

Ovarian Hyperstimulation by LH Leads to Mammary Gland Hyperplasia and Cancer Predisposition in Transgenic Mice

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Many risk factors for breast cancer are associated with hormonally regulated events. Although numerous mouse models of mammary cancer exist, few address the roles of hormones in spontaneous tumor formation. Here we report that transgenic mice that overexpress LH, resulting in ovarian hyperstimulation, undergo precocious mammary gland development. A significant increase in proliferation leads to ovary-dependent mammary gland hyperplasia. Transgenic glands morphologically mimic those of wild-type pregnant mice and expression levels of multiple milk protein genes are comparable with what is observed at d 14 of pregnancy. In

addition to sustained hyperplasia, spontaneous mammary tumors were observed with a mean latency of 41 wk, indicating that chronic hormonal stimulation causes mammary cancer. Although hormonally induced, these tumors lack expression of progesterone receptor, suggesting that following initiating events, the tumors may become hormone independent. This mouse model likely holds great potential as a tool for discovery of hormone-mediated mechanisms of breast cancer and identification of future targets for breast cancer prevention and treatment. (*Endocrinology* 143: 3671–3680, 2002)

DEVELOPMENT OF THE mammary gland is a hormonally regulated process. Estrogen, progesterone, and prolactin (PRL) mediate progression of the rudimentary ductal system present at birth to an extensive network following puberty and finally into a differentiated milk-producing organ during pregnancy and lactation (1). These same hormones also regulate the occurrence and timing of reproductive events that influence a woman's risk of developing breast cancer. An increase in risk is observed for individuals who exhibit early age at menarche, late age of menopause, late age at first full-term pregnancy or nulliparity (2–4). Conversely, late age at menarche, surgically induced menopause, and young age at first full-term pregnancy appear to have protective effects (5–7). Parity also confers protection against mammary cancer in rodents, and this effect can be mimicked by treatment with human chorionic gonadotropin (hCG) or combination therapy with estrogen and progesterone (8, 9). However, the mechanisms that contribute to this phenomenon are unknown (9, 10). Reproductive hormones are also implicated in both initiation and progression of mammary tumors. Treatment with tamoxifen, which acts as an estrogen antagonist in the breast, significantly reduces cancer recurrence and mortality in patients with estrogen receptor (ER)-positive breast tumors (11). In addition, tamoxifen treat-

ment can decrease the incidence of breast cancer in women at high risk of developing the disease (12).

Deciphering the complex signals that hormones convey to the mammary gland will lead to an increased understanding of the events surrounding tumor formation and growth that will ultimately spawn novel therapeutic strategies. Clearly, this will require development of appropriate model systems. Although tissue culture paradigms have utility for pathway dissection, it is not possible to reproduce the complex interactions that occur between different hormones and the multiple cell types of the mammary gland *in vivo*. In addition, although human studies are most relevant, they are not conducive to experimental manipulation or control. Hence, the development of animal models is most advantageous, allowing for hormonal alterations within a physiological background. The ability to manipulate the murine genome has made the mouse a powerful tool for establishing the roles of various genes in mammary gland development and carcinogenesis. Furthermore, several previously generated transgenic mouse models develop mammary tumors that are morphologically similar to human breast cancers (13).

To date, most mouse models of mammary cancer involve infection with the mouse mammary tumor virus or employ the mouse mammary tumor virus or whey acidic protein (WAP) promoters to target expression of oncogenes or protooncogenes to the mammary gland (14–17). Although useful, these models do not address the role of hormones in mammary tumor formation and progression. Other approaches, such as ovariectomy, hypophysectomy, pituitary isograft, and hormone injections have been used to determine the hormonal dependence of mammary

Abbreviations: BrdU, 5-Bromo-2'-deoxyuridine; DMBA, 7,12-dimethyl benz[*a*]anthracene; ER, estrogen receptor; hCG, human chorionic gonadotropin; LH β -CTP, transgenic mice; MIN, mammary intraepithelial neoplasia; OVX, ovariectomy; PR, progesterone receptor; PRL, prolactin; WAP, whey acidic protein; WDNM1, Westmead DMBA8 non-metastatic cDNA 1.

tumor cells injected into mouse mammary glands (18–20). In addition, increased PRL and progesterone levels induced by pituitary isografts enhance the ability of the carcinogen *N*-methyl-*N*-nitrosourea to cause mammary tumors in mice (21). Although each of these studies provides evidence for the role of hormones in tumorigenesis, they usually involve surgical manipulation, use of immortalized or transformed cell lines, treatment with superphysiological doses of hormone, or addition of carcinogens, all of which make translation to human carcinogenic processes difficult. More physiological evidence that PRL may be a key player in tumorigenesis is provided by the appearance of mammary tumors in a transgenic mouse model that expresses rat PRL under control of the metallothionein promoter; however, this approach does not address the potentially cooperative roles of estrogen and progesterone and detaches PRL from its conventional regulation and normal site of synthesis. Furthermore, although tumors were reported after 11 months of age, definitive tumor latency was not assessed (22).

Although the breast is not typically considered an LH-responsive tissue, receptors for LH/hCG have been found in human breast cancers and breast cancer cell lines. In addition, hCG can inhibit proliferation of some breast cancer cell lines in culture (23, 24). Although these data are intriguing, regression of the mammary gland on ovariectomy indicates that the predominant effect of LH *in vivo* is indirect through stimulation of estrogen production by the ovary (25). Furthermore, female ER knockout mice display significant defects in mammary gland development despite elevated levels of LH (26, 27). Increased breast cancer risk associated with high levels of estrogen suggests that LH activity may actually play a role in the pathology of this disease. Indeed, in recent clinical trials, ablation of ovarian estrogen through chronic treatment of breast cancer patients with GnRH analogs alone and in combination with tamoxifen has led to tumor regression and increased survival (28–30).

Transgenic mice (LH β -CTP) that overexpress LH from the gonadotropes of the anterior pituitary have been previously generated (31). Although male mice maintain normal levels of LH, females display levels of LH that are 5- to 10-fold higher than control animals (31). Consequently, estrogen (31), progesterone (32), and PRL (33) are elevated in female transgenic animals. These mice exhibit precocious puberty (34), anovulation (32), and infertility (31) and develop various ovarian pathologies in a strain-dependent manner (35). Because estrogen, progesterone, and PRL play imperative roles in mammary gland development and function, we investigated the impact of the LH-mediated alteration in the hormonal milieu on the mouse mammary gland. Herein we report that mammary glands of LH-overexpressing mice undergo precocious development and maintain an apparent pregnancy-like state of hyperplasia that is dependent on ovarian hormones. Furthermore, these mice develop spontaneous mammary tumors and display accelerated carcinogen-induced mammary tumorigenesis, compared with nontransgenic controls. We propose that the LH-overexpressing mouse may be a useful model for exploring the hormonal regulation of pathways involved in transformation of the mammary epithelium *in vivo*.

Materials and Methods

Animals

Mice harboring a transgene (LH β CTP) that confers overexpression of LH have been described previously (33). These mice have been maintained in the CF-1 genetic background. Studies described herein used either young mice from the CF-1 strain or mice from mixed genetic backgrounds containing CF-1 and C3H; CF-1 and FVB; or CF-1 and C57BL/6 components. No obvious strain-specific differences were observed in the morphology of the female transgenic mouse mammary glands. In addition, the ovaries of all young mice (less than 5 months of age) had numerous follicular cysts and all mice older than 5 months exhibited luteomas of pregnancy as previously described (31). Mice were housed in microisolator units with a 12-h light, 12-h dark cycle and given food and water *ad libitum*. Transgene genotyping was performed using PCR as previously described (33). All mouse studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Hormone measurements

Blood was collected from randomly cycling animals by cardiac puncture following asphyxiation with CO₂. Samples were obtained at various times of day. After clotting, sera were prepared by centrifugation and collection of supernatant. Sera were stored at –20 C until the time of assay. 17 β -Estradiol and progesterone levels were measured using RIA kits (Pantex, Santa Monica, CA) that have previously been validated for use with mouse sera (32, 36). Limits of detection are 10 pg/ml and 0.2 ng/ml, respectively. Each sample was assayed in duplicate. PRL serum levels were measured at the National Pituitary Hormone Center (Harbor-UCLA Medical Center Torrance, CA).

Morphological analyses of mammary tissue

Whole mounts of female mammary glands were generated using inguinal (no. 4) glands that had been immersed in Kahle's fixative for 2–4 h followed by overnight immersion in Carmine Alum stain (2% carmine, 5% aluminum potassium sulfate in water) at 4 C. Stained glands were dehydrated by graded ethanol washes, cleared with xylene, and mounted on glass slides with Permount (37).

For histological sections, thoracic (no. 2/3) or inguinal (no. 4) glands were removed and fixed in Kahle's fixative or 4% paraformaldehyde overnight. Tissue was embedded in paraffin and 5- μ m sections cut. Sections were deparaffinized in xylene, rehydrated with graded concentrations of ethanol, and rinsed in PBS. Sections not being used for immunohistochemical analyses were stained with hematoxylin and eosin.

Immunohistochemistry

Antigen retrieval was performed by boiling samples in 10 mM citrate buffer (38) for 10 min. Samples undergoing an immunoperoxidase reaction were treated for 20 min with 3% hydrogen peroxide in methanol to inactivate endogenous peroxidase activity. All incubations were carried out at room temperature unless otherwise indicated.

For detection of progesterone receptor (PR), samples were incubated with goat serum for 10 min before treatment with an anti-PR antibody (catalog no. A0098, 1:100 dilution, DAKO Corp., Copenhagen, Denmark) for 1 h. The rabbit IgG Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for detection of the PR antibody. Concentrations of reagents recommended by the manufacturer were used. After a PBS wash, samples were incubated for 30 min with the biotinylated goat antirabbit antibody (1:200 dilution), washed again with PBS, and then treated with the Vectastain ABC reagent for 30 min. The secondary antibody was detected by incubation with 3,3'-diaminobenzidine tetrahydrochloride solution (catalog no. D4168, Sigma, St. Louis, MO) for 2–5 min. Sections were counterstained with methyl green, dehydrated, cleared, and subsequently mounted with Permount.

For assessment of cells undergoing DNA synthesis, animals received an ip injection of 5-bromo-2'-deoxyuridine (BrdU) in dH₂O (10 mg/g body weight, Sigma) 2 h before being killed. After deparaffinization, rehydration, and antigen retrieval, samples were treated with 10% goat serum for 10 min. Following a 1-h incubation with mouse anti-BrdU

antibody (catalog no. 347580, 1:150 dilution, Becton Dickinson, Franklin Lakes, NJ), the samples were washed with PBS and treated with fluorescein isothiocyanate-conjugated goat antimouse antibody (catalog no. 115–095-003, 1:300 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h (39). Samples were mounted with Vectashield mounting media with propidium iodide diluted 1:4 in Vectashield mounting media (Vector Laboratories). The number of BrdU-positive epithelial cells was counted in 6–10 fields from each animal ($n = 3$ for both groups).

Apoptotic cells were detected using the TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products). Samples were treated in accordance with manufacturer's instructions. In short, samples were deparaffinized, hydrated, and then permeabilized with proteinase K (2 mg/ml). After inactivation of endogenous peroxidase activity and equilibration, biotinylated deoxynucleotide triphosphates were applied. After incubation for 1.5 h at 37°C, the reaction was terminated and samples were treated with a streptavidin-horseradish peroxidase conjugate. Signal was detected by incubation with 3,3'-diaminobenzidine tetrachloride for 2–10 min. Sections were counterstained with methyl green, dehydrated, cleared, and subsequently mounted with Permount. The number of apoptotic epithelial cells was counted in 10–12 fields per sample ($n = 3$ for both groups).

Gene expression profiling

Gene expression profiling was performed using Affymetrix Murine 11K GeneChips, which contain probes for 11,000 genes and expressed sequence tags. Samples were prepared in concordance with recommended protocols from Affymetrix. In short, total RNA was collected from mammary glands using TRIzol (Invitrogen, Carlsbad, CA) and mRNA was subsequently isolated with an Oligotex mRNA midi kit (QIAGEN, Chatsworth, CA). Following generation of double-stranded cDNA with the Superscript double-stranded cDNA synthesis kit (Invitrogen), a high-yield RNA transcript labeling kit (Affymetrix, Santa Clara, CA) was employed to create biotinylated cRNA. After purification with the RNeasy mini kit (QIAGEN, Chatsworth, CA), samples were hybridized to the chip and scanned with an argon-ion laser. Data were analyzed using Affymetrix software, including Microarray Suite, MicroDB, and Data Mining Tool (Affymetrix).

Ovariectomy

Transgenic female mice were either ovariectomized or received a sham surgery under avertin anesthesia at 4 months of age. The sham surgery involved a flank incision, exposure of the ovary, reinsertion of the ovary, and placement of a wound clip. Following a 21-d recovery period, mice were killed and thoracic (no. 2/3) mammary glands removed and prepared for histological examination as described above.

Carcinogen treatments

Transgenic and nontransgenic female mice were given 7,12-dimethyl benz[*a*]anthracene (DMBA) (1 mg/100 μ l corn oil) or corn oil by oral gavage at 5 wk of age. A second dose of either DMBA or vehicle was given 1 wk later. Mice were then palpated weekly for detection of developing mammary tumors. Tumor latency was measured using Kaplan-Meier analysis. Mice were killed on the appearance of tumors or if the animals appeared ill. Any mice that were obviously sick, but did not have overt mammary tumors, were censored from the study. These animals usually had either hydronephrosis (33) or pituitary adenomas (Nilson, J., unpublished observations) that were secondary to ovarian hyperstimulation caused by the LH β CTP transgene.

Analysis of milk protein gene expression

Mammary glands were collected from nontransgenic female mice at 9, 12, 14, 16, and 18 d post coitus or adult virgin transgenic and nontransgenic mice. Total RNA was isolated using Trizol (Invitrogen) according to manufacturer instructions. Northern blots containing 37.8 μ g total RNA for each sample were probed with [32 P]-end-labeled, oligodeoxynucleotide probes complementary to the murine β -casein (5' GTC TCT CTT GCA AGA GCA AGG GCC 3') or WAP (5' CAA CGC ATG GTA CCG GTG TCA 3') genes (40). Blots were hybridized in Quikhyb

solution (Stratagene, Cedar Creek, TX) for 2 h at 50°C followed by washing and exposure to x-ray film. To control for epithelial composition, blots were stripped and reprobed with a [32 P]dATP-labeled, randomly primed-probe to murine cytokeratin 8. Cytokeratin 8 template for probe production was generated by RT-PCR amplification of mammary gland RNA with the following primers: (forward, 5' GTGCCCAGTAC-GAGGACATT 3') and (reverse, 5' GTGAGTCCCCCATAGGATGA 3'). Blots were hybridized in Quikhyb solution at 50°C for 30 min, washed, and exposed to x-ray film.

Results

Mammary gland development is accelerated in LH β CTP mice

LH β CTP transgenic mice have elevated LH as early as postnatal d 14 (34), and, as a result, undergo precocious puberty, compared with their nontransgenic counterparts. Using age at vaginal opening as an indicator, LH β CTP transgenic mice enter puberty on postnatal d 21–22, at least 5 d earlier than nontransgenic littermates (34, 35). To assess whether early ovarian activity in LH β CTP transgenic had an impact on mammary gland morphogenesis, we examined glands from transgenic and nontransgenic mice at 3 and 5 wk of age. By 3 wk of age, accelerated development of the mammary gland was observed in transgenic mice, compared with age-matched nontransgenic controls (Fig. 1, A and B). In transgenic animals, primary ducts were elongated, nearly reaching the centrally located lymph node, and numerous alveolar buds were present. In contrast, nontransgenic mice displayed age-appropriate prepubertal ductal development without significant accumulation of alveolar buds. The accelerated growth of the transgenic mammary gland was more apparent at 5 wk of age (Fig. 1, C and D). Although the ductal network in nontransgenic animals had progressed through only half of the mammary gland fat pad, the entire fat pad of the transgenic mouse was filled with ducts that displayed abundant alveoli and a loss of terminal end buds. Although all animals were virgins, the morphological pattern observed in the transgenic females was remarkably similar to that observed at mid- to late pregnancy in normal mice (1).

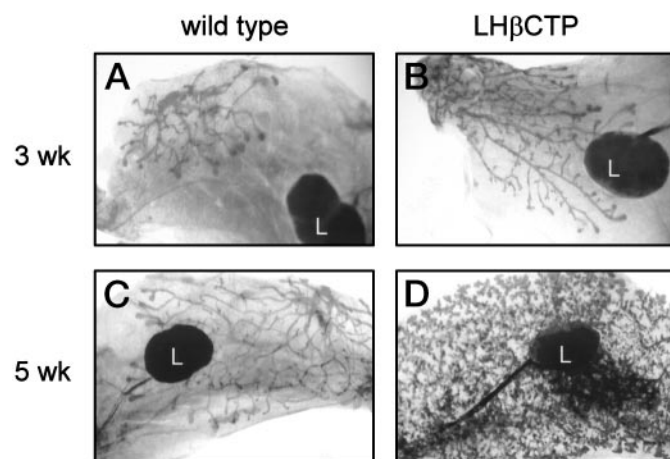


FIG. 1. Mammary gland development is accelerated in LH β CTP mice. Whole mounts of inguinal mammary glands (no. 4) were prepared from wild-type (A and C) or LH β CTP (B and D) mice that were either 3 (A and B) or 5 (C and D) wk old. L, Mammary gland associated lymph node.

The mammary glands in adult, virgin LH β CTP mice display a midpregnancy phenotype

In addition to precocious maturation, mammary glands from adult transgenic mice had extensive epithelial hyperplasia. As shown in Fig. 2, histological examination of mammary glands from 3-month-old transgenic mice revealed considerable alveolar development with accumulation of lipid droplets. Moreover, distended ducts were often observed with deposition of material within these ducts. Conversely, the mammary glands from age-matched, nontransgenic littermates displayed a limited accumulation of epithelial cells. The accumulation of epithelial cells in transgenic animals was due to a clear increase in proliferation. A 12-fold increase in the number of S-phase epithelial cells was measured by incorporation of BrdU ($P < 0.01$; Fig. 3, A–E). Further supporting an elevation in proliferative rate, expression profiling of mammary glands of LH-overexpressing mice revealed a 2.2-fold increase in Ki-67 mRNA levels, compared with their nontransgenic counterparts (average of data from two independent gene expression microarrays, data not shown). In contrast to the increase in proliferative rate, a compensatory change in the number of apoptotic cells was not observed (Fig. 3F).

The histological presentation of the mammary glands from adult, virgin transgenic mice supports the notion that these glands have undergone changes that normally accompany pregnancy. To further explore this possibility, we examined the expression pattern of molecular markers of pregnancy-induced differentiation within mammary glands of transgenic and nontransgenic mice. WAP, β -casein, and Westmead DMBA8 nonmetastatic cDNA 1 (WDNM1) are milk proteins whose corresponding mRNAs accumulate at specific stages of pregnancy (40). The earliest of these genes to be detected in pregnant mammary glands is WDNM1, followed sequentially by β -casein and WAP (40). To determine whether expression of the milk protein genes is activated in adult LH β CTP mammary glands, we compared expression levels in virgin transgenic and nontransgenic mice to those observed in glands collected from nontransgenic mice at various stages of pregnancy (Fig. 4). Expression of WAP and β -casein were nearly undetectable in mammary tissue collected from virgin, nontransgenic mice. In contrast, expression of these genes in LH β CTP transgenic mice was very

similar to that observed at d 14 of pregnancy in nontransgenic animals. A similar pattern was observed for WDNM1 (data not shown). Coupled with the morphological data described above, these gene expression data support the hypothesis that mammary glands in virgin transgenic animals with excessive LH have a phenotype that simulates midpregnancy.

Mammary gland hyperplasia in LH β CTP mice is dependent on ovarian input

Serum levels of LH have previously been reported to be 5- to 10-fold elevated in LH β CTP mice, compared with nontransgenic littermates (34). In response to high LH, increases in serum estrogen, progesterone, testosterone, and PRL were also observed in young animals (32–34). Because estrogen, progesterone, and PRL are known to impact mammary gland development, we measured serum levels of these hormones in mice at several different ages to identify the hormonal profile that supports the development of mammary hyperplasia and tumors in transgenic mice (Table 1). Significant increases in estrogen and progesterone levels were detected in LH-overexpressing mice as early as 5 wk of age, consistent with the extensive hyperplasia observed at this time. In addition, serum PRL levels were elevated; however, this was only observed later in life and may be due to chronic elevation in circulating estrogens, which increase lactotrope secretion of PRL (41). Although PRL levels in 20-wk-old transgenics were highly variable, ranging from 67 to 762 ng/ml; all animals examined at this age displayed significantly higher serum concentrations than age matched controls (5–7 ng/ml).

Although the mammary gland is not normally considered a target of LH action, the hyperplasia observed in LH β CTP mice could be due to direct stimulation of the gland by LH. Supporting this notion are studies by Russo *et al.* (42) and Srivastava *et al.* (43) that revealed a protective effect of hCG on chemically induced mammary cancers in rats. Because hCG binds the same receptor as LH (44), it is possible that this effect is mediated directly at the mammary gland. In addition, LH receptors have recently been identified in mammary epithelium (23, 24, 45). Thus, we sought to determine whether the mammary gland hyperplasia observed in LH β CTP transgenic mice was due solely to a direct action of LH on the mammary gland or required ovarian hyperstimulation caused by excess LH. We used an ovariectomy (OVX) paradigm to assess the relative importance of the ovary to mammary hyperplasia in LH-overexpressing mice. Although this approach eliminates ovarian factors, LH levels would actually increase because of the loss of steroid-negative feedback on transgene expression (46). Transgenic mice were OVX or sham operated and mammary glands collected 21 d following surgery. As shown in Fig. 5, mammary gland hyperplasia was persistent in sham-operated control mice. In contrast, loss of ovarian input caused complete regression of the gland. These data indicate that the ovary is an obligatory intermediate of LH action on the mammary gland in LH β CTP mice.

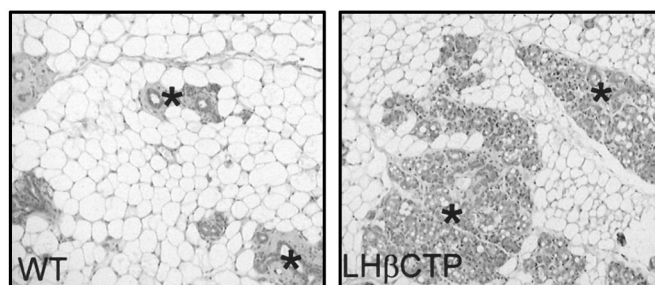
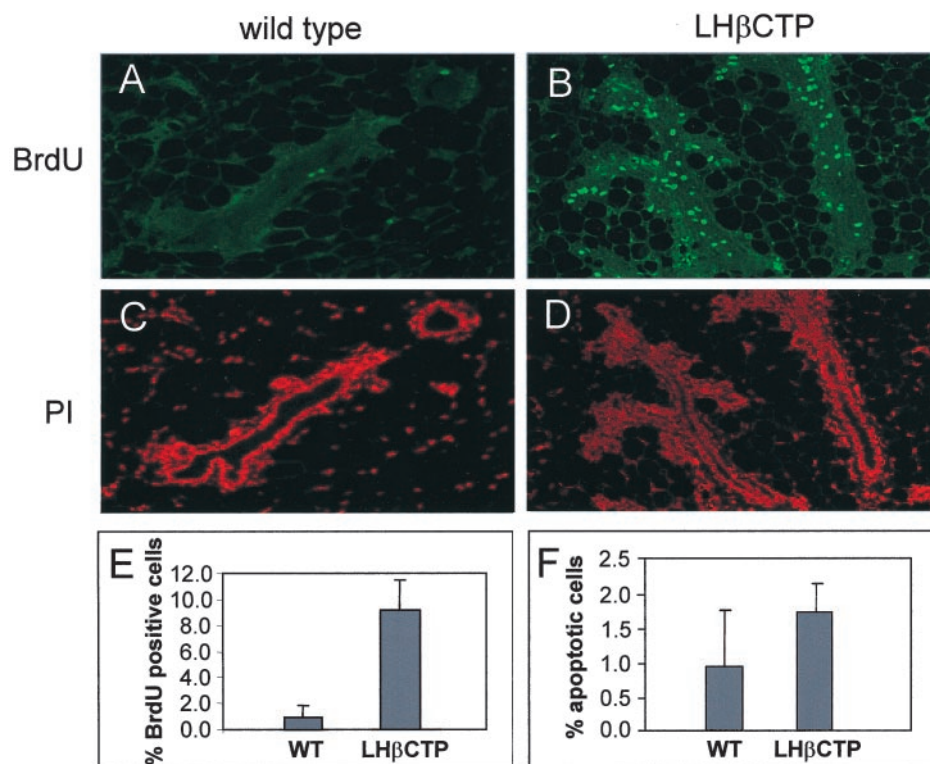


FIG. 2. The mammary glands in adult, virgin LH β CTP mice display a midpregnancy morphology. Histological sections of thoracic mammary glands (no. 2/3) from 4-month-old wild-type (WT) and LH β CTP mice (magnification, $\times 100$). Sections were stained with hematoxylin and eosin. Asterisks indicate collections of epithelial cells.

FIG. 3. The mammary glands of adult, virgin LH β CTP mice display an increase in epithelial cell proliferation but no change in apoptosis. Twelve-week-old wild-type (A and C) and transgenic (B and D) animals were injected with BrdU 2 h before being killed, and cells in S phase of the cell cycle were detected with an anti-BrdU antibody followed by a fluorescein isothiocyanate-conjugated secondary antibody (green, A and B). Sections were mounted with media containing propidium iodide (red, C and D), allowing for visualization of all nuclei (propidium iodide staining did not facilitate reliable assessment of apoptosis). E, Quantification of S-phase epithelial cells was done by calculating the percentage of BrdU-positive cells in 6–10 fields from each animal ($n = 3$ for both groups). The number of BrdU-positive cells was increased in the LH-overexpressing mice, compared with virgin wild-type mice ($P < 0.01$, Mann-Whitney mean-rank U test). F, Apoptotic cells were detected using a FragEL DNA fragmentation assay. The percentage of apoptotic cells was calculated for 10–12 fields per animal ($n = 3$ for both groups). Values represent the mean \pm SD. All pictures are magnified $\times 200$.



LH β CTP transgenic mice are predisposed to mammary cancer

Parity has a dual effect on breast cancer risk. Although pregnancy is associated with long-term protection against breast cancer, a short-term increased risk has been observed immediately following pregnancy (47, 48). With this in mind, we speculated that the LH β CTP mice might be predisposed to development of mammary cancer. In addition, we observed occasional spontaneous cancers in older transgenic mice but not in age-matched wild-type littermates. To directly assess whether the LH β CTP mice were particularly susceptible to developing mammary cancers, we treated virgin transgenic and nontransgenic controls with the mammary carcinogen DMBA. Female mice were treated with DMBA or corn oil at 5 and 6 wk of age, and tumor development was assessed by weekly palpation (Fig. 6). Tumor phenotype was evaluated using the criteria recommended by the Annapolis Pathology Panel (49). Transgenic mice that received DMBA developed invasive mammary carcinomas with squamous metaplasia (Fig. 7A) at a mean latency of 13.5 (± 3.8) wk after treatment. All transgenic mice developed tumors by 20 wk following the second DMBA treatment. In contrast, nontransgenic mice treated with DMBA developed mammary tumors at a much slower rate, with only 20% penetrance by 56 wk after carcinogen treatment. Lung metastases were also observed in most mice harboring DMBA-induced tumors. More importantly, transgenic animals treated with vehicle stochastically developed mammary tumors with 50% penetrance by 41 wk following corn oil administration, but control nontransgenic animals failed to develop tumors throughout the entire course of the experiment. Most of the spontaneous tumors identified in LH β CTP mice

were mammary intraepithelial neoplasias (MINs), exhibiting multiple layers of epithelial cells and atypical nuclear cytology but remaining within the confines of the basement membrane; both low- and high-grade MINs were observed (Fig. 7B). Lesions were multifocal, and histological examination indicated that they originated in the terminal ductal lobular unit of the mammary gland. In addition to the palpable tumors that were observed in older mice, nonpalpable low-grade MINs were identified in untreated, transgenic mice as young as 20 wk of age (Fig. 7C). The potential of these lesions to progress to malignancy was realized in a number of tumor-bearing animals that demonstrated invasive acinar or solid carcinomas with occasional lung metastases (Fig. 7D). Additionally, two animals developed spontaneous nipple adenomas. From these data, we conclude that LH β CTP mice are predisposed to mammary carcinogenesis, compared with their nontransgenic littermates.

Serum levels of estrogen, progesterone, and PRL were measured in tumor bearing transgenic mice and compared with age-matched wild-type control animals (Table 1). LH β CTP mice with tumors display elevated serum concentrations of all of these hormones relative to wild-type controls. Furthermore, serum levels of estrogen in tumor bearing mice are significantly higher than concentrations observed in tumor-free transgenic animals at any other age ($P < 0.01$, Mann-Whitney U test). Conversely, while tumor bearing mice demonstrate higher concentrations of progesterone than young transgenic animals, there is not a significant change relative to 20-wk-old LH β CTP mice. However, of note is the extensive variation observed in progesterone levels of tumor-bearing animals, which ranged from 110 to 3,000 ng/ml, but age-matched wild-type animals demon-

strated concentrations of 5–42 ng/ml. The most dramatic difference observed was in PRL levels of tumor-bearing animals, which ranged from 1,056 to 16,340 ng/ml, whereas wild-type animals at the same age displayed concentrations of 23–41 ng/ml.

Spontaneous mammary tumors from LH β CTP mice lack detectable PRs

Growth of mammary tumors can be classified as either hormone dependent or hormone independent. These assessments are based on response to hormone treatment (50). In humans, approximately one third of mammary tumors are hormone dependent and the remaining two thirds are independent. Conversely, almost all mammary tumors that develop in mice are hormone independent (50). One indication of the hormonal requirements for growth is the presence of receptors for estrogen or progesterone. Analysis of human breast tumors has revealed a significant correlation

between expression of ERs and PRs (51, 52). Furthermore, the presence of ER in human breast tumors usually indicates that the tumor will at least be initially responsive to chemical ablation of ovarian hormones using treatments such as tamoxifen and/or GnRH analogs (53, 54). Hence, determination of the receptor status can be important for predicting hormone dependency of tumors. Immunohistochemistry for PR was performed on spontaneous tumors from LH β CTP mice. Although adjacent normal epithelial cells express nuclear PR, tumor cells did not express detectable levels of this protein (Fig. 8). Because of the lack of a reliable antimouse ER antibody, consistent staining for ER in normal mammary tissue could not be achieved. However, expression of ER α mRNA was detected in control and transgenic mammary glands as well as tumors using RT-PCR analysis (data not shown). Although suggestive of the presence of ER in the tumor, these results should be viewed with caution, given the inability to determine the cellular origin of expression and the possibility of contamination with adjacent normal tissue.

Discussion

We have shown that persistent overexpression of LH from the pituitary of transgenic mice leads to precocious mammary gland development and ovary-dependent mammary hyperplasia. Hyperplasia is due to an increase in epithelial cell proliferation by an order of magnitude, compared with nontransgenic controls. In view of the fact that early puberty is a risk factor for breast cancer in humans (2, 55), it will be important to determine whether precocious puberty in the LH β CTP mice significantly contributes to their acquisition of mammary tumors. Mammary glands of adult LH-overexpressing mice display morphological similarity to those of mice at midpregnancy and also express the milk protein genes β -casein, WAP, and WDNM1. The pregnancy phenotype is intriguing, given the importance that pregnancy-related mammary gland changes play in the determination of breast cancer risk. Parity is known to impart a protective effect in humans as well as rodents (5, 56, 57). This phenomenon can be mimicked in rodents by treating with hCG, a placental hormone that binds to the LH receptor, or a combination of estrogen and progesterone; both paradigms render animals resistant to the tumorigenic effects of carcinogens (8, 58). These observations have spurred the proposition of hCG treatment for women who plan to delay their first pregnancy (59). The susceptibility of LH-

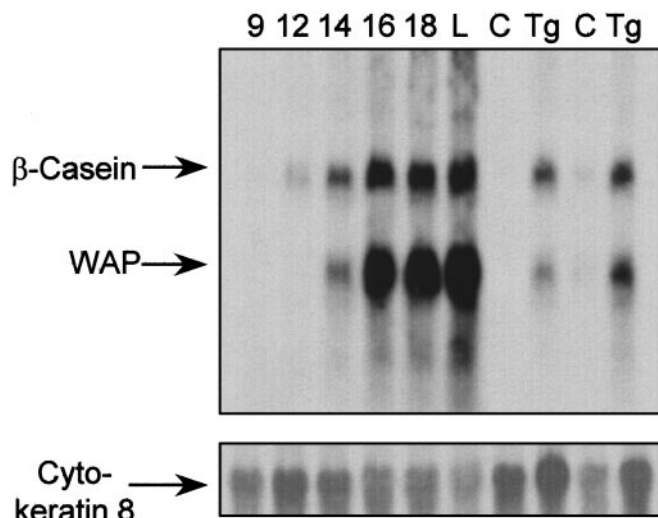


FIG. 4. The mammary glands in adult, virgin LH β CTP mice display a midpregnancy phenotype at the molecular level. Northern blots were performed with total RNA isolated from the mammary glands of virgin 4-month-old nontransgenic (C) and transgenic (Tg) littermates. For comparison, RNA was also collected from nontransgenic animals at d 9, 12, 14, 16, and 18 of pregnancy and d 1 of lactation (L). End-labeled oligonucleotide probes against WAP and β -casein were used simultaneously. To control for epithelial cell content, the blot was stripped and reanalyzed with a cytokeratin 8-specific probe.

TABLE 1. Serum levels of estrogen, progesterone, and PRL are increased in LH-overexpressing animals

		17 β -Estradiol (pg/ml)		Progesterone (ng/ml)		PRL (ng/ml)	
Age (wk)		$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n
5	WT	10.0 \pm 8.2	4	18.6 \pm 19.7	4	11.5 \pm 4.7	4
	CTP	75.3 \pm 40.5 ^a	3	31.9 \pm 26.4 ^b	3	31.3 \pm 40.0	4
10	WT	44.7 \pm 32.0	9	16.5 \pm 17.5	9	21.4 \pm 33.4	5
	CTP	71.5 \pm 24.5	6	433.4 \pm 385.5 ^b	6	56.2 \pm 39.2	5
20	WT	58.0 \pm 18.6	5	15.9 \pm 5.6	5	6.33 \pm 1.2	3
	CTP	148.0 \pm 74.1 ^b	8	316.0 \pm 163.1 ^b	8	237.8 \pm 260.1 ^a	6
41	WT	29.0 \pm 7.3	4	17.5 \pm 17.2	4	31.5 \pm 9.8	4
≥ 41 (with tumors)	CTP	280.6 \pm 177.8 ^{b,c}	8	603.1 \pm 986.8 ^b	8	4326.3 \pm 5160.4 ^b	8

Age matched samples were compared using the Mann-Whitney *U* test.

^a Indicates *P* < 0.05 when compared with age-matched wild-type control.

^b Indicates *P* < 0.01 when compared with age-matched wild-type control.

^c Indicates *P* < 0.01 when compared with tumor-free transgenic mice at any other age.

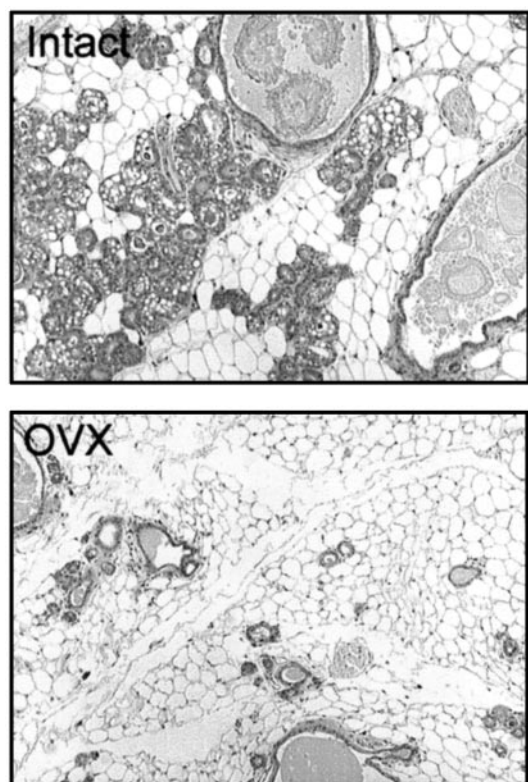


FIG. 5. Mammary gland hyperplasia in LH β CTP mice is dependent on ovarian input. Four-month-old transgenic mice were either OVX or sham operated (intact). Three weeks following the surgery, thoracic (no. 2/3) glands were collected, immersed in Kahle's fixative, sectioned, and stained with hematoxylin and eosin ($n = 3$ for each group; magnification, $\times 100$).

overexpressing mice to spontaneous tumor formation indicates that persistent exposure to this hormone may have detrimental effects. Therefore, the timing and duration of such treatment must be taken under serious consideration. An additional concern stems from the report that a transient increase in breast cancer detection is observed in women who have recently given birth; this most likely is due to the hormone-rich environment stimulated by pregnancy that would favor growth of previously transformed cells (47). Accordingly, manipulation of the hormonal milieu of a nulliparous woman may result in a short-term increase in breast cancer risk.

The molecular mechanisms underlying parity-mediated protection from breast cancer are unknown, although several hypotheses have been suggested. One possibility is that terminal differentiation of the mammary gland brought about by pregnancy or hormone treatment results in the removal of cells that are particularly susceptible to transformation (9). Alternatively, it has been postulated that parous animals display an altered hormonal milieu that modifies the biochemical profile of the mammary epithelia (10). Although the rat has been the animal model of choice for investigating the impact of hormonal manipulation, recent work has confirmed that parous mice demonstrate a resistance to carcinogen-induced tumorigenesis, providing evidence that the two rodent models are comparable (60). In contrast

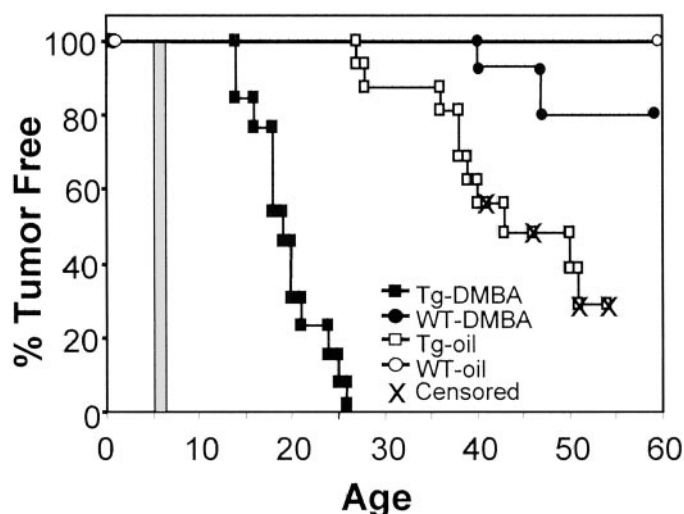


FIG. 6. LH β CTP transgenic mice are predisposed to mammary cancer. Transgenic and nontransgenic female littermates were treated with either DMBA or corn oil at 5 and 6 wk of age (gray line). Mammary tumor development was assessed by weekly palpation. Age is indicated in weeks. The following numbers of animals were used: wild type, DMBA treated = 15 (●); transgenic (Tg), DMBA treated = 13 (■); wild type, vehicle = 18 (○); transgenic, vehicle treated = 15 (□). Censored events (removal of an animal from the experiment because of death or sickness) are marked with an X. Spontaneous tumor latency in transgenic mice was found to be 41 wk using Kaplan-Meier survival analysis.

to the protection bestowed on mammary glands by pregnancy or estrogen/progesterone treatment, the pregnancy-like state of LH β CTP mice renders the mammary glands of these mice vulnerable to cancer formation rather than imparting protection. This discrepancy may stem from variations in the degree of hormone elevation, or they may be due to the chronic nature of hormone exposure in the LH-overexpressing mice, compared with the transient increase in hormone levels that occurs during pregnancy. It is also important to note that, although the mammary glands of LH β CTP mice mimic pregnancy morphologically, they may not have achieved parity. Perhaps the alterations observed in these mice are more similar to the changes caused by perphenazine (8), a dopamine antagonist that induces mammary gland differentiation but does not offer protection from carcinogens, than those induced by pregnancy or estrogen/progesterone treatment.

Interestingly, the hormone treatment paradigm that renders rats refractory to carcinogen-induced tumors triggers a 9-fold increase in estrogen and only a 4-fold increase in progesterone (8). Alternatively, perphenazine leads to increases in circulating PRL and progesterone concentrations but does not significantly alter estrogen levels (8). Although serum measurements cannot be directly compared, LH β CTP mice have only a modest increase (<3 -fold) in estrogen levels, compared with wild-type mice of the same age, but progesterone levels are more than 20-fold higher in tumor-free transgenic animals and PRL is nearly 40-fold higher in 20-wk-old transgenic mice, compared with age-matched wild-type controls. However, although the hormonal milieu of LH β CTP mice increases susceptibility to the carcinogen DMBA, treatment with perphenazine does not appear to sig-

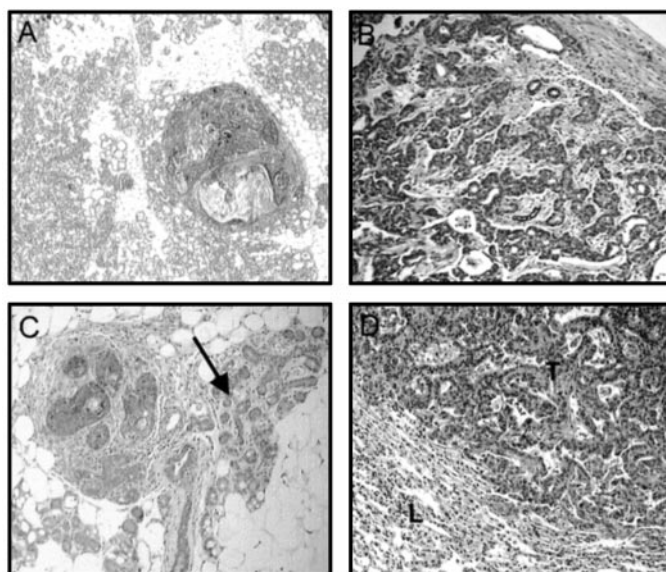


FIG. 7. DMBA-induced and spontaneous mammary cancers in LH β CTP mice. Hematoxylin and eosin-stained sections of tumors from LH β CTP mice (magnification, $\times 100$). A, Invasive mammary carcinoma with squamous metaplasia of transgenic mouse treated with DMBA. B, Spontaneous infiltrating mammary adenocarcinoma from LH β CTP mouse treated with corn oil. C, A nonpalpable *in situ* carcinoma identified in a 23-wk-old LH β CTP mouse. Arrow indicates adjacent normal mammary epithelial tissue. D, A lung metastasis identified in a transgenic female with a spontaneous mammary adenocarcinoma. L, Normal lung tissue; T, mammary cancer metastasis. All tissues were fixed with Kahle's fixative.

nificantly increase the rate of tumor formation in *N*-methyl-*N*-nitrosurea-treated rats. The increased tumor susceptibility of LH β CTP mice may also be due to the fact that they never reach the fully differentiated state of lactation. However, although lactation appears to enhance the protective effect of pregnancy in humans (61–64), rats that have undergone full-term pregnancy but not lactation demonstrate resistance to carcinogen-induced tumorigenesis, suggesting that lactation is not required for hormone-mediated protection in rodents (65). Finally, unlike wild-type mice that have achieved parity or received transient hormone treatment, the mammary glands of LH β CTP mice never undergo an involution-like event. The absence of tissue remodeling that is normally associated with this process may contribute to the tumor susceptibility of these animals. Further studies will be required to define the precise hormonal conditions that predispose the LH β CTP mice to cancer and, more importantly, to dissect the molecular mechanisms that govern both hormone-induced mammary tumorigenesis as well as protection from carcinogenic insults.

LH-overexpressing mice represent an autochthonous mammary tumor model in which the mice are susceptible to tumor induction by the carcinogen DMBA and also develop spontaneous mammary tumors with a mean latency of 41 wk. To date, *in vivo* studies of the hormonal components of breast cancer have often depended on external manipulations such as subcutaneous hormone pellets or pituitary isografts (8, 21). Persistent treatment with exogenous estrogen in this fashion leads to the formation of mammary tumors in rats; however, this effect is rarely observed in mice

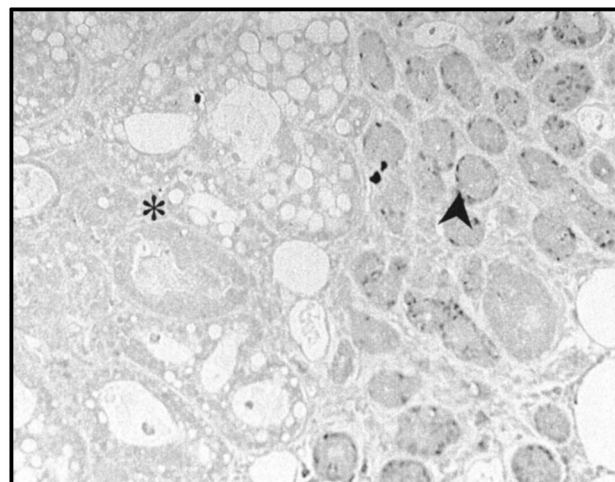


FIG. 8. Spontaneous mammary tumors of LH β CTP mice lack PR. Surrounding normal mammary epithelial cells (arrowhead) express PR, but the adjacent spontaneous adenocarcinoma does not (asterisk). Sections are counterstained with methyl green (magnification, $\times 100$). Dark staining within the tumor tissue is nonnuclear remnants of the methyl green counterstain. Results are representative of staining performed on five different animals with tumors.

(50). In contrast, continual administration of medroxyprogesterone acetate causes mammary adenocarcinomas in BALB/c virgin female mice (66). This effect of progesterone appears to be strain specific. Lastly, transgenic mice expressing PRL under the control of the metallothionein promoter have been constructed, but these mice express the transgene primarily in the liver, a tissue that does not normally secrete PRL (22). In contrast to these approaches, LH β CTP mice provide a model for studying the pathology that occurs as a result of chronic hyperstimulation of the intact pituitary-gonadal axis. Given the dramatic increase in serum PRL levels observed in LH-overexpressing mice with mammary tumors (Table 1), it is conceivable that this hormone plays a significant role in the tumorigenic process. However, several lines of evidence suggest that PRL may require additional inputs to cause tumor formation. In particular, the PRL-overexpressing mice described above develop mammary tumors but with an extended latency, compared with that observed in the LH β CTP mice (22). Furthermore, although pituitary isografts, which cause an increase in PRL levels and a subsequent increase in progesterone production by the ovary, enhance susceptibility of the mouse mammary gland to carcinogen-induced tumorigenesis, they rarely lead to the formation of spontaneous mammary tumors (21, 67). One possibility is that PRL functions to promote growth of transformed cells, leading to the formation of a discernible tumor. In support of this notion, many mice with mammary tumors were also found to contain functioning pituitary prolactinomas (Nilson, J., personal communication). Extensive studies using dopamine agonists, such as bromocriptine (41), or crosses with PRL-deficient mice (68) will be required to assess the specific role that PRL plays in tumorigenesis in this model.

Spontaneous mammary tumors of LH-overexpressing mice do not display detectable expression of PR. In human breast tumors, absence of PR would strongly suggest that the

tumor lacks expression of ER as well (51, 52). Although definitive correlation studies of steroid hormone receptors have not been done in rodent models, most mouse mammary tumors display hormone-independent growth (50). Absence of PR suggests that the tumors of LH-overexpressing mice may be hormone independent; however, given the indeterminate status of ER, verification of hormone-independent growth by ovariectomy of tumor-bearing animals will be required to assess hormone dependency of established tumors.

Similarities observed between mammary glands of transgenic and pregnant mice, including overall morphology and expression of milk proteins, prompted us to assess the molecular resemblance of these tissues on a global scale. Gene expression profiling revealed many genes that behave similarly in transgenic and pregnant glands, compared with virgin wild-type glands (Milliken, E., manuscript in preparation). Presumably, these genes are regulated, directly or indirectly, by the altered hormonal milieu present in both transgenic and pregnant animals. On the other hand, there are also a number of genes that are differentially expressed between these two samples. Differentially expressed genes will be of significant interest, given the fact that the LH-overexpressing mice display an increased susceptibility to carcinogen-induced mammary cancer, but pregnancy actually imparts protection (69). Expression profiling also indicates that the dramatic increase in the proliferative index of LH β CTP mammary glands (Fig. 3, data not shown) can be accounted for, at least in part, by changes in several cell cycle regulatory genes. mRNA levels of cyclins A, B1, and B2 as well as cyclin dependent kinase-1 are increased in mammary glands from LH-overexpressing animals, compared with wild-type age-matched controls, but expression of an inhibitor of cell cycle progression, p18, is decreased (data not shown). Further assessment of the significance of these changes is currently underway.

In summary, we have reported that the mammary glands of mice overexpressing LH undergo precocious development and acquire spontaneous tumors in the absence of forced protooncogene overexpression. Molecular profiling of the mammary glands of these mice throughout development using global approaches such as gene chip expression analyses should provide further insight into the mechanisms of hormone-mediated hyperplasia and tumorigenesis. These mice may also be of use for testing prospective hormonally relevant anticancer drugs. Revealing signaling pathways that are important in hormone-induced tumorigenesis may also lead to the identification of potential therapeutic targets and strategies for treatment of hormone-induced human breast cancers.

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