional differentiation during secretory initiation and activation.

Little is known about the program of altered gene expression that drives these developmental events. To screen for key members of this program, we combined transcript profiling with two contrasting models of prolactin action: an *in vivo* model in which prolactin action is ablated specifically in the mammary epithelial cells (MECs) and the Scp2 cell model of augmented prolactin action. These models provide multiple contrasts, such as negative prolactin action *vs.* positive prolactin action, whole tissue *vs.* cultured cells, and proliferative phase *vs.* secretory activation phase, and offer a highly selective set of overlapping criteria that we have exploited to reduce the normally large set of genes produced by transcript-profiling experiments to a small and focused set of prolactin-regulated genes, which were validated by quantitative PCR. Two of these genes, Socs2 and Elf5 (E74-like factor 5), are shown by genetic complementation to rescue the developmental defects seen in $Prlr^{+/-}$ and $Prlr^{-/-}$ mammary glands, respectively, demonstrating their crucial roles in mediating the developmental signal delivered by prolactin.

RESULTS

Identification of Elf5 and Socs2

Both the epithelium and stroma of the murine mammary gland express the Prlr (14, 15), but Prlr is only required in the epithelium for lobuloalveolar development (13). We transcript profiled wild-type mammary fat pads cleared of endogenous epithelium, or wild-type mammary fat pad cleared of endogenous epithelium and transplanted with $Prlr^{+/+}$ or $Prlr^{-/-}$ epithelium, all at 2, 4, and 6 d of pregnancy. The comparison of $Prlr^{+/+}$ to $Prlr^{-/-}$ material provides a very large contrast in prolactin action, much greater than can be achieved using prolactin treatment of wild-type epithelium. This strategy also allowed epithelial patterns of gene expression to be distinguished from genes expressed in the entire gland. The use of transplantation removes the confounding effects of the loss of the Prlr from the endocrine system, as the only cells carrying the null mutation of the Prlr occur in the mammary epithelium of our experimental animals. It also prevents the lymph node diluting the mammary RNA pool. A small selected list of genes from this experiment has been previously published (13), and the complete list of epithelial genes that decreased in $Prlr^{-/-}$ epithelium is supplied in supplemental Fig. 1 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. To further increase the discriminatory power of our analysis we have added a model of positive prolactin action. SCp2 cells grown on matrigel were treated for 48 h with insulin and hydrocortisone, and either with or without prolactin. Supplemental Fig. 2 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. lists the genes that increased in response to prolactin. To distill a small set of genes for further investigation we combined the Scp2 cell data with that of the mammary epithelial transplants. We screened for genes that showed decreased expression at any day in the $Prlr^{-/-}$ epithelial transplants, that increased expression in one of the two SCp2 transcript profiles, and that exhibited epithelial expression at any day in the $Prlr^{-/-}$ epithelial transplants. This three-way selection criteria resulted in a small set of genes. Quantitative RT-PCR was used to verify the expression patterns of this small set, allowing the exclusion of a number and resulting in a small validated set that comprised the milk proteins caseins α, κ, and β, Expi (extracellular proteinase inhibitor), the ets transcription factor Elf5, and Plet-1 (placenta-expressed transcript 1) (Table 1). Plet-1 is expressed poorly in humans due to degradation of its splice acceptor and donor sequences (16) and so was not investigated further. Elf5 was chosen from this set for further analysis.

In the Scp2 cells only five genes showed a sufficiently robust increase of expression in response to

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Table 1. Genes Selected by Searching for Probe Sets that Showed Epithelial-Specific Expression, Decreased in Glands Formed from $Prlr^{-/-}$ Compared with $Prlr^{+/+}$ Epithelium, and Increased in Response to Prolactin Treatment of Scp2 Cells

Probe Set ID	Sequences Derived from	Gene Title	Gene Symbol	Function
103051_at	X93037	Extracellular protein kinase inhibitor	Expi	Milk protein
96030_at	M36780	Casein-alpha	Csnα	Milk protein
99065_at	M10114	Casein-kappa	Csnκ	Milk protein
99130_at	X04490	Casein-beta	Csnβ	Milk protein
97413_at	AI121305	Placenta-expressed transcript 1	Plet-1	Unknown
103283_at	AF049702	E74-like factor 5	Elf5	Transcription

Expressed sequence tags not shown.

prolactin to allow detection in both experimental replicates. We believe this was due to variation in culture conditions between replicates, which serendipitously identified only the most robust changes in gene expression in response to prolactin. This set consisted of four milk proteins (caseins β, α, and κ and Expi) and Socs2. Examination of the transplant data showed that Socs2 decreased expression in response to the loss of the Prlr (average –1.8-fold), but was only called decreasing at d 4 by MAS4. Socs2 did not show epithelial expression as a signal was detected in the stroma. Given these data indicating prolactin regulation of Socs2 expression, and our previous demonstration of the involvement of the family member Socs1 in prolactin-directed mammary development (17), we chose to also analyze the role of Socs2 as a mediator of the mammary response to prolactin.

Expression of Elf5 and Socs2 in the Transcript-Profiling Experiments

We used quantitative PCR to examine Elf5 and Socs2 expression in the transcript-profiling experiments. Elf5 expression was lower in glands formed from Prlr$^{-/-}$ epithelium compared with Prlr$^{+/+}$ epithelium at all times, and was absent from the mammary stroma. Elf5 levels increased in Scp2 cells when they were treated with prolactin (Fig. 1A). Socs2 expression was also decreased in response to a loss of the Prlr from the mammary and was increased by prolactin treatment of Scp2 cells. Socs2 expression was clearly seen in both the epithelium and stroma (Fig. 1B). We sought to demonstrate that these genes are essential members of the prolactin-directed program of gene expression that results in mammary development during pregnancy by a genetic complementation approach.

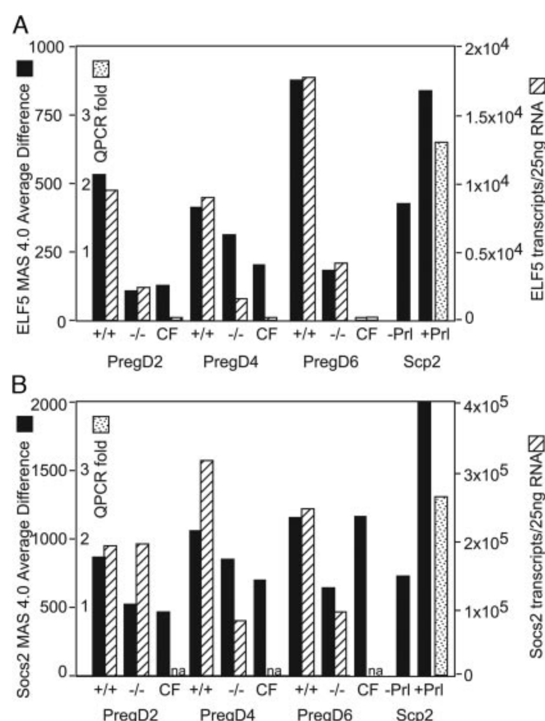

Fig. 1. Candidate Genes Selected by Transcript Profiling
Expression pattern of Elf5 (panel A) and Socs2 (panel B) in the transplants and Scp2 experiments by quantitative RT-PCR. *Solid bars* show average difference, the MAS4 measurement of gene expression level. *Hatched bars* show gene expression measured by quantitative PCR using an absolute quantification method that reports transcripts per microgram of total RNA. *Stippled bars* show gene expression measured by quantitative PCR using a relative method that reports fold change relative to control. CF, Cleared fat pad; Preg, pregnancy; QPCR, quantitative PCR.

Socs2 Expression during Mammary Development

We examined the pattern of Socs2 expression in the mammary gland using *in situ* hybridization. Socs2 was expressed throughout mammary ontogeny, with intense staining of epithelial cells, particularly during pregnancy and lactation (Fig. 2). In contrast, the sense control showed little background staining (Fig. 2A). Socs2 expression was not restricted to the epithelium as a moderate signal intensity was detected in the surrounding stroma, adipocytes, and vasculature. Socs2 expression increased in pregnant samples compared with virgin.

Loss of Socs2 Restores Lactogenesis in Prlr$^{+/-}$ Females

To determine whether loss of the negative regulator Socs2 could rescue the failure of lactation observed in Prlr$^{+/-}$ mice, we generated females that were null for Socs2 and heterozygous for the Prlr gene (Socs2$^{-/-}$, Prlr$^{+/-}$) by interbreeding the respective targeted mice, both of which are on a C57Bl6 background. On this genetic background, failure of lactation occurs in 100% of Prlr$^{+/-}$ females (18). Whole-mount and histological analyses of mammary glands from wild-type animals (Socs2$^{+/+}$ Prlr$^{+/+}$) revealed normal lobuloalveolar development (Fig. 3, A and B) whereas Socs2$^{+/+}$ Prlr$^{+/-}$ females (four of four) showed markedly reduced lobuloalveolar development and failed lactation (Fig. 3, C and D). In contrast, deletion of both Socs2 alleles resulted in complete rescue of lactation in Socs2$^{-/-}$ Prlr$^{+/-}$ females (seven of seven), with all pups surviving. There was no evidence of rescue by deletion of a single Socs2 allele, because pups of Socs2$^{+/-}$ Prlr$^{+/-}$ mothers contained little milk in their stomachs. Western blot analysis to measure milk protein expression in mammary gland lysates revealed that Socs2 deficiency led to restoration of whey acidic protein, α- and β-casein production, comparable to that seen in wild-type mammary glands (Fig. 4A). Analysis of Stat5 activation showed that heterozygous loss of the Prlr greatly reduced Stat5 phosphorylation and that this was restored in animals that also carried a loss of the Socs2 gene (Fig. 4B) These results demonstrate that loss of Socs2 rescued the lactational

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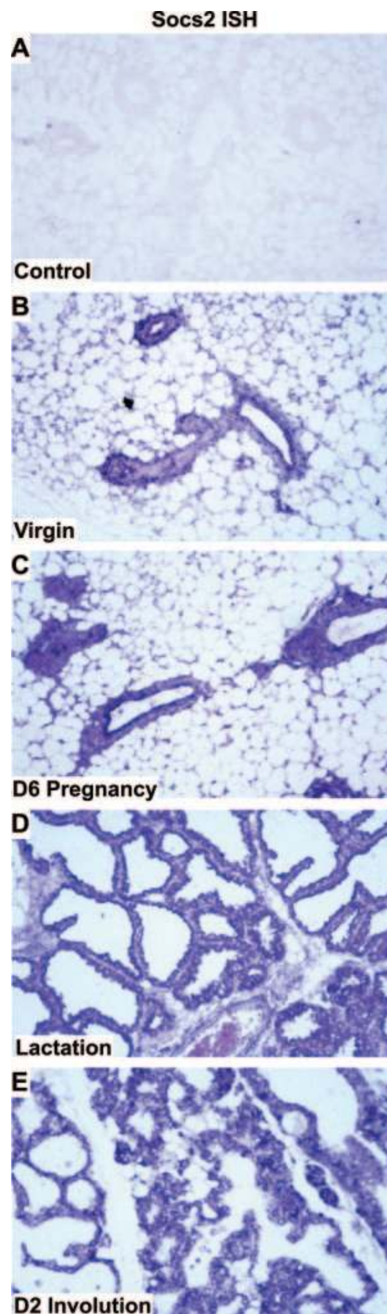

Fig. 2. Mammary Socs2 Expression Increases during Pregnancy and Lactation

In situ hybridization was used to examine the pattern of Socs2 expression during the indicated stages of mammary gland development. Sense control hybridizations (panel A) showed no signal. Socs2 was seen at increased levels during pregnancy and lactation (compare panel B with panels C and D) in the epithelium with a weaker signal in the stroma. Socs2 expression remained high during early involution (panel E). ISH, *In situ* hybridization.

failure produced by loss of a single Prlr allele via a mechanism involving Stat5 activation, establishing Socs2 as an attenuator of prolactin signaling in the mammary gland *in vivo*.

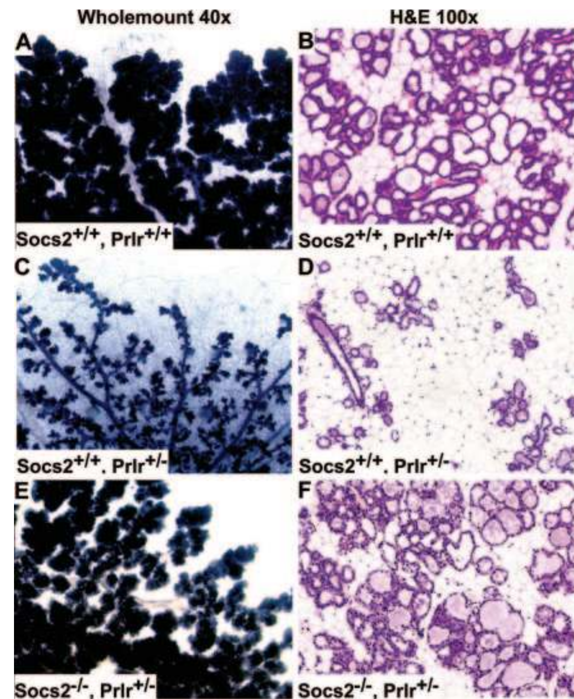

Fig. 3. Null Mutation of the Socs2 Gene Rescues Mammary Gland Development in $Prlr^{+/-}$ Mice

C57Bl6 mice carrying null mutations of Socs2 or Prlr were crossed, and mammary gland development was compared among the resulting genotypes at 1 d post partum by wholemount (*left panels* A, C, and E) or H&E histology (*right panels* B, D, and F).

Elf5 Expression in Mammary Gland and Breast

Examination of Elf5 expression by quantitative RT-PCR showed that a massive increase in Elf5 levels occurs in the mammary gland during pregnancy. In the virgin mammary gland, Elf5 was expressed at levels within the same order of magnitude as other epithelial tissues, approximately 10^4 copies per 25 ng total RNA (Fig. 5A, *bars*). Pregnancy induced a very large increase in Elf5 expression to a peak of about 600,000 transcripts per 25 ng total RNA at d 2 of lactation. Levels remained high throughout lactation. These RNA levels were mirrored by a large increase in Elf5 protein, from low levels that were inconsistently detectable by Western blot in virgin and early pregnant glands to high levels during lactation. Involution of the mammary gland induced by pup removal at d 15 of lactation had an immediate effect on Elf5 protein levels, which became undetectable within a day and preceded the fall in mRNA expression (Fig. 5A, *blot*). Immunohistochemistry showed that Elf5 was located predominantly in the nuclei of the keratin 18-expressing luminal mouse MECs, with approximately half of these cells clearly positive for the protein (Fig. 5B). Elf5 was not expressed in cells located at the basal membrane that expressed high molecular weight keratin. A similar pattern of expression was seen in normal human breast (data not shown).

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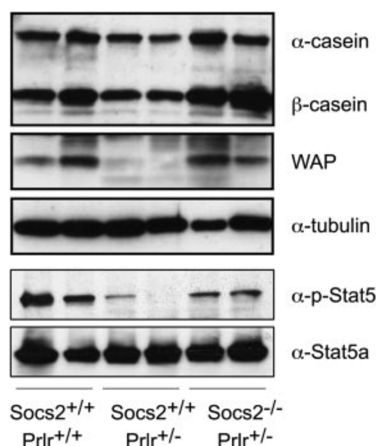

Fig. 4. Null Mutation of the Socs2 Gene Rescues Milk Protein Expression and Mammary Stat5 activation in $Prlr^{+/-}$ Mice

$Prlr^{+/-}$ mice showed a failure of mammary development associated with reduced milk protein synthesis (*top panels*) and Stat5 phosphorylation (*lower panels*); however, mice that were $Prlr^{+/-}$ and $Socs2^{-/-}$ showed near-normal levels of milk protein synthesis and Stat5 phosphorylation. p-Stat-5, phosphorylated Stat5; WAP, whey acidic protein.

Reexpression of Elf5 Restores Normal Development to $Prlr^{-/-}$ Mammary Epithelium

To investigate whether Elf5 could compensate for loss of the Prlr in the mammary epithelium, we reexpressed Elf5 in $Prlr^{-/-}$ MECs by infection with the PolyPOZ retrovirus (19) encoding Elf5. We then transplanted the resulting heterogeneous population of infected and uninfected MECs into the mammary fat pad (previously cleared of endogenous epithelium) of immunocompromised $Rag1^{-/-}$ animals. As a control we infected and transplanted $Prlr^{-/-}$ MECs with empty polyPOZ. The animals were mated 12 wk after the transplant, and the mammary glands collected at 1 d post partum. The experiment was repeated on four separate occasions. In total we attempted to reconstitute nine control $Prlr^{-/-}$ mammary glands using empty polyPOZ-infected MECs. Of these, five glands were successfully reconstituted, and all showed the typical $Prlr^{-/-}$ defect of stalled lobuloalveolar development after the formation of alveolar buds (Fig. 6A). Hematoxylin and eosin (H&E) histology showed the formation of alveolar buds but no lobuloalveoli (Fig. 6B), and the lumen of these structures stained very weakly with an antibody directed against mouse milk (Fig. 6C). We successfully reconstituted 28 mammary glands from a total of 38 attempts using $Prlr^{-/-}$ MECs that we had infected with Elf5-polyPOZ. Of these, 10 showed restoration of alveolar morphogenesis with examples seen in all four experiments. The pattern of restoration was chimeric, comprised of completely rescued lobules and areas that showed varying degrees of incomplete rescue. The areas of incomplete rescued varied from the induction of small single al-

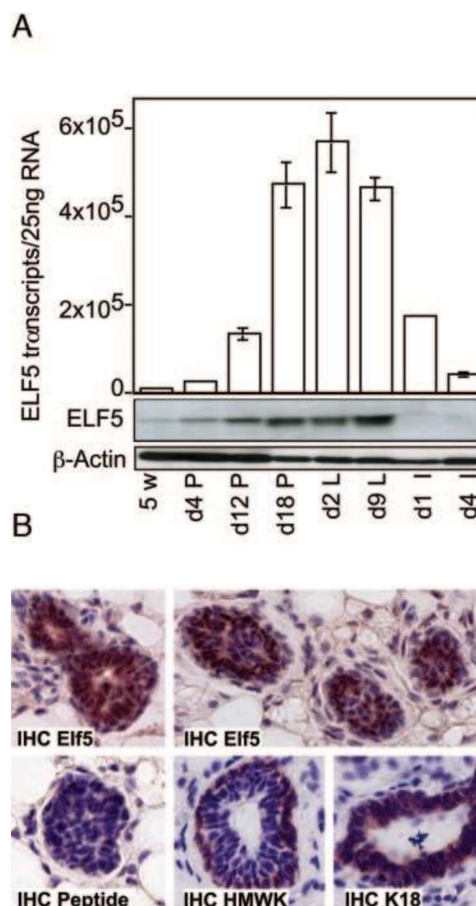

Fig. 5. Elf5 Expression during Mammary Gland Development

A, The level of Elf5 was measured by quantitative RT-PCR using an absolute method, and by Western blot at various stages during mammary gland development as indicated. B, Immunohistochemistry was used to examine the sites of Elf5 expression in the mammary gland at d 12 of pregnancy. An Elf5-blocking peptide was used as a control. Luminal cells and basal cells were distinguished by the expression pattern of keratin 18 (K18) and high-molecular weight keratin (HMWK) respectively. IHC, Immunochemistry.

veoli along the ducts, to lobules that appeared normal but smaller in size than those seen in endogenous glands. Figure 6D shows an example that combines complete rescue (area immediately to the *left of the arrow*) with incomplete rescue, seen as ducts covered with single alveoli (*arrow*). In this example both regions were connected via a common ductal network. H&E histology showed that the large lobules contained oil droplets and colostrum (Fig. 6E), and these lobules stained intensely with the antibody raised against mouse milk (Fig. 6F). The ducts covered with single alveoli (Fig. 6, A–C), were more developed than anything present in $Prlr^{-/-}$ glands as they showed multiple single alveoli (Fig. 6E, *arrow*), but the lumen of these structures did not stain strongly with the antimilk antibody (Fig. 6F, *arrow*). Figure 6, G–I, shows a $Prlr^{-/-}$ gland displaying complete rescue in most ar-

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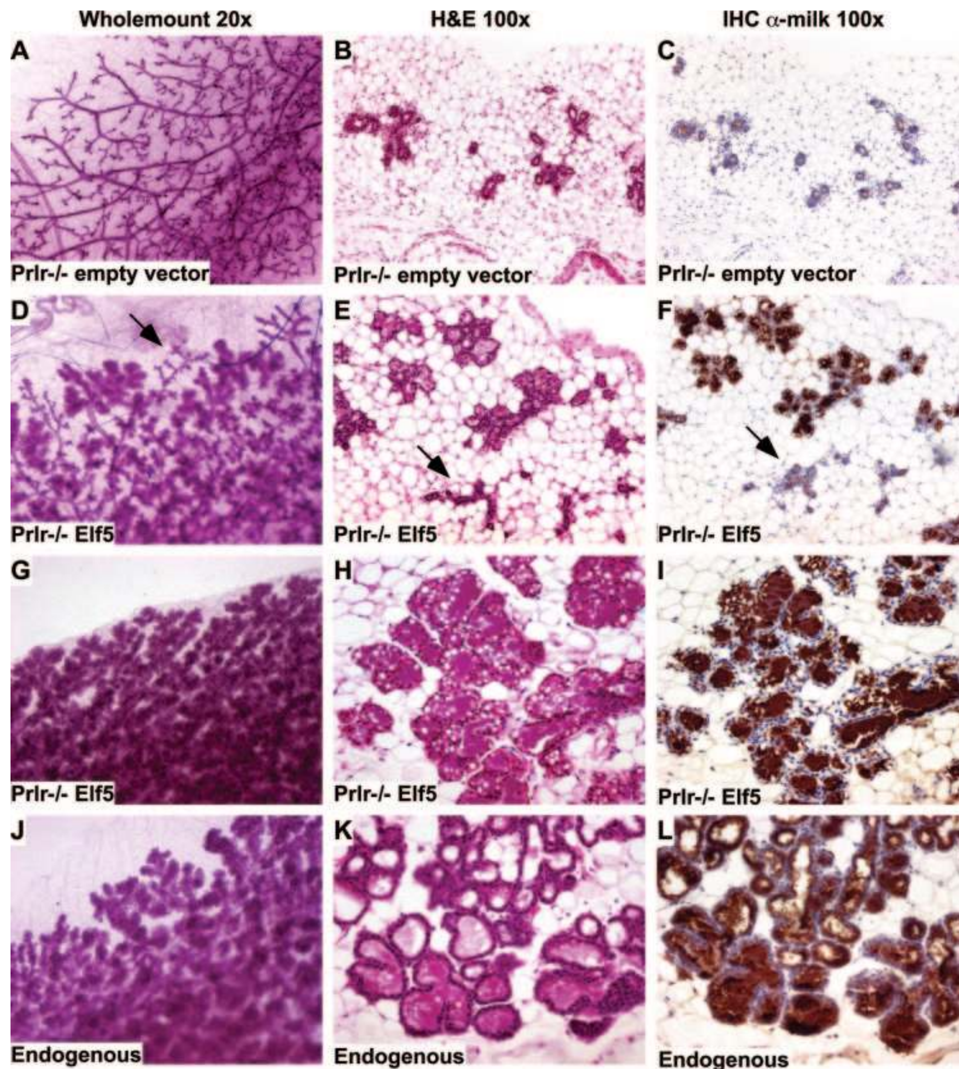

Fig. 6. Expression of Elf5 in $Prlr^{-/-}$ Mammary Gland Rescues Mammary Gland Development

$Prlr^{-/-}$ MECs were infected with the polyPOZ retrovirus and then transplanted to the cleared mammary fat pad of host $Rag1^{-/-}$ mice and made pregnant 12 wk after transplant. A–C, Use of polyPOZ without the Elf5 construct (empty vector) had no effect on the failure of development seen in $Prlr^{-/-}$ transplants during pregnancy in all cases. D–F, Use of a polyPOZ Elf5 construct resulted in a rescue of mammary gland development that showed a chimeric pattern of complete rescue mixed with partial rescue (*arrows*). G–I, Some glands showed near-complete rescue that mimicked development seen in the endogenous glands. J–L, Endogenous glands. IHC, Immunohistochemistry.

eas, but scanning of the tissue showed occasional small areas with structures identical to those indicated by *arrows* in Fig. 6, D–F. In examples showing the least degree of rescue, the whole mounts appear as a ductal tree covered in single alveoli, similar to that indicated by *arrow* in 6D, with a just small portion containing lobules that stain intensely with the antimilk antibody (data not shown). Of the 10 rescued glands, two showed a chimeric rescue similar to Fig. 6, D–F, four showed almost complete rescue similar to Fig. 6, G–I, and four showed a chimeric rescue with just a small region of lobules. In all cases, development had proceeded past the production of the sparse alveolar buds that characterize the $Prlr^{-/-}$ defect. H&E staining showed that the lobules that had formed in these cases often had smaller alveoli with smaller lumens than wild-type transplants or endogenous glands. Milk staining showed that these lobules synthesized milk at wild-type levels but that the single alveoli located along the ducts showed less milk protein expression. This is presumably due to development of a mammary tree from a heterogeneous population of infected and uninfected MECs, which have contributed to a different extent to the developing epithelium. Where the ratio of cells expressing high levels of Elf5 is high, lobuloalveolar development proceeds further. Our vector contained a LacZ marker under the control of an internal ribosome entry site (IRES). We were able to visualize a weak Lacz signal in MECs after PolyPOZ infection, which showed the cultures to be a mixture of

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Elf5-expressing and nonexpressing cells, but we were unable to successfully visualize LacZ in the resulting transplants. The use of an IRES results in much lower expression of the marker compared with the test gene, and so we believe that lack of LacZ sensitivity prevented visualization. RT-PCR investigation of $Prlr^{-/-}$ glands that showed rescued development by infection with PolyPOZ-Elf5-LacZ retrovirus demonstrated the presence of LacZ in these glands.

We examined the level of Elf5 expression in these glands. In $Prlr^{-/-}$ glands infected with the empty vector, endogenous Elf5 was seen as a weak nuclear signal of the luminal epithelial cells with a columnar shape (Fig. 7, A and B). About half of the luminal epithelial cells stained positive for Elf5. In $Prlr^{-/-}$ glands showing partial rescue of alveolargenesis, higher levels of Elf5 were detected in the nuclei, which now showed an oval shape, and weak cytoplasmic Elf5 staining was seen (Fig. 7, C and D). Still higher levels of nuclear and cytoplasmic Elf5 were detected in areas of complete rescue that stained strongly for milk (Fig. 7, E and F). Both the level of expression and the nuclear/cytoplasmic localization were similar to the levels seen in endogenous glands, where Elf5 was seen as a strong cytoplasmic signal and an intense red-brown staining of large rounded nuclei that completely obscured the hematoxylin nuclear stain (Fig. 7, G and H). Use of a peptide block or no Elf5 antibody (Fig. 7, I and J) showed the nuclear and cytoplasmic staining to be specific but showed the adipocyte margin staining to be nonspecific. Thus, complete rescue of lobuloalveolargenesis by infection with an Elf5-producing retrovirus was associated with restoration of Elf5 expression and subcellular distribution in a way that mimicked the level and pattern seen in endogenous glands. Incomplete rescue was associated with a conversion of columnar to oval nuclei and a level of Elf5 expression intermediate between $Prlr^{-/-}$ and endogenous levels.

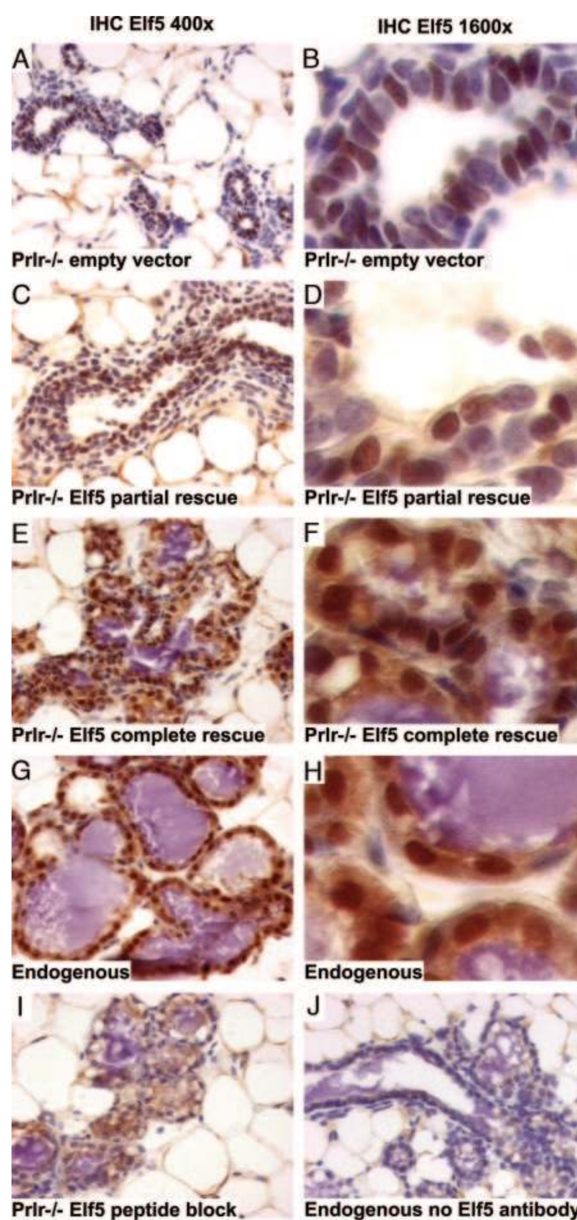

Fig. 7. Expression of Elf5 in $Prlr^{-/-}$ Glands Rescued by Reexpression of Elf5

Immunohistochemistry was used to examine Elf5 levels. A and B (*top row*), Elf5 expression in $Prlr^{-/-}$ epithelium infected with empty PolyPOZ. Weak nuclear staining is seen. C and D, A region of partial rescue showing increased Elf5 expression over $Prlr^{-/-}$ in the nuclei. Note change in nuclear shape. E and F, A region of complete rescue showing a further increase in nuclear Elf5 level and expression in the cytoplasm, rounding of the epithelial nuclei, multiple alveoli with lumen formation, and the synthesis of milk protein (*purple* color in the lumen). G and H, Elf5 levels in endogenous glands show identical histological features and levels and patterns of Elf5 staining as produced by PolyPOZ in panels E and F. I and J, Controls demonstrating nuclear Elf5 staining to be specific but Elf5 staining of the adipocyte margins and alveolar lumen to be nonspecific. IHC, Immunohistochemistry.

DISCUSSION

From our transcript-profiling experiments we chose to focus on Socs2 and Elf5. We report here that both Socs2 and Elf5 can recapitulate prolactin function *in vivo* by genetic complementation using Prlr-deficient mammary epithelium.

Signaling initiated by cytokine receptors via the Jak-Stat pathway is attenuated via three mechanisms, the protein inhibitor of activated STAT proteins, which prevent Stat dimerization or DNA interaction, the SH2-containing protein tyrosine phosphatases, which dephosphorylate activating tyrosine phosphorylations, and the Socs proteins, which are transcribed in response to cytokine signaling and which interact with the receptors or the receptor-associated Jak kinase to prevent Stat activation and to promote degradation via the proteasome (20). Knockout of Socs2 in mice re-

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sulted in a loss of growth control. The long bones and nose to tail length is increased, and organs appear normal, but are larger (although in proportion) due to increased cell numbers with increased collagen deposition, especially in the organ ducts and vessels (21). Socs2 has not previously been implicated in the attenuation of prolactin action in the mammary gland *in vivo*, and it is now clear that both Socs1 (17) and Socs2 perform this function via modulation of Stat5 phosphorylation. Although the studies of Flint and colleagues (22) show that GH treatment could enhance alveolar development in $Prlr^{+/-}$ mice, it further suppressed secretory activity and prevented lactation. Because we observed normal lactation, we can discount a GH-based mechanism of Socs2 rescue of $Prlr^{+/-}$ development.

Elf5 is an ETS transcription factor, a large and diverse family homologous to the *v-ets* oncogene encoded by the E26 avian erythroblastosis virus, with a conserved DNA-binding domain of the winged helix-turn-helix superfamily. They are involved in cell proliferation, differentiation (23), and carcinogenesis (24). Most ETS factors are expressed in MECs (25), where PEA3 influences branching morphogenesis (26) and is implicated in the initiation of ERB2-positive breast cancer in humans (27, 28) and mice (29). ETS2 in the fat pad is necessary for development of tumors initiated by ERRB2 in the mammary epithelium (30) and is necessary for anchorage-independent growth of breast cancer cell lines (31). The Elf subfamily consists of five members (Elf1 to -5). Elf5 acts as an activator of transcription in the mouse (32) and human (33). Elf5 directly activates a GGAA site in the whey acidic protein promoter (34). It has been suggested that Elf5 has a negative regulatory domain that inhibits DNA binding (33). Elf5 is located on human chromosome 11p13–15 (32), a region of the genome known to experience loss of heterozygosity in some breast cancers. Elf5 mRNA expression is also lost in a number of breast cancers compared with adjacent normal tissue (35). Elf5 is a key regulator of lobuloalveolar development, as demonstrated by the formation of lobules capable of milk production after retroviral reexpression of Elf5 in Prlr knockout mammary epithelium. Not only were morphologically normal alveoli produced, histological examination showed correct cellular architecture, the formation of lipid droplets within the cells of these alveoli, and milk production, demonstrating that the secretory initiation phase had been entered. Rescued portions of Prlr knockout glands showed greatly increased expression of Elf5. The conclusion that Elf5 is a key regulator of mammary development is supported by our recent finding that heterozygous null mutation of the Elf5 gene caused lactational failure (36). Analysis of the effect of complete loss of Elf5 has been prevented by early embryonic lethality of homozygous Elf knockout mice (36).

It is surprising that a single transcription factor can produce such a comprehensive rescue of development in $Prlr^{-/-}$ mammary epithelium. A caveat here is that the retroviral-transplantation model does not allow secretory activation to be evaluated, because the mammary tree is not connected to the nipple and so the gland undergoes engorgement-induced involution post partum. Therefore we do not know whether Elf5 alone can rescue lactation in $Prlr^{-/-}$ mammary gland to a level sufficient for pup survival. Prolactin has a pro-proliferative effect during alveolargenesis and an additional differentiation action during secretory activation. Thus, although Elf5 can substitute for the proliferative action of prolactin, our data allow no conclusions regarding the differentiative and lactogenic actions of this hormone. Because Elf5 alone can rescue alveolar development, it is very likely that Elf5 mediates mammary development in response to many of the hormones and growth factors known to be essential for this process. Given this, it is also likely that the regulation of Elf5 will involve the interaction of the signaling pathways activated by many of the hormones controlling mammary development and will not be solely regulated by the Prl/Prlr/Jak2-Stat5 pathway. Our findings demonstrate that expression of Elf5 is sufficient to do the work of building the lobuloalveoli, and it will intriguing to discover whether Elf5 expression can rescue mammary development in knockout models of other pathway members, such as Stat5, and in other nonpathway members, such as progesterone receptor. Placing Rank ligand relative to Elf5 will also shed further light on the composition of this pathway.

Only two genes have been reported to recapitulate prolactin action by genetic complementation. Retroviral reexpression of Igf2 has been shown to allow partial development of Prlr knockout mammary epithelium during pregnancy (37), and heterozygous loss of Socs1 has been demonstrated to fully rescue lactational failure in Prlr heterozygous glands (17). We can now add reexpression of Elf5 in Prlr knockout mammary epithelium and homozygous loss of Socs2 from Prlr heterozygous mammary epithelium, to this small list of genes that have been demonstrated by complementation assays to recapitulate prolactin action.

MATERIALS AND METHODS

Mice, Tissue Recombination, and Epithelial Transplantation

All animal experimentation was conducted under the supervision and within the guidelines of the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee. Glands for transcript profiling were prepared by clearing both the fourth mammary fat pads from a $Rag1^{-/-}$ mouse of endogenous mammary epithelium and then transplanting $Prlr^{-/-}$ or $Prlr^{+/+}$ epithelium (C57BL6×129SVPas) to either fourth mammary fat pad of the same animal (38). The animals were then aged for 12 wk. Animals were mated and checked for vaginal plugs in the morning. At 2, 4, and 6 d after observation of a plug the mammary glands were collected, frozen in liquid nitrogen, and stored at −70 C. The $Socs2^{-/-}$ and $Prlr^{+/-}$ mice used for the interbreeding study were inbred C57Bl6 and generation 6 backcrossed to C57Bl6, respectively.

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SCp2 Cell Differentiation Assay

SCp2 MECs (39) were passaged in DMEM-F12 medium containing DMEM-HAM, 10% fetal calf serum, and insulin 5 μg/ml (Sigma Chemical Co., St. Louis, MO). Briefly, 7×10^5 cells were plated in 35-mm dishes precoated with extracellular matrix (ECM) (Matrigel; Collaborative Research) in DMEM-F12 containing 2% fetal calf serum and insulin (5 μg/ml) (Sigma). The next day, cells were washed twice, and then placed in differentiation media containing DMEM-F12 plus insulin (5 μg/ml), hydrocortisone (1 μg/ml), and prolactin (3 μg/ml) a kind gift of Dr. A. Parlow, National Hormone and Pituitary Program. Cells were induced to differentiate for 48 h (10 dishes). Untreated cells (10 dishes) were incubated with insulin (5 μg/ml) and hydrocortisone (1 μg/ml) but not prolactin. Cells were harvested directly for RNA extraction using Trizol (Life Technologies, Gaithersburg, MD).

Target Preparation, GeneChip Hybridization, and Scanning

Total RNA was prepared from frozen mammary glands by homogenization with a Polytron in Trizol for 30 sec, chloroform extraction, and isopropanol precipitation. RNA was further purified using QIAGEN RNeasy Mini Kit (QIAGEN, Chatsworth, CA). The cRNA targets were generated as recommended by Affymetrix (Santa Clara, CA) and hybridized to MG-U74A GeneChips (Affymetrix). The GeneChips were scanned using the GeneArray Scanner, and the hybridization intensities and fold change between experiments was obtained using Microarray Suite 4.0 (Affymetrix) and the MGU74A mask.

GeneChip Analysis and Database Interrogation

Data were analyzed using Microarray Suite 4.0 (Mas4, Affymetrix) Mas5, and Spotfire visualization software. Known genes were searched in PubMed for relevance to mammary gland development. Expressed sequence tags were associated with known genes or other expressed sequence tags by querying Unigene (http://www.ncbi.nlm.nih.gov/UniGene/) and verified by ClustalW alignment in Macvector (Oxford Molecular, Inc., Palo Alto, CA). Further sequence and structural information, ontologies, and human orthologs were obtained from Resourcerer (http://pga.tigr.org/tigr-scripts/magic/r1.pl) and Netaffx (https://www.affymetrix.com), and exons were identified in the mouse genome by searching the Ensembl database (http://www.ensembl.org/Mus_musculus/).

Quantitative PCR

Total RNA was prepared using the Trizol method. RNA was reverse transcribed using AMV reverse transcriptase (Promega Corp., Madison, WI). PCR primers were designed using Macvector so that the product spanned an intron. The PCRs were performed in a LightCycler using the FastStart DNA master SYBR Green I enzyme mix (Roche Clinical Laboratories, Indianapolis, IN) in a 10-μl reaction volume. Absolute quantification was performed by comparing transcript levels in samples to a standard curve constructed by performing serial dilutions of PCR product purified using QIAquick Gel Extraction Kit (QIAGEN) and analyzed using the Second Derivative Maximum method (Roche). All data were normalized to expression of the housekeeping gene β-actin.

Immunohistochemistry

Mammary glands were dissected from mice, rinsed in cold PBS, and fixed overnight in 10% neutral buffered formalin or for 2 h in 4% paraformaldehyde at 4 C. Tissues were processed and embedded in paraffin wax and cut in 5-μm sections onto Superfrost Plus slides (Menzel-Glaser, Singapore). Antigen retrieval used DAKO (DAKO, Carpinteria, CA) Target Retrieval Solution, pH 9.9, in a boiling water bath for 20 min followed by H_2O_2 treatment. Counterstaining was performed with hematoxylin and 1% acid alcohol incubation with primary antibody. Elf5 immunohistochemistry was performed using the Elf-5 (N-20) affinity-purified goat polyclonal antibody (sc-9645) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) at a dilution of 1:300, and a secondary biotinylated horse antigoat (Vector Laboratories, Inc., Burlingame, CA), LSAB+ (DAKO) and was detected using Liquid DAB+ Substrate Chromogen (DAKO). The antimilk (1:12,000) primary antibody (Accurate Chemical & Scientific Corp., Westbury, NY) was incubated for 30 min, and bound antibody was detected using the Envision System (DAKO) and 3,3′-diaminobenzidine Plus (DAKO) as substrate. Rabbit IgG without antibody provided the negative technical control. The anticytokeratin 18 mouse monoclonal (Research Diagnostics, Flanders, NJ), and the high-molecular weight keratin mouse monoclonal antibody (DAKO) were used at 1:100 and were biotinylated with the DAKO ARK kit antibody and detected using LSAB+/DAB as above.

In Situ Hybridization

Full-length mouse SOCS2 cDNA was cloned into Bluescript SKII (Stratagene, La Jolla, CA). Antisense and sense riboprobes were generated using T3 or T7 RNA polymerase (Promega) with digoxigenin-UTP (Roche). Standard *in situ* hybridizations were performed as described elsewhere (22).

Western Blotting

Mouse mammary gland protein lysates were prepared by grinding the tissue into a fine powder in liquid nitrogen and subsequent solubilization in 1% TEB [150 mM NaCl; 5 mM EDTA, 50 mM Tris (pH 7.5); 0.1% Nonidet P-40] supplemented with complete protease inhibitor (Roche, Mannheim, Germany), 10 mM sodium fluoride, and 1 mM sodium orthovanodate. Protein lysates (50 μg) were separated by SDS-PAGE. After transfer, filters were blocked and incubated with rabbit polyclonal antiserum raised against mouse milk-specific proteins (Accurate Chemical & Scientific Corp.) or anti-α-tubulin monoclonal antibody (Sigma). Antibodies specific for a-phospho-STAT5 (Upstate Biotechnology, Inc., Lake Placid, NY) and a-STAT5a (Santa Cruz Biotechnology) were also used. Antibody binding was visualized with peroxidase-conjugated antirabbit or antimouse (Amersham Pharmacia Biotech, Arlington Heights, IL) using the enhanced chemiluminescence system (Amersham).

Retroviral Infection of MECs

Mouse Elf5 cDNA was isolated from mammary gland cDNA by PCR and cloned into the retroviral vector polyPOZ [a gift from Dr. T. Dale, (19)]. *Elf5*-IRES-*LacZ*-polyPOZ and *LacZ*-polyPOZ ecotropic retroviruses were packaged in Phoenix-Eco cells (a gift of Philip Achacoso and Garry Nolan, Stanford University Medical Center, Stanford, CA) by transient transfection using FuGENE-6 Reagent (Roche). Viral supernatant was harvested by filtration through a 0.45-μm filter. Primary mouse MECs were harvested from mammary glands of 11- to 13-wk-old virgin *Prlr*$^{-/-}$ mice. Briefly, the no. 4 mammary glands were dissected out under sterile conditions, finely chopped, and subjected to three to four rounds of collagenase (10 mg/ml) digestion in 2.5% fetal bovine serum/HEPES-buffered RPMI 1640. The purified epithelial cells were plated in DMEM: Ham's F12 (GIBCO) supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 5 μg/ml hydrocortisone, and 10 ng/ml cholera toxin (all additives from Sigma). Primary MECs were sub-

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jected to four rounds of retroviral infection by addition of viral supernatant plus 8 μg/ml polybrene. 0.5–1 $\times$ 10^6 MECs were injected into the no. 4 mammary fat pad of a 3-wk-old *RAG1*$^{-/-}$ recipient female mouse prepared as described above.

Whole-Mount and H&E Histology

The mammary glands were fixed in 10% formalin solution, defatted in acetone stained in carmine alum (0.2% carmine, 0.5% aluminum sulfate) or hematoxylin, dehydrated in ethanol followed by SlideBrite, and then cleared in methyl salicylate. Hematoxylin and eosin staining used 4-μm sections of mammary glands incubated in two changes of SlideBrite (SASKO, Stuttgart, Germany), hydrated, stained with hematoxylin, washed in water, dehydrated, stained with eosin, and dehydrated in ethanol and SlideBrite before mounting with Britemount.

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