

# Estrogen receptor- $\alpha$ expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice

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The estrogen receptor- $\alpha$  (ER $\alpha$ ) is a critical transcription factor that regulates epithelial cell proliferation and ductal morphogenesis during postnatal mammary gland development. Tissue recombination and transplantation studies using the first generation of ER $\alpha$  knockout (ERKO) mice suggested that this steroid hormone receptor is required in the mammary stroma that subsequently exerts its effect on the epithelium through additional paracrine signaling events. A more detailed analysis revealed that ERKO mice produce a truncated ER $\alpha$  protein with detectable transactivation activity, and it is likely that this functional ER $\alpha$  variant has masked the biological significance of this steroid receptor in the mammary epithelium. In this article, we describe the generation a Cre-lox-based conditional knockout of the ER $\alpha$  gene to study the biological function of this steroid receptor in the epithelial compartment at defined stages of mammary gland development. The mouse mammary tumor virus (MMTV)-Cre-mediated, epithelial-specific ablation of exon 3 of the ER $\alpha$  gene in virgin mice severely impaired ductal elongation and side branching. The conditional knockout resulted in ablation of the ER $\alpha$  protein, and the progesterone receptor (PR), whose expression is under the control of ER $\alpha$ , was largely absent. The whey acidic protein (WAP)-Cre-mediated deletion of ER $\alpha$  during successive gestation cycles resulted in a loss of ductal side-branching and lobuloalveolar structures, ductal dilation, and decreased proliferation of alveolar progenitors. These abnormalities compromised milk production and led to malnourishment of the offspring by the second lactation. These observations suggest that ER $\alpha$  expression in the mammary epithelium is essential for normal ductal morphogenesis during puberty and alveologenesis during pregnancy and lactation.

conditional knockout | mammary gland

Estrogen receptor (ER) is a transcription factor that regulates the genetic program of cell cycle progression and growth in healthy and cancerous mammary glands in response to circulating ovarian hormones. Of the two receptor forms (ER $\alpha$  and ER $\beta$ ), ER $\alpha$  is considered the primary receptor for mammary gland development and function. The mammary gland is unique compared with every other organ of the body in that the bulk of its development and differentiation occurs postnatally. After birth and until puberty, the mammary gland is dormant and rudimentary. At puberty, the mammary gland develops rapidly in response to changes in circulating hormone levels. Terminal end buds (TEBs) that are composed of multiple layers of cuboidal epithelial cells begin to form at the termini of primitive ducts. The TEBs invade the fat pad and give rise to a branching network of ducts that end at the edge of the mammary fat pad. Growth of the ductal network ceases at this stage until further stimulation during pregnancy (1).

The role of ER $\alpha$  in mammary gland development was directly established by the seminal studies of Korach and colleagues in their genomic ER $\alpha$  knockout (ERKO) mouse model (2, 3). Later studies, however, showed that these genomic ERKO mice,

in which the ER $\alpha$  gene was disrupted by the insertion of a neomycin resistance gene (neo) into the first coding exon, are hypomorphic for ER $\alpha$  in that substantial ER $\alpha$  function is retained (4). Circulating prolactin (PRL) levels were reduced in hypomorphic ER $\alpha$  females, and there was a lack of mammary gland development beyond the prepubertal stage (2). Restoration of PRL levels by pituitary isograft normalized mammary gland development, which could be prevented by ovariectomy. Likewise, exogenous estradiol and progesterone induced normal ductal elongation and TEB formation in hypomorphic ER $\alpha$  females. Thus, the observed phenotype was in part attributable to abnormal pituitary and ovarian hormone levels in the animals, whereas ER $\alpha$  function was largely retained in the mammary gland.

The mammary gland consists of multiple cell types including luminal and basal epithelial cells, stromal cells, adipocytes, and vascular endothelial and smooth muscle cells. In the mammary glands of rodents, both the epithelium and stroma express ER $\alpha$  (5–7). In an effort to dissect the complex paracrine and autocrine regulation of mammary gland development by estrogen, Cunha *et al.* pioneered a technique of tissue recombination *in vivo* consisting of various combinations of stromal and epithelial compartments from hypomorphic ER $\alpha$  and WT mice (8). The results from this and subsequent studies (9) suggested that stromal ER $\alpha$  was necessary for mammary gland development, whereas epithelial ER $\alpha$  was dispensable. This suggested role of ER $\alpha$  in the mammary gland of rodents appears to contradict the finding that tamoxifen treatment of breast cancer patients targets ER $\alpha$  in the cancer cells themselves and not the stroma of the breast. This issue is further substantiated by the fact that these targeted therapies are effective in the treatment of metastatic breast cancer cells, which are epithelial in origin and interact with stromal cells of distant organs. Thus, the conclusions drawn from the tissue recombination experiments using the hypomorphic ER $\alpha$  mice require reevaluation. Toward this end, a more recent ER $\alpha$  knockout ( $\alpha$ ERKO) mouse model has been developed in which exon 3 of the ER $\alpha$  gene was deleted without any detectable expression of ER $\alpha$  transcript (10). Mammary glands from genetic  $\alpha$ ERKO mice were normal before puberty. After the onset of puberty, however, TEBs remained absent and

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Abbreviations: Cre, Cre-recombinase; ER, estrogen receptor; ERKO, ER knockout; MEC, mammary epithelial cell; MMTV, mouse mammary tumor virus; P, progesterone; PCNA, proliferating cell nuclear antigen; PI-MEC, parity-induced mammary epithelial cell; PR, progesterone receptor; PRL, prolactin; TEB, terminal end bud; WAP, whey acidic protein.

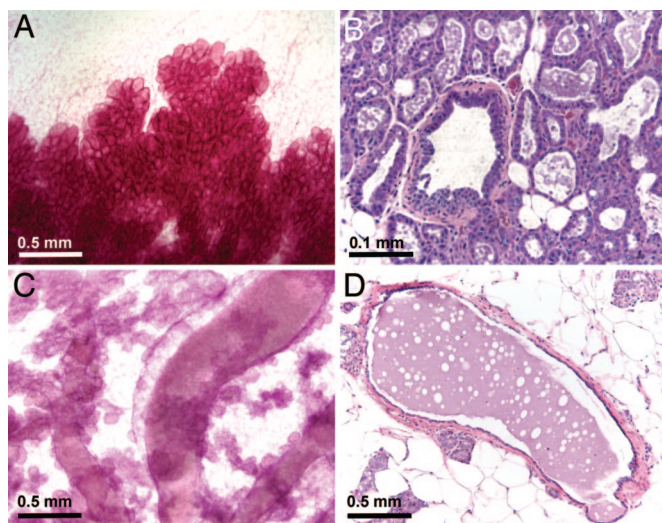
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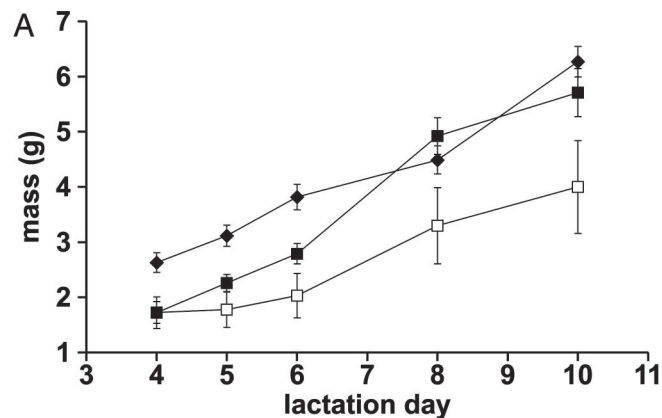


**Fig. 3.** Dilatation and inadequate branching of ducts in WAP-ERKO mice during second lactation. Whole mounts (A and C) and H&E staining (B and D) were used to analyze the morphology of the mammary glands at second lactation day 1 of the WAP-ERKO females (C and D) and ER $\alpha^{fl/fl}$  controls (A and B). Note different magnifications in B and D, emphasizing grossly enlarged ducts in mutant mice.

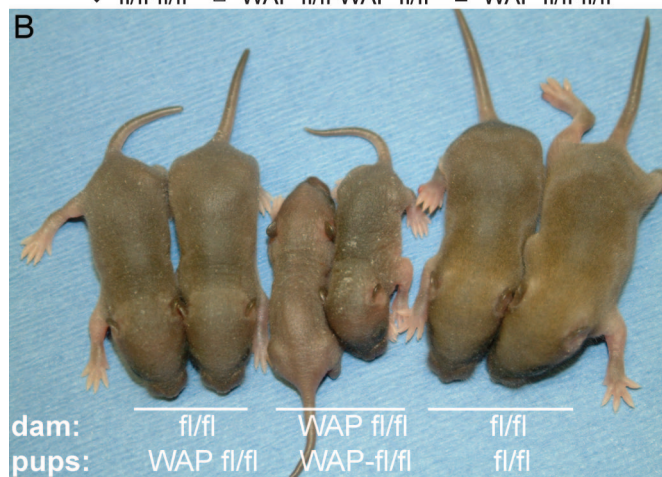
expression of Cre recombinase in the mammary glands of WAP-Cre mice (data not shown). Hypomorphic  $\alpha$ ERKO mice are characterized by increased circulating levels of estrogen and reduced levels of progesterone and PRL, all of which are required for normal mammary gland development, differentiation, and lactation (17). Using commercial ELISA kits, we found that serum estradiol and progesterone levels are within the normal physiological range in both virgin WAP-Cre/ER $\alpha^{fl/fl}$  and pregnant WAP-ERKO mice compared with ER $\alpha^{fl/fl}$  mice ( $n = 3$  females per group, data not shown). In a cell-based assay, in which the proliferation of PRL-dependent mammary epithelial cells is quantified (18), we found that serum PRL levels were also normal in the conditional knockouts (data not shown).

To determine the impact of ER $\alpha$  conditional deletion on mammary gland development, we examined the inguinal mammary glands (no. 4) from WAP-ERKO and ER $\alpha^{fl/fl}$  females at various stages of successive pregnancy and lactation cycles. During the first lactation, a mosaic phenotype was detected in the mammary glands of WAP-ERKO females. Many secretory alveoli were of normal size. However, alveolar growth was less extensive in various parts of the mammary gland of lactating WAP-ERKO females, and we also observed a mild defect in tertiary ductal branching during the first pregnancy and lactation (SI Fig. 7). In contrast, ductal structures were obscured and not directly visible because of the normal alveolar expansion in ER $\alpha^{fl/fl}$  control mice during the first and also the second pregnancy (Fig. 3 A and B). However, during the second pregnancy cycle, WAP-ERKO females exhibited aberrantly dilated ducts with few side-branches that were filled with milk-like secretions (Fig. 3 C and D). We observed strikingly fewer lobular alveoli in WAP-ERKO mice compared with ER $\alpha^{fl/fl}$  control females (Fig. 3C). The diameters of engorged WAP-ERKO ducts (Fig. 3D) were an order of magnitude larger than ER $\alpha^{fl/fl}$  control ducts (Fig. 3B).

Unlike transplant models that are not suitable for lactation studies because of the lack of a nipple connection, the WAP-Cre-based, mammary-specific knockout of ER $\alpha$  allowed us to assess the importance of epithelial ER $\alpha$  for the growing offspring. Using the conditional knockout mice, we observed that ER $\alpha$  deficiency profoundly impaired the normal growth of the

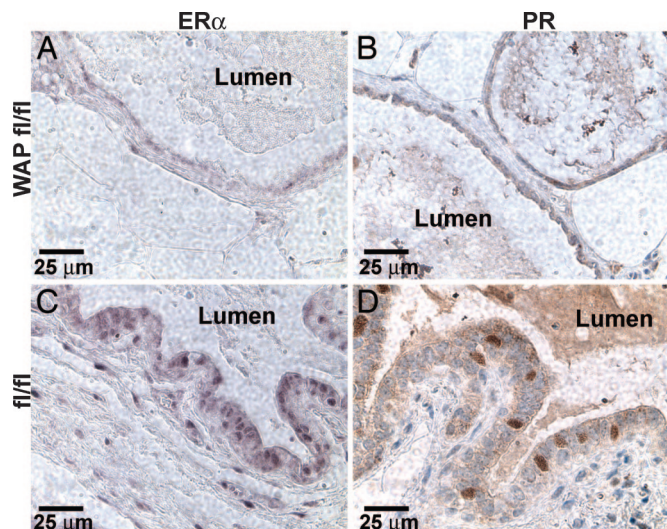


◆ fl/fl-fl/fl □ WAP fl/fl-WAP fl/fl ■ WAP fl/fl-fl/fl



**Fig. 4.** Growth retardation in WAP-ERKO pups from the second litter is attributable to mother's genotype. (A) WAP-Cre/ER $\alpha^{fl/fl}$  pups nursed by their WAP-ERKO mothers (WAP $^{fl/fl}$ -WAP $^{fl/fl}$ ,  $n = 5$ ) were significantly smaller than control ER $\alpha^{fl/fl}$  pups nursed by ER $\alpha^{fl/fl}$  mothers (fl/fl-fl/fl). Beginning on day 4 of lactation, a subset of WAP-Cre/ER $\alpha^{fl/fl}$  pups was fostered with lactating ER $\alpha^{fl/fl}$  mothers (WAP fl/fl-fl/fl). After 4 days of fostering by an ER $\alpha^{fl/fl}$  dam (lactation day 8), there was no longer a statistically significant difference between WAP-Cre/ER $\alpha^{fl/fl}$  and ER $\alpha^{fl/fl}$  pup mass ( $n = 4$ ). Data are presented as the mean  $\pm$  1 standard deviation. (B) Pairs of representative pups nursed for 10 days by birth mother or for 4 days by birth mother followed by 6 days of nursing by foster dam as in A.

offspring. Approximately one-third of the pups nursed by WAP-ERKO mothers were malnourished during the first lactation period. The average body weight of 21-day-old pups was  $\approx 17\%$  less than that of pups from similar-sized litters nursed by ER $\alpha^{fl/fl}$  females (10 g versus 12 g,  $n = 8$ ,  $P < 0.001$ ). The growth retardation of the pups was more severe during the second lactation. At this time, the average weight of 6-day-old pups nursing on a WAP-ERKO mother was  $\approx 50\%$  that of control pups nursing on ER $\alpha^{fl/fl}$  dams (Fig. 4). The growth retardation of the offspring from WAP-ERKO females was fully rescued by fostering them with ER $\alpha^{fl/fl}$  mothers (Fig. 4). Milk harvested from WAP-ERKO females contained normal concentrations of WAP (SI Fig. 8), which is critical for offspring nourishment (19). This suggests a defect in milk production and delivery attributable to abnormal glandular architecture rather than production of inferior-quality milk in WAP-ERKO nursing females. Accordingly, the volume of milk harvested from nursing WAP-ERKO females during the second lactation cycle was less than half that of ER $\alpha^{fl/fl}$  females ( $n = 2$  per group, data not shown).



**Fig. 5.** Loss of ER $\alpha$  and PR immunohistochemical staining in WAP-ERKO (WAP *fl/fl*) but not ER $\alpha^{fl/fl}$  (*fl/fl*) mammary glands of the second lactation, day 1. Nuclei of multiple luminal epithelial cells in control mammary glands were positive for ER $\alpha$  and PR (C and D), which were absent in the enlarged ducts of WAP-ERKO mice (A and B).

Based on the proposed role of WAP-Cre-expressing, parity-induced mammary epithelial cells (PI-MECs) that survive the first gestation cycle and function as alveolar progenitors during subsequent pregnancies, we reasoned that progressive loss of ER $\alpha$ - and downstream progesterone receptor (PR)-mediated signaling in the mammary gland with successive pregnancies was responsible for the observed mammary gland defects and the resulting offspring malnourishment. Progressive loss of ER $\alpha$  was verified by immunohistochemical staining of WAP-ERKO mammary glands during the second lactation (Fig. 5). Nuclear ER $\alpha$  was nearly undetectable in the epithelium of abnormally enlarged mammary ducts of biparous WAP-ERKO mice (Fig. 5A). Accordingly, expression of PR, which is a transcriptional target of ER $\alpha$  in mammary epithelial cells, was similarly absent (Fig. 5B). The loss of ER $\alpha$  and PR only occurred after lactation because WAP-Cre/ER $\alpha^{fl/fl}$  virgin mice displayed robust nuclear staining throughout the mammary gland luminal epithelium (SI Fig. 9A and B). Multiple nuclei of ductal and lobular epithelial cells of control ER $\alpha^{fl/fl}$  expressed ER $\alpha$  and PR in the first day of the second lactation (Fig. 5C and D). The expression of epithelial ER $\alpha$  in uteri of WAP-Cre/ER $\alpha^{fl/fl}$  2-month-old virgin and involuted primiparous 5-month-old mice was also analyzed. The uterine epithelial expression patterns of ER $\alpha$  in both groups of WAP-ER $\alpha^{fl/fl}$  mice were similar to age-matched ER $\alpha^{fl/fl}$  controls (data not shown), confirming the restriction of WAP-Cre recombinase expression to the mammary epithelium. Importantly, ER $\alpha$  expression in stromal cells was abundant in the mammary glands and uteri of lactating WAP-ERKO females (SI Fig. 10), and the pattern of stromal staining in both tissues was similar to control ER $\alpha^{fl/fl}$  mice (data not shown). Next, we analyzed the expression of the proliferating cell nuclear antigen (PCNA) to determine the possible cause of the ductal dilation and loss of secretory alveoli. By day 1 of the second lactation, ductal and alveolar epithelial cells from control ER $\alpha^{fl/fl}$  mammary glands robustly expressed PCNA. In contrast, PCNA expression was diminished in the enlarged ducts and alveoli of WAP-ERKO mammary glands. The number of nuclei of mammary epithelial cells in lactating WAP-ERKO mice that were PCNA-positive was approximately half of that observed in control ER $\alpha^{fl/fl}$  mammary glands (data not shown). Thus, the dilation was not attributable to increased proliferation of ductal epithelial cells,

whereas the loss of alveoli may be attributable in part to impaired ER $\alpha$ -dependent proliferation of luminal epithelial cells.

## Discussion

We found that progressive loss of ER $\alpha$  in mouse mammary glands during gestational cycles results in loss of lobuloalveoli, impaired ductal side-branching, and inadequate milk delivery. MMTV-Cre-mediated excision of ER $\alpha$  occurs in multiple organs shortly after birth. The resulting mammary gland phenotype of arrested ductal growth at the prepubertal stage was similar to the genomic ER $\alpha$  knockout (11). Our data indicate that early and complete loss of ER $\alpha$  throughout the mammary epithelium prevents the formation of TEBs and severely impairs ductal elongation. Studies that used tissue recombination and the ER $\alpha$  hypomorphic model (8, 9) suggested that ER $\alpha$  was dispensable in the mammary epithelium. The authors proposed a predominant role of ER $\alpha$  signaling in the stroma, which created much doubt in the scientific community about the legitimacy of mice to model ER $\alpha$ -based breast cancer prevention, because the mammary stroma in humans express little ER $\alpha$ . Then again, our findings in the Cre/lox-based conditional knockout model of the ER clearly indicate that ER $\alpha$  is essential in the epithelial compartment of the murine mammary gland. Therefore, our proposed model, which emphasizes the significance of ER $\alpha$  signaling in the epithelium, might also suggest that tamoxifen has a direct effect on the growth of premalignant lesions in selected ER $\alpha$ -positive murine mammary cancer models. Recent studies by Medina *et al.* demonstrate that tamoxifen had a profound impact on the prevention of mammary tumorigenesis in the p53 knockout transplant model (20). The ER $\alpha$ -expressing breast cancer models in combination with the ER $\alpha$  conditional knockout model will be invaluable for studying ER signaling in premalignant and cancerous lesions of the mammary gland.

Essential functions of ER $\alpha$  were primarily associated with ductal elongation rather than lobuloalveolar formation (8). Recent studies by Mallepell *et al.* (11) establish a role for ER $\alpha$  in mammary epithelial cells during puberty, and these observations are in complete agreement with the phenotypic analyses in the MMTV-Cre-based conditional knockout mice. However, the transplant model by Mallepell *et al.* (11) and the MMTV-ERKO mice provide limited insights into the role of ER $\alpha$  signaling at later stages of mammogenesis. Using WAP-ERKO mice, we were able to specifically ablate ER $\alpha$  in duct termini and alveolar units after ductal elongation was completed. Also, these mice permitted us to study the loss of ER $\alpha$  during multiple pregnancies and lactation cycles. Successive gestational cycles drove a progressive loss of ER $\alpha$  in the mammary epithelial cells (MECs). In summary, our observations clearly indicate that ER $\alpha$  signaling is required for ductal elongation. Furthermore, ER $\alpha$  is equally important for pregnancy-induced tertiary branching and the proliferation and maintenance of differentiating alveolar cells (i.e., WAP-Cre is first activated during the second half of pregnancy when alveolar cells assume an advanced differentiation profile).

Paracrine signaling between neighboring cells within the mammary gland can compensate for lack of ER $\alpha$  (and thus PR) in specific mammary epithelial subtypes. A 10:1 ratio of ER $\alpha$ -competent to ERKO transplanted MECs reconstituted normal mammary gland development and induced participation of ERKO MECs in all epithelial compartments: luminal and basal cells and cap and body cells of TEBs (11). A 1:1 ratio of PR-competent to PR-deficient transplanted MECs similarly rescued mammary gland development (21). We have found that alveolar abnormalities can emerge very early after initiation of ER $\alpha$  excision from MECs during the first lactation, uncovering a greater stringency for adequate ER $\alpha$  and PR signaling during reproduction that may have been overlooked or not feasible with previous transplantation approaches. More importantly, our observation of the critical role of ER $\alpha$  and PR during lactation



was unexpected because estradiol and progesterone levels decline sharply after birth because of the loss of the corpus luteum.

Expression of the *WAP* gene continues throughout lactation, and the highest expression is restricted to differentiated luminal MECs. Expression declines rapidly with weaning, but a subpopulation of hormonally responsive alveolar MECs resists apoptosis and survives involution (15, 16). These PI-MECs are predominantly located within terminal ducts and alveolar units of involuted mammary glands (i.e., the murine equivalent of terminal duct lobular units in the human breast). Using genetic labeling, it has been shown that PI-MECs are able to self-renew, and they serve as alveolar progenitors in successive pregnancies. The increasing penetrance of the abnormal phenotype in multiparous WAP-ERKO females might suggest that PI-MECs critically require intrinsic ER $\alpha$  signaling to numerically expand during successive gestation cycles and that the ablation of ER $\alpha$  can inhibit the capacity of this epithelial subtype to self-renew. Because PI-MECs possess features of multipotent stem cells upon transplantation into the cleared fat pad of recipient mice (refs. 15 and 16 and L. A. Matulka and K.-U.W., unpublished data), the ER $\alpha$  conditional knockout mice might be a valuable tool to address in future studies the importance of ER $\alpha$  signaling in multipotent progenitors and mammary epithelial stem cells.

## Materials and Methods

**Generation of Conditional ER $\alpha$  Knockout Mice.** The mouse ER $\alpha$  genomic clone from 129/sy ES cells containing exon 3 of ER $\alpha$  was a kind gift from J.-Å. Gustafsson (Karolinska Institute, Stockholm, Sweden). The 9.2-kb BamHI fragment containing exon 3 consists of nucleotides 655–845 and amino acids 156–218 and encodes the first zinc finger of the DNA binding domain (22). The floxed phosphoglycerine kinase-Neo cassette, a kind gift from P. Sanford and T. Doetschman (University of Cincinnati), was cloned into the Eco47III site, and a loxP site followed by a BamHI site was introduced at the NheI site. An HSV-TK expression cassette was subcloned into the HpaI site as the negative selection marker. The 12.2-kb targeting vector linearized at the PmlI site was electroporated into 129/SvOla ES cells (Fig. 1). ES cells containing a targeted ER $\alpha$  allele were identified by 5' and 3' outside PCR. The 5' outside primers were: 5'-AGCAAGGGAAAACAAAACCTGTGT-3' (forward) and 5'-AGTCATAGCCGAATAGCCTCTCCAC-3' (reverse). The 3' outside primers were: 5'-CTATCAGGACATAGCGT-TGGCTACC-3' (forward) and 5'-AATGAGAGAGGAC-CAGCGATCTTAT-3' (reverse). Genotyping on tail DNA was performed by PCR using the following primers: 5'-TGGGTT-GCCCATAACAATAAC-3' (forward), and 5'-AAGAGAT-GTAGGGCGGGAAAAG-3' (reverse). The size of the amplified DNA fragment was 1,280 bp in ER $\alpha^{fl/fl}$  and 1,200 bp in WT mice as separated on a 1.3% agarose gel. ER $\alpha^{fl/fl}$  mice were bred with transgenic mice expressing the Cre enzyme in the mammary tissue under the control of the MMTV long-terminal repeat and the WAP gene promoter as described in ref. 15. Cre-mediated excision of the ER $\alpha$  gene was verified by genotyping PCR of DNA extracted from tail clips, using the same primers described above.

**Blood/Tissue Collection and Analyses.** Mice were killed in a CO<sub>2</sub> chamber according to Institutional Animal Care and Use Committee and institutional protocols. Blood was collected from the ventricle of the heart with a 23-gauge needle. After clotting at room temperature for 2 h, the blood was centrifuged, and the serum was collected and stored at  $-80^{\circ}$  C. Mammary glands were dissected, mounted on glass slides, and fixed and stained in carmine alum solution as described for whole-mount analysis (2). For immunohistochemical analysis, tissues were fixed overnight in 10% neutralized buffered formalin (Richard-Allan Scientific, Kalamazoo, MI; catalog no. 9400-5), processed, and embedded in paraffin according to standard procedures by the University of Cincinnati Comparative Pathology Core Laboratory. H&E staining was performed as described in ref. 23. Serum estradiol and progesterone levels were measured in collected serum according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI; catalog nos. 582251 and 582601). PRL levels were determined by a cell-based bioassay as described in ref. 18. SDS/PAGE and Coomassie blue staining of WAPs in the milk of lactating ER $\alpha^{fl/fl}$  (+/+) and WAP-ERKO (-/-) females were performed as described in ref. 19.

**Immunohistochemistry.** Slides were boiled in 1 $\times$  citrate buffer (pH 6.0) for 20 min for antigen retrieval. Immunostaining with ER $\alpha$  (1:150; Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-542) and PR (1:250; Santa Cruz Biotechnology; catalog no. sc538) antibodies were performed as described in ref. 24. PCNA (1:1,000; Santa Cruz Biotechnology; catalog no. sc-56) IgG was used with the Histo-mouse-MAX kit (Zymed Laboratories, South San Francisco, CA; catalog no. 87-9551) to detect protein expression according to the manufacturer's instructions. Biotinylated secondary antibody (goat anti-rabbit IgG) and the avidin-biotin blocking kit were from Invitrogen (Carlsbad, CA; catalog nos. 50-235Z and 00-4303) and Vectastain ABC kit was from Vector Laboratories (Burlingame, CA). Digital photomicrographs were acquired with a Nikon (Tokyo, Japan) Microphot-FXA microscope and SPOT software, Version 4.0.9 (Diagnostic Instruments, Sterling Heights, MI).

**Statistical Analysis.** Data were compared by a one-way ANOVA, followed by a one-tailed Student's *t* test to evaluate levels of significance at a 95% confidence interval. Differences were determined to be statistically significant when  $P < 0.01$  unless otherwise noted.

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