Neonatal Experiences Differentially Influence Mammary Gland Morphology, Estrogen Receptor α Protein Levels, and Carcinogenesis in BALB/c Mice

Allison L. Boyd1, Ayesha Salleh1, Brent Humber2, Janet Yee1,2,3, Ladislav Tomes5, and Leslie R. Kerr1,2,4

Abstract

Prevention of breast cancer can be achieved with a better understanding of the factors contributing to normal breast development. Because the breast develops postnatally, alterations in the development and lifetime activity of the neuroendocrine system may set up an environment that increases cancer risk. The present study examined how two neonatal experiences over the first 3 weeks of life influence normal and malignant mammary gland development in female BALB/c mice. Following puberty, both brief (15 minutes) and prolonged (4 hours) daily maternal separations of newborn mice accelerated mammary gland development relative to nonseparated mice. Despite similar mammary gland morphologies between mice exposed to these two neonatal separation experiences, only mice exposed to prolonged maternal separation bouts showed a higher incidence and faster onset of mammary tumorigenesis following adulthood carcinogen [7,12-dimethylbenz[a]anthracene] administration. Molecular analysis of estrogen receptor α (ERα) and p53, two proteins that have been implicated in breast cancer, revealed that for mice exposed to prolonged neonatal maternal separation bouts, mammary gland ERα protein levels were upregulated in a transcription-independent manner. On the other hand, p53 expression in mammary glands of adult mice was not differentially influenced by neonatal experiences. Our findings show that chronic, moderate psychosocial stress during the neonatal period increases the expression of ERα protein and promotes mammary tumorigenesis in adulthood. Cancer Prev Res; 3(11); 1398–408. ©2010 AACR.

Introduction

Prevention of breast cancer can be achieved with a better understanding of the factors that contribute to normal breast development. Some studies examining cancer etiology suggest that the external environment of the offspring, such as the mother's diet or psychosocial stressors, can alter the hormonal microenvironment of the developing mammary gland, which may influence mammary gland development and the risk of carcinogenesis (1–3). Relevant to the present study, neonatal experiences, especially challenging or stressful experiences, have been shown to cause long-term developmental changes in the brain, influencing behavioral (e.g., coping) and physiologic (e.g., hormonal) profiles of rodents, primates, and humans (4–7). Because the mammary gland, like the brain, completes its development postnatally, it is possible that through neuroendocrine effects, neonatal experiences can also influence the development of mammary tissue, increasing or decreasing the likelihood of mutagenic events. Elaboration of the factors that influence mammary gland development is crucial to our understanding of the etiology of breast cancer.

It has been established that in laboratory rodents, exposure to the chronic, moderate stressor of long bouts (e.g., 4 hours) of maternal separation (LMS) over the first 2 to 3 weeks of life can permanently alter brain development, causing exaggerated hormone reactivity to stressors later in life (8–11). Alternatively, the chronic but mild stress experienced by pups exposed to brief periods (e.g., 15 minutes) of maternal separation (BMS) over the same neonatal period often results in attenuated hormonal responses to stressors in adulthood (7, 12–14). Importantly, neuroendocrine regulation not only influences circulating stress hormone levels (e.g., corticosterone in rodents) but can also alter the circulating levels of mammogenic reproductive hormones through the well-established interactions between the limbic-hypothalamic-pituitary-adrenal (LHPA stress) and hypothalamic-pituitary-gonadal (HPG; reproductive) axes (15). Hormonal disturbances during key developmental periods early in life can create predispositions to abnormal breast growth and therefore breast cancer risk (16).
There have been only a handful of studies that have examined how neonatal environments affect the development of the breast and fewer still relating these changes to breast cancer risk. To date, only one study has shown that compared with nonhandled rats, neonatal handling decreased the incidence of carcinogen-induced mammary tumors (3); however, the molecular and morphologic profiles that underlie this altered cancer risk have yet to be elucidated. We suggest that the enduring changes in LHPA and HPG axis activity, which result from brief (BMS) and prolonged (LMS) separation events (8, 15), can differentially alter the development of hormone-responsive tissues such as the breast. Neonatal manipulations of maternal-offspring interactions therefore represent a valuable model to study the relationship between early-life stress and both normal and carcinogen-induced mammary gland development.

Murine models examining normal and malignant mammary gland development allow for the direct comparison of molecular profiles in mammary glands both before and after carcinogenesis, providing invaluable insight into cancer etiology. Many studies relating molecular factors to breast cancer risk rely on end-point tumor analyses that make it challenging to dissociate tumor-initiating changes from products of tumorigenesis (17, 18). It is estimated that an individual cell requires the accumulation of 5 to 10 genetic or epigenetic events before neoplastic transformation occurs (19) and then another 10 to 15 years (in humans) for a neoplastically transformed cell to progress to a clinically detectable tumor (20). Due to the significant delays between cancer-predisposing events, cancer initiation, and cancer detection, the factors involved in cancer risk need to be assessed long before cancer diagnosis is possible.

The objective of the present study was to assess the impact of early-life experiences on molecular and morphologic profiles of the developing mammary gland and to relate these changes to the risk of mammary tumorigenesis in BALB/c mice. To model mild and moderate early-life stress, we used the well-characterized neonatal manipulations of daily brief (BMS; 15 minutes) or long (LMS; 4 hours) maternal separations for the first 3 weeks of life, as well as mice that did not experience maternal separation [typically reared (TR)] as environmental controls. At the developmental stages of puberty [postnatal day (PND) 30] and young adulthood (PND 60), mammary glands from mice reared in each of these environmental conditions were examined for mRNA and protein levels of both estrogen receptor α (ERα) and tumor-suppressor p53. These factors were chosen as they are considered to play important roles in normal and malignant mammary gland growth (16, 21, 22). Changes in the molecular profiles of mammary epithelial cells may influence the morphology of the mammary gland, which can also be used to infer cancer risk. For example, high relative proportions of undifferentiated, proliferative epithelial structures [terminal end buds (TEB) in rodents] relative to more differentiated, mature terminal structures [lobular alveolar units in rodents (LAU)] have been suggested to represent a higher predisposition toward carcinogenesis (23). Accordingly, we evaluated the morphologic structure of mammary glands from BMS, LMS, and TR mice at puberty (PND 30), young adulthood (PND 60), and later adulthood (PND 220). Finally, to relate early-life environment-induced molecular and morphologic changes to the risk of mammary cancer, mice reared according to each neonatal condition were administered a known carcinogen [7,12-dimethylbenz(a)anthracene (DMBA)] at PND 60, and mammary tumor incidence and invasiveness were assessed.

Materials and Methods

Animals

Following acclimatization (22 ± 2°C; 12:12 hours light/dark; lights on at 7 a.m.), BALB/c mice (Charles River Laboratories) were housed in groups of one male to two to three females for breeding. Once pregnant, female mice were housed individually with ad libitum food and water. All offspring were semirandomly cross-fostered among dams and across treatment conditions. From PND 2 to 22, offspring were exposed to daily neonatal manipulations of either brief (15 minutes; BMS), protracted (4 hours; LMS), or no (TR; typically laboratory reared) maternal separation (see Supplementary Material for a full description of the procedure). On PND 23 to 25, pups were weaned and housed in same-sex, same-litter groups with two to four mice per cage. Following this point, only females were used in this study.

Tissue collection

Mammary glands were collected at approximately PND 30, 60, and 220 (±2 PND for each age point), and only when mice were in estrus. Moreover, for pubertal mice (PND 30), mammary glands were collected at the estrus phase of the first estrous cycle (first estrus is an index of both pubertal maturation and the start of adult functioning of the HPG axis; ref. 24). Mammary glands used for analysis of RNA (seventh and eighth thoracic glands) or protein expression (second and third thoracic glands) were stored at −80°C until required. Mammary glands used for morphologic and immunohistochemical analyses (fourth and ninth inguinal glands) were fixed overnight and then underwent carmine alum staining or immuno-histochemical procedures.

Determination of stage of estrous cycle

Beginning on PND 27, female mice were examined daily to detect the estrous cycle stage. Female mice were lavaged with physiologic saline (0.9% NaCl) to obtain vaginal smears for observation under a light microscope. Proestrus was identified when there was a distribution of small, round nucleated, cornified epithelial cells with few to no leukocytes. Estrus was determined through the presence of numerous and clumped large cornified cells
with degenerated nuclei. Metestrus was determined by the presence of many leukocytes and few cornified cells, and the diestrus smear was composed of many leukocytes. Mice were sacrificed in the estrus stage to control for fluctuations in hormone levels. The remaining mice were terminated on PND 60 or 220 and also during the estrus stage of their cycle.

**Mammary gland morphology**

Carmine-stained whole mounts were analyzed under a dissecting microscope (Wild) to evaluate the numbers of age-relevant mammary gland structures. A trained observer blind to the experimental treatment group counted the total numbers of mammary gland structures distal to the central lymph node. Structures were assessed based on morphologic criteria provided in ref. (25); see representative examples in Fig. 1. At PND 30 and PND 60, terminal structures were evaluated, as the appearance of TEBs occurs near PND 30 and TEBs typically mature into terminal LAUs near PND 60 (generally between 9 and 12 weeks of age; ref. 26). Lateral alveolar development occurs later than terminal alveolar development, beginning around 12 weeks of age with variable degrees of development occurring among nulliparous mice (26). Therefore, lateral alveolar buds (LAB) were counted in mammary glands of mice at PND 220. Branch points were counted in mammary glands of all ages as branches can arise from either ductal bifurcation in younger mice or lateral budding in older mice (26).

**Western blots**

Membranes were incubated with primary antibodies [1:300 for ERα (MC-20) and 1:300 for actin (I-19), Santa Cruz Biotechnologies; 1:750 for p53 (CM-5), NovCastra Laboratories] and then with appropriate secondary IgG antibodies [1:200, Santa Cruz Biotechnologies]. Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and the same total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies). Total protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad), and then with appropriate secondary IgG antibodies (1:200, Santa Cruz Biotechnologies).

**Immunohistochemistry**

Staining for ERα was done on paraffin-embedded mammary gland sections (6 μm). Following deparaffinization, antigen retrieval, and a 30-minute incubation with 6% goat or rabbit serum, tissue sections were incubated with ERα (MC-20; 1:60) or actin (I-19; 1:50) primary antibody (Santa Cruz Biotechnologies). Then, appropriate secondary IgG antibodies (1:150; Santa Cruz Biotechnologies) were applied. Counts were expressed as the percentage of nuclei that positively stained for ERα, relative to the total number of nuclei. For each neonatal manipulation group, tissue sections from either five (PND 30) or eight (PND 60) mice were counted under a light microscope (Weiss), with a total of 500 to 1,250 nuclei counted for each mammary gland.

**Quantitative reverse transcriptase-PCR**

Total RNA was extracted from mammary glands (RNeasy Lipid Tissue Mini Kit; Qiagen), and an on-column DNase digestion was done (Sigma). This was followed by an RNA quantification and integrity assessment (NanoDrop spectrophotometer, Thermo Fisher Scientific) and first-strand cDNA synthesis with M-MulV reverse transcriptase (Fermentas). An Applied Biosystems 7900HT real-time PCR machine was then used to quantify the cDNA (see Supplementary Materials for primer sequences and cycling parameters). Relative gene expression was calculated using the ΔΔCt method. The expression of the β-actin gene for each sample was used as the normalizer, and the gene expression in the TR sample was used as the calibrator.

**Carcinogen administration**

Following neonatal manipulations, cages of female mice were randomly assigned to either receive carcinogen (n = 15 animals in each neonatal treatment group) or vehicle (n = 10, 10, and 9 animals for BMS, LMS, and TR, respectively). Beginning at PND 60, mice were orally gavaged with either 0.1 mL DMBA (10 mg/mL) dissolved in vehicle (safflower oil) or 0.1 mL of vehicle alone, once per week for 6 weeks. This is an established DMBA administration procedure for mice that results in at least 60% mammary tumor development (27). Approximately 1 month after the start of DMBA/vehicle administration, mice were weighed biweekly and monitored for tumor incidence and growth by manual palpation and caliper measurements. Tumor size in cm² was calculated using the following equation: (width² × length)/2. Data were collected up to 300 days after the first DMBA administration. A DMBA-treated mouse was terminated once tumor burden reached 10% of the animal’s body weight, if the animal experienced rapid weight loss, or if other indicators of suffering were apparent. At this point, the estrous phase was determined by vaginal lavage and all mammary glands were collected. Any other abnormalities observed during dissection were noted (e.g., enlarged spleen, other tumors). Mammary glands from vehicle-treated mice were collected along with those of DMBA-treated mice in a 2:3 ratio.

**Histopathologic evaluation of tumors**

Paraffin-embedded tumors were sectioned (6 μm) and stained with Harris’ hematoxylin and eosin Y. Two to four stained sections of each mammary tumor were evaluated for lesional type and invasion by a pathologist (LT). Immunohistochemistry with myoepithelial cell stains [p63 (4A4) and actin (I-19); 1:50; Santa Cruz Biotechnologies] was used to confirm the nature of the lesion.

**Statistical analyses**

All data are presented as means ± SEM. Statistical comparisons were done using one-way ANOVAs (morphologic
data), repeated-measures ANOVAs (quantitative PCR data, using replicate experimental runs as a within-subjects factor), or factorial ANOVAs (Western blot data using experimental run as a second factor; immunohistochemistry data using tissue compartment as a factor). Where indicated, data were first log-transformed if parametric assumptions were violated, or nonparametric Kruskal-Wallis tests were used if transformation was not possible. Cox-Mantel tests were used to evaluate survival and mammary tumor incidence data between groups as of 300 days following the first DMBA gavage. The analysis of mammary tumor incidence used time to tumor detection for complete responses and time to death for censored responses, whereas the survival analysis used time to death for all complete responses. The software package Prism (GraphPad) was used for analyses of

![Fig. 1. Mammary gland whole mounts of BMS (brief separation), LMS (prolonged separation), and TR (control) mice at PND 30, 60, and 220. Scale bar, 500 μm. A, at puberty (PND 30), the number of TEBs (closed arrow) was similar across neonatal conditions (n = 11 BMS, 12 LMS, and 11 TR). By early adulthood (PND 60), mammary glands of BMS and LMS mice had significantly fewer TEBs relative to TR mice, implying accelerated maturation of TEBs into terminal LAUs (n = 14 BMS, 19 LMS, and 15 TR). B, at PND 220, mammary glands of BMS and LMS mice had significantly more LABs (open arrow) and ductal branching relative to mammary glands of TR mice (n = 10 BMS, 10 LMS, and 9 TR). Data are presented as means ± SEM. *, P < 0.05. PND 60 TEB data were log-transformed before statistical analysis.](https://cancerpreventionresearch.aacrjournals.org)
survival and mammary tumor incidence. All other analyses were done using Statistica version 6.1 (StatSoft).

**Results**

**Neonatal maternal separation causes precocious mammary gland development following puberty**

Mammary gland morphology was first assessed in animals at puberty onset (at PND 30), a time when the formation of TEBs occurs. As may be expected at such an early stage of pubertal development, the number of TEBs was not affected by either mild or moderate early-life stressors (i.e., BMS or LMS manipulations; Fig. 1A). However, in young adult (PND 60) mice, the number of TEBs was influenced by maternal separation events. By early adulthood, TEBs have guided the expansion of the ductal epithelial network to the distal boundaries of the fat pad and have begun to mature into morphologically distinct terminal LAUs (28, 29). Relative to TR mice, those mice that experienced maternal separation events (BMS and LMS) had fewer immature TEB structures (Fig. 1A), despite having similar numbers of branch points at this age (which is indicative of similar total terminal structures across groups; data not shown). This suggests early maturation of TEBs into terminal LAUs in neonatally manipulated mice. Additionally, the mammary glands of BMS and LMS mice at PND 60 had limited LABs compared with no lateral budding in the mammary glands of TR mice (data not shown). Similar to our observation of accelerated mammary gland development in BMS and LMS mice at young adulthood (PND 60) is our finding that the mammary glands of older adult (PND 220) BMS and LMS mice had increased branching (due to the emergence of tertiary branches) and a greater number of LABs compared with mammary glands of TR mice (Fig. 1B). The timing of puberty onset and body weight were similar among all neonatal conditions (data not shown); therefore, none of the morphologic differences could be attributed to alterations in either of these factors.

**ERα, but not p53, protein expression is increased in mammary glands of adult LMS mice**

As may be expected because ERα protein expression in the mouse mammary gland tends to be influenced...
following the surge of ovarian hormones at puberty (30), no significant differences were observed in ERα protein expression levels in mammary glands of BMS, LMS, or TR mice that had just reached puberty (Figs. 2A and 3A). However, at young adulthood (PND 60), ERα expression in the mammary glands of LMS mice was more than 200% greater than the expression levels in TR mice and 30% greater than the expression levels in the mammary glands of BMS mice (Fig. 2A). As links have been made between mammary gland ERα levels and mammary tumor susceptibility (31–33), it is possible that the increased ERα protein expression in LMS mice may represent a predisposition to mammary tumorigenesis. Immunohistochemical analyses of PND 60 mammary glands (Fig. 3A and B) support the Western blot data (LMS > BMS > TR) and also show that although a main effect of tissue compartment in ERα protein expression patterns was observed (epithelial > stromal, \( P < 0.0001 \)), the expression patterns were similar among neonatal conditions.

Like pubertal (PND 30) ERα expression, protein expression levels of the tumor-suppressor p53 were similar in the mammary glands of pubertal BMS, LMS, and TR mice (Fig. 4A). However, p53 proteins levels in mammary glands of young adult mice (PND 60) were also similar across neonatal conditions (Fig. 4A). This is in contrast to the ERα protein levels in mammary glands of young adult mice, which were differentially affected by neonatal experience.

**Transcript levels of ERα and p53 are not altered in mammary glands of pubertal or young adult LMS mice**

ERα transcript expression levels were found to be similar in mammary glands of PND 60 BMS and LMS mice (Fig. 2B). This was unexpected due to the significantly higher ERα protein expression in mammary glands of PND 60 LMS mice relative to BMS mice (Fig. 2A). The fact that the ERα transcript levels in the mammary glands of BMS and LMS mice did not reflect the ERα protein levels observed between these samples suggests that the increase in ERα protein levels observed in the mammary glands of LMS mice relative to BMS mice may be due to posttranscriptional events. Like the protein levels, the transcript levels of p53 in the mammary glands of young adult (PND 60) BMS and LMS mice were similar (Fig. 4A and B).

**Neonatal manipulations differentially alter mammary carcinogenesis**

Although the overall survival rates (Fig. 5A) were similar among all mice due to malignancies in other tissue types, the incidence of mammary lesions was most pronounced in the LMS condition as this group had the greatest number and fastest onset of mammary lesions (significantly relative to the BMS condition and marginally relative to the TR condition; Fig. 5B). Three hundred days after the first carcinogen administration (i.e., PND 360), 20% of BMS, 53% of LMS, and 20% of TR mice had developed palpable mammary lesions (expressed as percentages of DMBA-treated animals; Fig. 5C). One BMS mouse and
one LMS mouse had multiple mammary lesions (each of these mice had lesions in two separate mammary glands). One BMS mouse and two LMS mice that were terminated due to significant mammary tumor burden were also found to have ovarian tumors at necropsy. Other DMBA-induced causes of death in mice that did not develop mammary tumors consisted of gastrointestinal tumors (BMS = 2; LMS = 1), skin lesions (BMS and TR = 1 each), an ovarian tumor (TR = 1), and hematologic malignancies (BMS = 3; TR = 2).

Evaluation of the invasive potential of hematoxylin and eosin–stained tumor specimens indicated that many of the lesions contained cribriform or papillary areas, and that there was a considerable spectrum of invasiveness among mammary tumors (Fig. 5D). Only 1 of the 15 BMS mice was found to have an invasive mammary carcinoma, whereas 4 of the 15 DMBA-treated LMS mice had lesions that were classified as invasive. All mammary lesions found in the DMBA-treated TR mice were classified as invasive. Of the remaining mammary lesions (two BMS and five LMS mice), most were classified as in situ carcinomas or proliferative benign lesions, except one mammary tumor of an LMS mouse that was determined to be a phyllodes tumor (of mesenchymal origin but also containing epithelial cells). In addition to having shorter mammary tumor latencies, LMS mice had the greatest number of invasive mammary carcinomas. This again suggests that neonatal manipulations differentially altered mammary carcinogenesis; the chronic stress of prolonged maternal separation early in life increased mammary tumor risk in female BALB/c mice relative to the mild stress of brief maternal separation. The increased mammary tumor risk of LMS mice could be related to higher ERα expression in LMS mice at the time of carcinogen administration (PND 60).

Discussion

We have shown for the first time that the early-life experiences of either brief (BMS) or prolonged (LMS) maternal separation differentially influence the risk of carcinogen-induced mammary tumors, which may be related to molecular and morphologic differences between the mammary glands of these mice. Specifically, mammary tumorigenesis was significantly enhanced in LMS mice relative to BMS mice within 300 days following DMBA administration. LMS mice also had the highest incidence of invasive mammary carcinomas out of any experimental group. Our findings that the LMS group had both the fastest onset of mammary carcinogenesis and the highest mammary gland ERα levels at the time of carcinogen administration complements studies that have shown delayed onset of mammary tumors in ERα-knockout mice (33).

It has been established that neonatal manipulation paradigms can cause enduring functional changes to both the LH/HPA (34) and the HPG (35) axes. We suggest that neonatal experience–induced alterations in circulating hormone levels can influence developmental processes of the mammary gland, ultimately contributing to changes in gene expression and/or protein levels and cancer risk. The similar timing of puberty onset among all
mice as well as the morphologic similarities in the mammary glands of pubertal BMS, LMS, and TR mice suggest that the prepubertal levels of circulating ovarian steroid hormones were comparable. However, others have shown that neonatal handling alters hormone secretion patterns of the adult rat estrous cycle (e.g., reduced estradiol secretion relative to nonhandled controls during proestrus; ref. 35). The hormonal variations during the estrus cycle influence the balance of proliferation and apoptosis in the mammary epithelium (36). Alterations in estrus cycle regulation due to early-life stress may therefore be most apparent in the mammary gland morphology of older animals, as they have had the cumulative effects of many estrus cycles on the growth dynamics of the mammary epithelium. This possibility is consistent with our morphologic observations of adult mouse mammary glands, which show increasing differences with age between mice experiencing maternal separation and those that did not. That is, relative to mammary glands of young adult (PND 60) TR mice, mammary glands of BMS and LMS mice at PND 60 had fewer immature structures (i.e., TEBs) and related premature appearance of LABs. These morphologic differences among the mammary glands of the BMS, LMS, and TR mice were even more striking at PND 220, with the mammary glands of BMS and LMS mice having extensive development of side branches and LABs compared with the mammary glands of TR mice. In the absence of tertiary structures (e.g., side branches and LABs), it would be expected that the lower number of proliferative TEBs in the mammary glands of BMS and LMS mice at PND 60 would be protective against carcinogenic insult (23). However, the presence of side branches and LABs has been linked to higher rates of both alveolar and ductal proliferation (36), suggesting a higher global index of proliferation (and therefore, possibly, cancer risk) in the mammary glands of young adult BMS and LMS mice despite the early maturation of TEBs into LAUs.

Although both young adult BMS and LMS mice had mammary gland morphology indicating increased
proliferative potential and therefore cancer risk, only LMS mice suffered from a significantly higher incidence and shorter latency of carcinogen-induced mammary tumors. This emphasizes the importance of not only morphologic relationships with respect to cancer risk but also functional relationships related to protein expression because differential regulation of proliferation-related signaling pathways (e.g., ERα) could increase or decrease the opportunities for abnormal cellular growth. In addition to similarities in puberty onset and pubertal mammary gland morphology, PND 30 ERα protein expression was similar in the mammary glands of BMS, LMS, and TR mice. However, at early adulthood (PND 60), mammary gland ERα protein expression significantly differed among neonatal conditions, with the highest mammary gland ERα protein expression in LMS mice. Naturally, ERα protein levels gradually increased in the murine mammary gland once estrous cycling begins with the surge of ovarian steroid hormones at puberty (30). Thus, the postpubertal increase in mammary gland ERα protein levels may have been amplified in LMS mice (relative to BMS and TR mice) due to altered HPG axis functioning. Previous studies using postpubertal social isolation as a stressor have shown stress-related effects on mammary tumorigenesis despite unaltered ERα protein levels (37, 38). It is clear that the perceived intensity and the timing of the stressor with respect to development are important factors that modulate the effects of stress on mammary tumor risk. It is possible that mild stressors such as brief bouts of maternal separation during important developmental periods may cause transient molecular changes in the very early stages of mammary gland development (e.g., during isometric mammary growth) that ultimately have a protective effect on mammary gland development, whereas more extreme stressors such as prolonged maternal separation could cause long-term molecular changes in mammary tissue with negative implications for future tumor risk. Specific mechanisms governing these possible effects in our mouse tumor model are currently being investigated, including those related to differences in circulating hormone levels and possible epigenetic events.

As previous studies have suggested that the loss of ERα protein expression delays the onset of mammary tumor formation (33), the high levels of ERα protein expression in the mammary glands of LMS mice at the time of carcinogen exposure (PND 60) may be related to the elevated mammary tumor incidence observed in these mice. Interestingly, the higher levels of ERα protein in the mammary glands of LMS mice relative to other groups was not reflected at the mRNA level; ERα transcript levels in the mammary glands of BMS and LMS mice did not differ. This suggests that the differences observed in ERα protein levels are caused at a point following transcription, such as increased translation rates of ERα protein or decreased degradation rates of ERα protein in the mammary glands of LMS mice compared with BMS mice. For example, LMS mice could have high levels of ERα-stabilizing chaperones, such as heat shock protein 90, which would result in slower ERα degradation rates and therefore higher ERα protein levels without an increase in ERα transcript expression levels (39). High ratios of ERα protein to ERα transcript levels, which were observed in the mammary glands of LMS mice, have also been reported in primary human breast tumors (40).

In contrast to the ERα protein expression data of the present study, expression levels of p53 protein and mRNA were not differentially influenced by early-life rearing experiences at either age examined (PND 30 or PND 60). It is possible that disruption of p53 protein expression might not be an early step in tumor initiation in this model, although some sort of interference with p53 function may be a necessary step for eventual tumor promotion (as this is a commonly observed event in tumorigenesis; refs. 21, 41). Expression of p53 protein in all tissues is typically low, with an increase observed in response to cellular stress or damage (41). Any mechanism that prevents the increase of p53 levels in response to cellular damage or that interferes directly with p53 activity could impair the tumor-suppressor function of p53, with the same ultimate result as genetic mutations or decreased expression levels. Therefore, other molecular changes that result from BMS and LMS manipulations may have the potential to affect p53 activity and influence cancer risk. For example, ERα protein, which was significantly upregulated in LMS mice, has been shown to suppress p53-mediated transcriptional activity (42, 43). Although alterations in p53 protein expression may not be an early step in cancer predisposition or initiation in this model, alterations in the expression or activity of this protein could be a later consequence of other neoplastic changes in the mammary gland induced by early-life stress.

Although breast cancer is normally detected later in life, the initiation of cancer occurs many years before clinical detection is possible, and predispositions toward cancer-initiating events may be established even sooner (i.e., during mammary gland development). Here, we show that chronic, moderate early-life stress in the form of prolonged maternal separation causes both morphologic and molecular changes in the mammary glands of adult mice, resulting in the higher incidence of carcinogen-induced mammary tumors. The mechanisms governing these changes are currently being investigated. This study highlights the importance of understanding the contributions of early-life environment to developmental events in the breast that can lay the foundation for future breast cancer risk.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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