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Original Research

Mouse Mammary Gland Is Refractory to the Effects of Ethanol after Natural Lactation

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Ethanol is a dietary factor that dose-dependently increases breast cancer risk in women. We previously have shown that ethanol increases mammary epithelial density through increased branching after dietary exposure during puberty in CD2/F1 mice. To extend these studies to parous mice in a breast cancer model, we used a transgenic mouse model of human parity-associated breast cancer, the FVB-MMTV-Her2/Neu mouse, which overexpresses wildtype EGFR2, resulting in constitutive activation of growth signaling in the mammary epithelium. Here we describe the short-term effects of ethanol feeding on progression through involution. Mice were fed diets supplemented with 0%, 0.5%, 1%, or 2% ethanol for 4, 9, or 14 d starting on day 21 of lactation (that is, at the start of natural postlactational involution). Unlike peripubertal mice exposed to ethanol, postlactational dams showed no changes in body weight; liver, spleen, and kidney weights; and pathology. Ethanol exposure had no effect on mammary gland lobular density and adipocyte size throughout involution. Likewise, the infiltration of inflammatory cells and serum oxidized lipid species were unchanged by diet, suggesting that ethanol feeding had no effect on local inflammation (leukocyte infiltration) or systemic inflammation (oxidized lipids). In conclusion, ethanol exposure of parous dams had no effect on mammary gland structure or the regression of the lactating mammary gland to a resting state. The period of involution that follows natural lactation appears to be refractory to developmental effects of ethanol on mammary epithelium.

Abbreviations: HOTE, hydroxyoctadecatrienoic acid; OXO-ODE, oxo-octadecadienoic acid; 9-HODE, 9-hydroxyoctadecadienoic acid.

Breast cancer affects more than 180,000 women yearly in the United States, and more than 40,000 women each year die of the disease.[1] Although the incidence rate seems to have stabilized during the last decade and a decline in the mortality rate has been noted, breast cancer remains a major health problem. Despite a great deal of dietary research in breast cancer chemoprevention, meta-analysis of epidemiologic studies and clinical trials has shown that the only dietary modifier of human breast cancer risk is alcohol consumption.[59] Breast cancer risk increases by approximately 10% for every drink consumed daily, and these effects are seen for both pre- and postmenopausal breast cancer.[45,59] Approximately 1% to 2% of breast cancers are estimated to be due to light drinking,[45] based on the increased incidence seen at even low levels of consumption,[8] independent of type of alcohol consumed.[2] Whether lifelong exposure is necessary for the deleterious effects of ethanol to be manifest is unknown, as is whether it is beneficial to modify ethanol consumption during periods of rapid postnatal developmental remodeling of the breast, for example puberty or postlactational involution. Previous studies have shown that short-term prenatal exposure of rats to ethanol (less than 2 wk) can affect long-term mammary glandular development and subsequent breast cancer risk in chemical carcinogenesis models.[15,35] Because chemoprevention is, by nature, a lifestyle change in a healthy population to decrease cancer risk over a lifetime, pulsing of interventions at developmental windows, preferably postnatal, is potentially a more expedient chemopreventive approach than lifelong exposure. However, to address the question of the effects of developmental timing of risk exposures, it is critical to advance appropriate animal models that can be used to test and refine clinically relevant interventions. Specifically, animal models are needed that can manifest the deleterious effects of ethanol on mammary gland development and differentiation.

The primary animal model that has been used to compare effects of dietary (orally administered) ethanol on mammary gland structure and tumorigenesis is the peripubertal rat, which shows increased epithelial proliferation and susceptibility to chemical carcinogenesis.[47-50] In mice, pulsed exposure to dietary ethanol during puberty induces structural alterations in the mammary gland, increasing ductal branching and epithelial density.[23] In women, increased breast density has clearly been associated with breast cancer risk.[6] Although puberty is an attractive target for early strategies of breast cancer prevention, puberty is variable in onset and duration, potentially comprising years. In contrast, a more accessible target for chemopreventive dietary changes is the relatively short (1 to 2 wk) period of postlactational involution. This defined period of rapid glandular remodeling has recently

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been recognized as contributing to the significantly increased breast cancer risk that occurs postpartum and can endure for years after each completed lactogenic cycle.[21] Although early parity does indeed induce lifelong protection against breast cancer, postlactational involution has recently been identified as a critical developmental time point that can induce a short-term increase in type II parity associated breast cancer in terms of development, progression, and metastasis.[43] Therefore, the brief window of postlactational involution may represent a sensitive target for dietary intervention which could be used to alter risk of parity-associated breast cancer in women. Ingestion of protective compounds or avoidance of negative compounds (such as ethanol) during this developmental window may have a disproportionate effect on altering parity-associated breast cancer risk.

The purpose of the current study was to test the hypothesis that ethanol exposure during involution, a time of rapid mammary stromal vascular and epithelial remodeling, induces short-term or long-lasting effects on mammary gland structure in a way that can increase mammary tumor risk, for example, increased mammary gland density by increased epithelial branching.[9] To test this hypothesis, we used the FVB/N-Tg(MMTVneu)202Mul/J transgenic mouse model, which overexpresses wildtype (unmutated) rat EGFR2 under control of the progesterone-responsive MMTV promoter in mammary epithelial cells; expression is detected in salivary gland and lung also.[14] The overexpressed growth factor receptor–tyrosine kinase acts in a constitutively activated manner to stimulate the growth of multifocal mammary adenocarcinomas, similar to 20% to 30% of human breast cancers.[13,33,36,51,52] Tumors develop after prolonged latency, appearing as early as 4 mo but with a median of 6 to 7 mo, thereby mimicking the indolent growth kinetics of human breast cancer.[58] This transgenic mouse model is also a model for high-frequency spontaneous metastasis to the lung.[14] Lastly, the acceleration of tumor growth by pregnancy makes this mouse model a potential tool for studying parity-associated breast cancer. We selected this strain so that we could compare short-term effects of ethanol on progression through involution (described here) with long-term effects of ethanol on Her2/neu-mediated tumorigenesis in future studies. This current report presents the effects of ethanol exposure on the process of mammary gland involution and stromal vascular remodeling, including indices of inflammation.

Materials and Methods

Animals. SPF FVB/N-Tg(MMTVneu)202Mul/J mice (hereafter referred to as Her2/Neu mice) were purchased from Jackson Labs (Bar Harbor, ME). To obtain lactating dams for involution studies, mice were mated inhouse by using a 2:1 ratio of female to male rats. Female rats were 70 to 85 d of age at mating. Mice were caged individually for 1 wk after mating, and dams remained caged individually after weaning until euthanasia. Pups were weaned at day 21 of age and then caged in single-sex groups of 2 to 5 mice. All mice were maintained in conventional housing (open-top caging), provided aspen shavings for bedding, and fed standard rodent diet (no. 2018, 18% Protein Rodent Diet, Harlan Teklad, Madison, WI) until the ethanol-containing diet was initiated (dams only). The mice were maintained on a 12:12-h light:dark cycle in 30% to 70% relative humidity with a room temperature of 68 to 72 °F (20.0 to 22.2 °C). Mice were housed in accordance with the standards set by the NIH and the University at Buffalo IACUC.

Dietary model of chronic ethanol exposure. At 21 d of lactation, dams were distributed by similar weights into 4 dietary groups, receiving liquid Modified Clifford Koury Folate-deficient Liquid Diet containing: microcrystalline cellulose, 94.59 g/L; corn oil (stab, 0.15% BHT), 25.33 g/L; salt mix no. 210020, 12.67 g/L; sodium acetate, 2.05 g/L; L-amino acid premix, 44.55 g/L; vitamin mix no. 317759, 2.53 g/L; folic acid premix, 5 g/kg; succinyl sulfathiazole, 2.53 g/L; choline chloride, 0.51 g/L; and xantham gum, 3 g/L (Dyets, Bethlehem, PA).[5] Maltose dextran was 58.20 g/L in the base diet, and an additional 94.69, 87.31, 79.93, or 65.17 g/L was added to diets containing 0%, 0.5% (5.26 mL/L), 1% (10.53 mL/L), or 2% (21.05 mL/L) ethanol, respectively, to render diets isocaloric as described.[23] Diets were received in powder form and stored at 4 °C in the dark until use. Ethanol was purchased as 95% ethyl alcohol from Pharmco (Brookfield, CT). Capped 30-mL graduated sipper feeding tubes (catalog no. 900012, Dyets) supported by feeding tube holders (catalog no. 901100, Dyets) were used to deliver liquid diet as described.[23] Mice were weighed, and 20 mL of liquid diet was provided daily, with any variations in consumption recorded.

After 4, 9, or 21 d of mammary involution, mice were euthanized by cervical dislocation (to avoid stress to the animal that could affect oxidative stress and inflammatory cytokine status[18]) followed by cutting of the diaphragm. Mice were weighed, and vaginal smears were performed to determine estrous cycle stage. Blood was collected from the thoracic cavity after piercing the heart and was allowed to coagulate for 20 min at room temperature before spinning at top speed in a microfuge; the supernatant sera aliquoted and stored at −80 °C until use. Contralateral abdominal mammary glands (gland no. 4) were dissected for whole-mount preparation or placed into 4% phosphate-buffered formalin for paraffin embedding. Inguinal mammary glands, uterus, ovaries, kidney, spleen, and liver were dissected, weighed, and divided into weighed fractions for RNA or protein extraction by snap-freezing in dry ice with 70% ethanol and storing at −80 °C or placed into formaldehyde for paraffin-embedding and sectioning.

Analysis of paraffin sections. The contralateral abdominal mammary gland (right gland no. 4) from each mouse was fixed in phosphate-buffered formalin for preparation of hematoxylin-and-eosin–stained paraffin sections. The lymph node of each mammary gland was included within the plane of section, as a point of reference. Three slides were made from each gland, and each was stained with one of the following stains: hematoxylin and eosin, Masson trichrome (to visualize collagen deposition), and May–Grunwald Giemsa (to visualize mast cells). The slides stained with hematoxylin and eosin were used for the adipocyte, nuclei, and ductule counts. Each slide was electronically scanned by using an Aperio Imager (Vista, CA) with a 20× objective. Images were accessed through a web-based link provided through Roswell Park Cancer Institute (Buffalo, NY).

Analysis of adipocyte size. To image adipocyte relipidation during involution, clusters of 10 unilocular (white) adipocytes were analyzed for cross-sectional diameter. The longest diameter of each adipocyte was measured (in micrometers) by using Aperio ImageScope Spectrum software (Vista, CA). An average of four 20×-objective fields containing 10 clusters of 10 adipocytes were assessed per mouse mammary gland. To control for variation across the mammary gland, regions proximal to the lymph node were sampled (that is, within 2 lymph node diameters). Data then were combined on a per-mouse basis for comparison.

Analysis of local adipose inflammation. Leukocyte infiltration of the paucicellular adipose tissue was assessed by determining number of nuclei per adipocyte cluster imaged above.[41] An average of four 20×-objective fields containing 10 clusters of 10 unilocular adipocytes were assessed per mammary gland section. Data then were combined on a per-mouse basis for statistical analysis.

Analysis of lobular regression. Lobular regression was assessed over time by quantifying the number of acini per lobule. An average of 5 fields per mouse mammary gland was analyzed under a 10×-objective field of view. Ten lobules were analyzed per field. To control for variation across the mammary gland, lobules proximal to the lymph node (that is, within a region equivalent to 2 lymph node diameters) were selected for analysis. Data then were combined on a per-mouse basis for statistical comparison.

Lipid analyses of sera. Serum linoleic acid and its oxidation products 9-hydroxy-octadecadieneoic acid (9-HODE) and 9-oxo-octadecadieneoic acid (OXO-ODE) were measured simultaneously by liquid chromatography–mass spectroscopy. Serum samples were spiked with 10 ng deuterated 9-HODE (d4-9-HODE, Cayman Chemical, Ann Arbor, MI). Total lipids were extracted from 100 µL serum by using hexane:isopropanol (3:2). Extracts were dried under nitrogen, reconstituted in 1 mol/L ethanolic sodium hydroxide, and rotated for 60 min to hydrolyze lipid esters. Samples then were neutralized to pH 2.5 to 3.5 with 1 mol/L HCl, and total fatty acids were reextracted into hexane:methyl-tertiary butyl ether (4:1 v/v) and dried under nitrogen. Samples were dissolved in ethanol and analyzed by liquid chromatography–mass spectroscopy. Chromatographic analysis was performed by using a LC2010A system (Shimadzu, Columbia, MD) with 2 pumps (catalog no. LC-10ADvp), low-volume mixer, autosampler (catalog no. SIL-HT), column oven (catalog no. CTO-10AC), and diode array detector (catalog no. SPD-M10A). Spectrographic analysis was performed by using a 2010A system (Shimadzu). Chromatography was accomplished by using a binary gradient of acetonitrile and water starting at 30% water for 0 to 10 min, followed by a linear gradient to 100% acetonitrile at 20 min, which was maintained for 20 min until the end of run. The flow rate was 0.3 mL/min, and the analytical column was a SupelcoSil column (length, 25 cm; inner diameter, 3.0 mm; particle size, 3 µm; catalog no. catalog no. LC18-ODS, Sigma–Aldrich, St Louis, MO) with 2-cm guard column. Detection was performed by using electrospray ionization in negative ion mode. Selected ion monitoring was performed at 295 m/z for 9-HODE, 299 m/z for the d4-9-HODE internal standard, 293 m/z for oxo-ODE, and 279 m/z for linoleic acid. Quantification was done by internal standard methodology using d4-9-HODE.

Quantitation of mast cells. May–Grunwald Giemsa stain was used to visualize mast cells, as previously described.[41] Mast cells were quantified by identifying large, dark-purple cells within the stroma of regressing lobules; five lobules were quantified per mouse. Mast cell number was normalized to lobular area, determined by using the Map Area function of the Spectrum Analysis program (Aperio). Mast cells per lobular area then were averaged on a per-mouse basis, with 3 mice analyzed per group.

Statistical analysis. Statistical analyses were performed by using SAS version 9.2 (SAS Institute, Cary, NC). Effects of dietary group on organ weights at euthanasia, whole-mount and histologic analyses of lobular complexity, and percentage areas were analyzed by using 2-factor ANOVA with interaction. The Tukey method was used to control for experiment-wise type I error rate at the 0.05-level for pairwise comparisons. Weights were analyzed with data binned by week, as well as on a daily basis. To analyze the effects of the 4 dietary treatments on the weights of individual pair-fed mice over time, global contrast was performed to determine significance at each time point. For time points determined to be significant, repeated-measures ANOVA (body weight and dietary consumption) were performed by using time as a third variable; a P value of less than 0.05 was defined as being significant. Pairwise comparisons were performed in the event of significant effects.

Results

Ethanol exposure during involution: systemic effects. Mouse dams undergoing mammary involution consumed equivalent volumes of isocaloric liquid diets, regardless of ethanol content (Table 1). Body weight did not differ significantly between dietary groups or time points (Table 1). Organs were weighed at the time of euthanasia to determine whether ethanol feeding led to organ enlargement, as previously described in peripubertal mice fed ethanol diets.[23] Table 2 shows that ethanol had no effect on liver, kidney, or spleen size, both in terms of absolute weight and when normalized as percentage of body weight. A trend toward liver enlargement was neither significant nor dose-dependent. Histologic analysis of liver sections revealed no frank histopathology proceeding to local inflammation or fibrosis in any dietary group. Intracellular vacuolation was increasingly evident in the hepatocytes of mice fed increasing amounts of ethanol (data not shown). This finding was consistent with intracellular lipid accumulation; however, definitive lipid staining could not be performed on paraffin-embedded (and therefore, solvent-extracted) tissue sections.

Serum lipids were analyzed for lipid peroxidation, to assess whether ethanol altered the systemic inflammatory environment associated with involution. No diet-related effect on serum lipid peroxidation was seen (Table 3). A time-dependent decrease in peroxidation was significant ($P < 0.05$) for hydroxyoctadecatrienoic acid (HOTE) in the control (0% ethanol) group between days 9 and 14 (Table 3). When dietary groups were pooled to assess whether these trends were consistent, absolute amounts of linoleic acid and T-HODE were found to have increased significantly ($P < 0.05$) over time postweaning, whereas concentrations of OXO-ODE and HOTE were unaltered (Figure 1). When oxidized lipids were compared as percentages of total linoleic acid, T-HODE increased ($P < 0.05$) from day 4 to day 9. In contrast, relative amounts of OXO-ODE and HODE were decreased ($P < 0.05$) at day 21 (Table 4).

Ethanol effects on involution process: epithelial and adipose tissue. Postlactational involution is marked by 2 opposing activities: regression of the lobular epithelial compartment and expansion of the adipose compartment. To determine whether either of these processes was affected by ethanol exposure, we performed systematic histomorphometric analysis of hematoxylin-and-eosin–stained paraffin-embedded mammary gland sections.

Figure 2 A shows that, as expected, the average number of terminal ductules per lobule decreased ($P < 0.05$) as involution proceeded. This decrease was not affected by ethanol exposure. In addition, mast cell number was analyzed per unit area in regressing lobules (Figure 2 B) but failed to reveal a diet-specific effect at any given time point. As expected, mast cell number tended to increase throughout involution, and this increase was significant ($P < 0.05$) for the mice fed 0.5% ethanol: mast cell number was significantly

Table 1. Daily food consumption (mL) and body weight (g) of mice after ethanol consumption for 1 wk

	% Ethanol in diet			
Duration of feeding (d)	0%	0.5%	1%	2%
Consumption				
4	18.52 ± 1.21	18.28 ± 1.41	17.96 ± 0.66	18.09 ± 1.26
9	18.43 ± 0.98	19.38 ± 0.25	19.42 ± 0.39	19.72 ± 0.14
14	19.10 ± 0.36	19.49 ± 0.29	19.65 ± 0.13	19.17 ± 0.25
Body weight				
4	24.29 ± 0.28	24.97 ± 0.61	25.47 ± 0.40	25.97 ± 0.39
9	24.59 ± 0.60	24.64 ± 1.14	25.33 ± 0.87	25.73 ± 1.28
14	25.31 ± 1.28	25.49 ± 1.22	26.32 ± 0.84	26.09 ± 1.49

Data are presented as mean ± SEM (n = 6 mice per time point, except for day 4 for the 0.5% ethanol group [n = 5]).

Table 2. Absolute (g) and relative (%; normalized to total body weight) tissue weight at time of euthanasia

	% Ethanol in diet			
	0%	0.5%	1%	2%
Absolute organ weight				
Liver	1.17 ± 0.11	1.33 ± 0.09	1.28 ± 0.11	1.29 ± 0.14
Kidney	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.16 ± 0.01
Spleen	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Relative organ weights				
Liver	4.77 ± 0.27	5.34 ± 0.44	4.95 ± 0.40	5.06 ± 0.41
Kidney	0.64 ± 0.03	0.67 ± 0.05	0.67 ± 0.04	0.64 ± 0.03
Spleen	0.36 ± 0.03	0.39 ± 0.08	0.36 ± 0.04	0.36 ± 0.02

Data are presented as mean ± SEM (n = 6) of organ weights on day 21 after weaning from the group that received the 14-d dietary treatment.

Table 3. Effect of dietary treatments on serum lipid peroxidation (%) throughout involution as a function of total serum linoleic acid

		% Ethanol in diet			
	Duration of ethanol feeding (d)	0%	0.5%	1%	2%
9-HODE	4	1.58 ± 0.45	1.26 ± 0.30	1.50 ± 0.95	1.46 ± 0.27
	9	2.51 ± 1.02	2.29 ± 0.23	3.24 ± 0.59	2.50 ± 0.39
	14	2.72 ± 0.66	2.26 ± 0.36	2.07 ± 0.46	2.20 ± 0.25
OXO-ODE	4	4.61 ± 1.33	4.55 ± 0.62	4.87 ± 1.05	4.74 ± 0.90
	9	4.60 ± 1.50	5.14 ± 1.44	5.56 ± 1.73	6.00 ± 1.88
	14	3.14 ± 1.10	2.70 ± 0.78	3.14 ± 1.08	2.75 ± 0.75
HOTE	4	0.79 ± 0.40[a]	0.80 ± 0.50	0.60 ± 0.27	0.40 ± 0.18
	9	0.75 ± 0.34[a]	0.70 ± 0.35	0.73 ± 0.24	0.82 ± 0.39
	14	0.47 ± 0.24[b]	0.43 ± 0.24	0.33 ± 0.14	0.53 ± 0.32

Data are presented as mean ± 1 SD (n = 3 mice per group). Different superscript letters in within a column indicate a significant (P < 0.05) difference between those values within that group.

(P < 0.05) greater in mice fed 0.5% ethanol for 14 d than mice fed 0.5% ethanol for 4 d. In mice fed 2% ethanol, the mast cell number was already maximal by day 9; however, because of large variation from lobule to lobule, this trend was not statistically significant.

Masson trichrome staining revealed numerous blue-green–stained collagen fibrils throughout within the lobule, beginning as early as day 4 of involution, in all dietary groups (Figure 3, left panels). At day 4 of involution, 3 of the 5 mice fed 1% and 3 of the 5 fed 2% ethanol retained distended lobules (Figure 3 G and J), whereas none of the 6 mice fed 0% and none of the 5 mice fed 0.5% ethanol (Figure 3 A and B) retained active lobules at this time. In contrast, at later time points in involution (for example, day 9), all lobules showed lumenal collapse and loss of most secretions (Figure 3, middle panels). At day 21 of involution, mice from all dietary groups showed

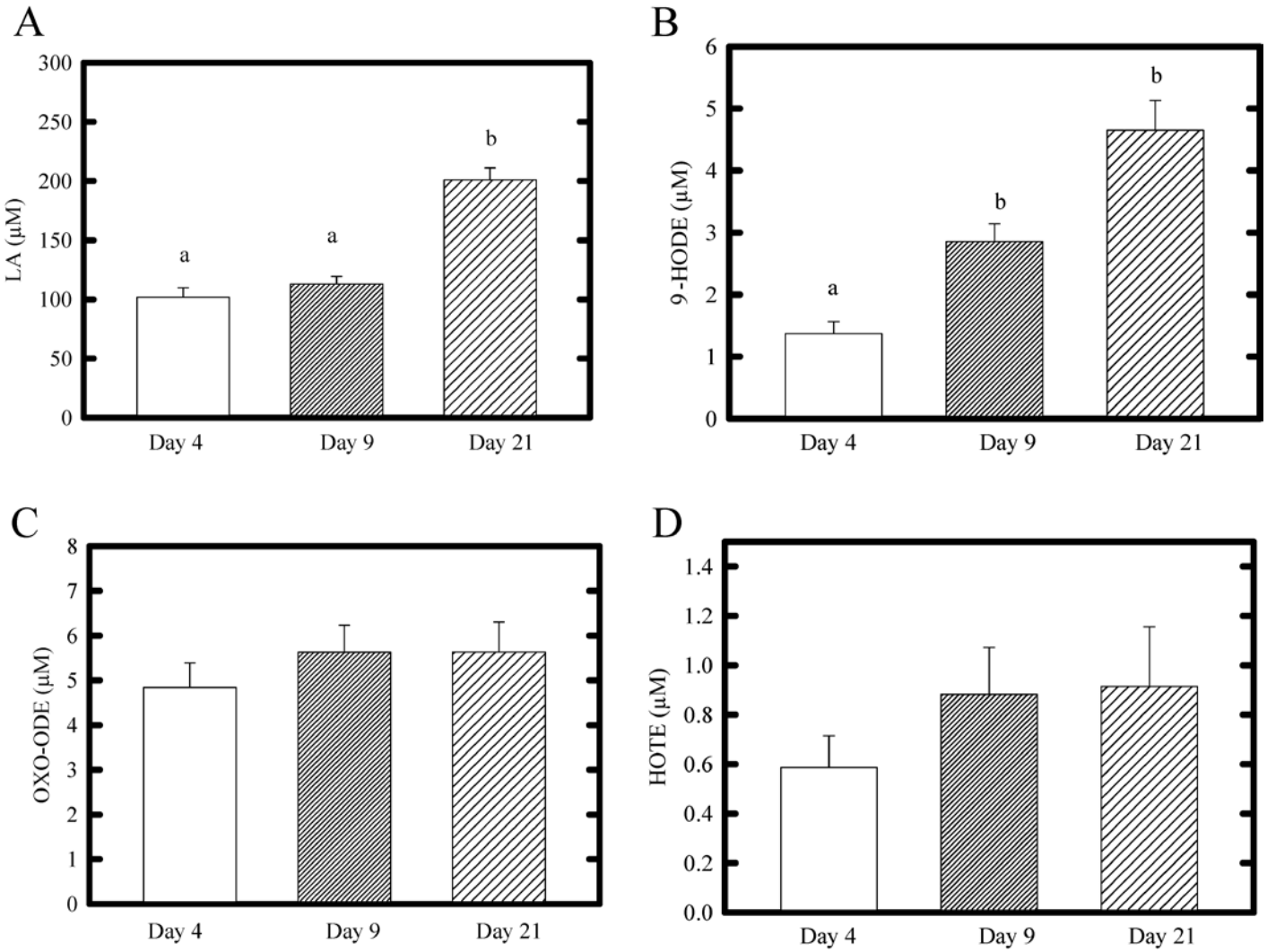

Figure 1. Involution causes shifts in total and oxidized lipids in serum. (A) Absolute levels of linoleic acid are increased at day 21 of involution, compared with days 4 and 9. Whereas (B) 9-HODE levels are increased on days 4 and day 9 of involution, absolute levels of (C) OXO-ODE and (D) HOTE are not altered by involution. Data are presented as mean ± SEM (n = 12 mice) of all diets combined by time point. Different letters indicate a significant ($P < 0.05$) between values.

collapsed alveolar structures containing intensely stained cells (Figure 3, right panels).

Within the adipose compartment, adipocytes tended to gradually increase in size throughout involution in all groups (Figure 4 A); this trend was significantly ($P < 0.05$) different between time points in the control and 1% ethanol groups. Adipocytes also increased in abundance, occupying the majority of the mammary gland area at day 21 (data not shown). In contrast to the changes that occurred in the epithelial compartment (Figure 2 B), remodeling of the adipose compartment was not inflammatory, as no change in infiltrating cells was seen as involution progressed (quantified as nuclei per field, Figure 4 B). Similar to adipocyte relipidation, cellular infiltration within the adipose compartment was unaffected by ethanol consumption.

Discussion

Involution represents a readily accessible short-term target for lifestyle chemopreventive intervention to decrease type II parity-associated breast cancer risk, a devastating and aggressive form of breast cancer that predominantly affects young women within a decade of their previous pregnancy.[21] One successful application of this strategy is demonstrated by ingestion of antiinflammatory drugs during acute involution (weaning after 10 d of lactation); this regimen modifies postinvolution mammary gland structure and is protective against tumor growth and, more importantly, tumor progression in a preclinical model.[20] We hypothesized that ethanol ingestion during this developmental period would disproportionately alter mammary gland structure to increase risk (for example, by increasing epithelial and stromal density).

However, this current study yielded the unexpected finding that after natural lactation (lasting 21 d), mice are largely refractory to developmental effects of ethanol on mammary gland structure. Ethanol feeding did not affect lobular regression in terms of the number of ductules per lobule at later time points, although at day 4, lumenal secretions in distended lobules were more frequent in mice fed 1% and 2% ethanol. The significance of this

Table 4. Effect of involution on serum lipid peroxidation (%), normalized to total serum linoleic acid

Duration of ethanol feeding (d)	% 9-HODE	% OXO-ODE	% HOTE
4	1.58 ± 0.24[a]	4.61 ± 0.43[a]	0.79 ± 0.16[a,b]
9	2.51 ± 0.29[b]	4.60 ± 0.72[a]	0.75 ± 0.14[a]
14	2.72 ± 0.21[b]	3.14 ± 0.41[b]	0.47 ± 0.11[b]

Data are presented as mean ± 1 SD (n = 12 mice; all diets grouped together by day). Different superscript letters in a column indicate a significant (P < 0.05) difference between those values within that group.

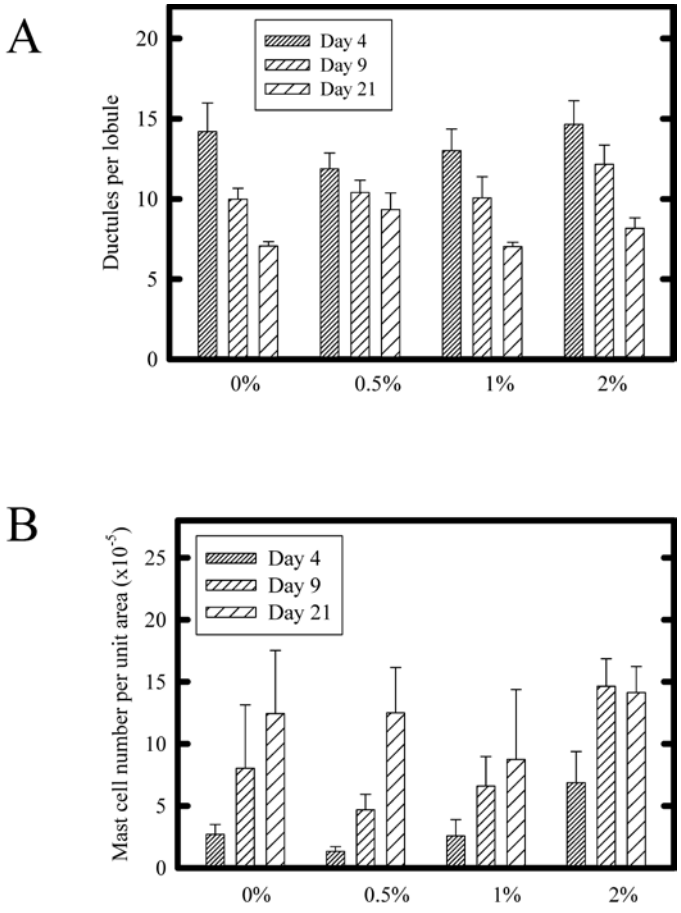

Figure 2. Effects of ethanol exposure on lobular regression or mast cell recruitment during involution. (A) The numbers of ductules per lobule on days 4, 9, and 21 after weaning were compared between mice fed diets containing 0%, 0.5%, 1%, or 2% ethanol for 4, 9, or 14 d, respectively. Lobule complexity decreases over time, with no significant effect of diet observed. (B) Mast cell number per unit area is unaffected by ethanol consumption throughout involution. Data are presented as mean ± SEM (n = 6 mice per time point, except for day 4 for the 0.5% ethanol group [n = 5]).

observation is unclear, given that this retained secretion was gone in all dietary groups by day 9 and day 21, suggesting no long-term effect of ethanol on mammary gland structure. All other indices of involution (local and systemic inflammation and adipose stromal recovery) were unaltered by ethanol feeding.

We previously observed that short-term feeding of mice with ethanol at puberty induces changes to mammary gland structure, increasing branching and epithelial density.[23] In the current study, involution was selected as a time period of intervention for several reasons: (1) ethanol consumption is more common in women postweaning compared with puberty; (2) the time period of involution is more condensed than is puberty, in terms of clinically applicable pulsed intervention; (3) postlactational involution is associated with risk of type II parity associated breast cancer;[20] and (4) involution is a sensitive developmental time period in which multiple changes in gene expression have been identified which can independently give rise to altered developmental recovery and enhanced tumorigenesis.[17,26,29,37,42,44,57]

The general lack of effect of ethanol on involution in our study was unexpected but perhaps not surprising in retrospect. Both rat and mouse pups begin to ingest solid food as early as 12 d of age, allowing the dam to gradually decrease milk output.[16] This event suggests that the model of natural weaning is more similar to extended human breast feeding that persists well past the age of 6 mo, when the infant first begins to shift to solid foods. Using a time period of natural weaning for these studies may have been protective and counterbalanced any deleterious effects of ethanol feeding. In agreement with this concept, meta-analysis shows that extended, but not short-term, lactation is protective for breast cancer in women,[11] and a more recent study concludes that extended breast feeding is more specifically protective for aggressive, estrogen-receptor–negative breast cancers.[32]

In contrast to the current study, many previous studies of involution demonstrated a deleterious effect of involution on mammary gland remodeling and tumorigenic properties, using removal of mouse or rat pups at day 10 of weaning, a time period of peak lactation. Using a model of weaning at day 10 of lactation, multiple rodent studies have shown an influx of inflammatory cells including M2 macrophages, increased collagen proteolysis and deposition of fibrillar collagen, and elevated levels of inflammatory cytokines.[10,25,31,40,53] These changes in the day 10 lactation–involution breast microenvironment have clearly been shown to enhance tumor cell progression.[20] However, early mouse weaning may be a better model for the human weaning times of approximately 6 wk used by many women returning to the workforce, following a typical maternity leave in the United States.

We chose to focus on ethanol intervention during natural weaning at day 21 lactation, rather than weaning at day 10 lactation, for several reasons. First, because day 21 of lactation is the natural time for pups to be weaned, we could mitigate the stress from early separation, such as altered patterns of feeding and sleep, elevated corticosteroid levels, changes in heart rate and core body temperature, as well as acute mammary gland discomfort.[28] These effects could potentially confound our results by altering steroid hormone signaling in the mammary epithelium. Second, our previous studies in rat mammary gland showed the expected rapid influx of inflammatory cells during involution following 21 d of lactation, concomitant with mammary gland engorgement, stromal remodeling, and rapid induction of apoptosis in the lobular epithelium,[22] similar to that described for involution after short-term lactation in this mouse model.[39]

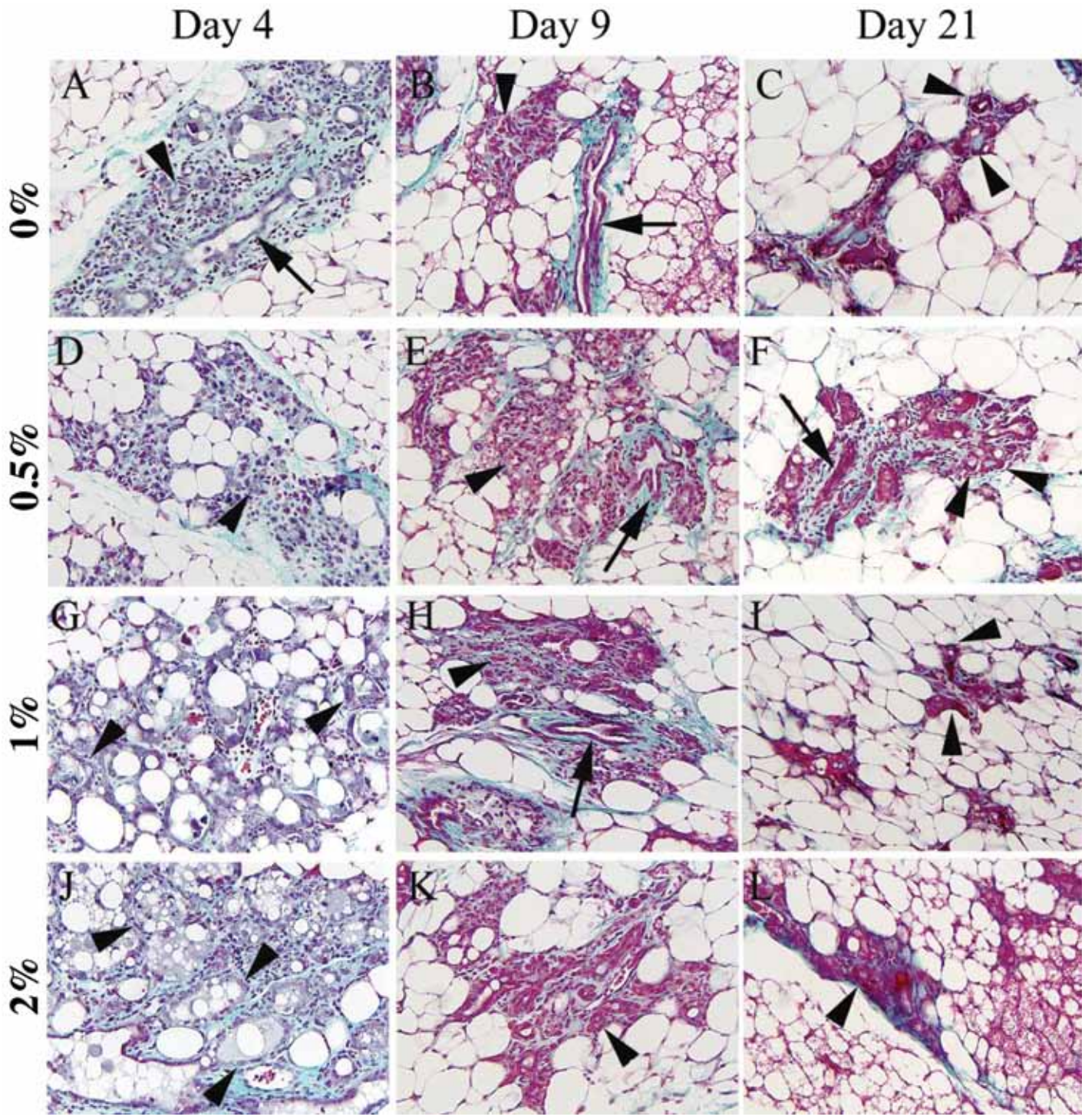

Figure 3. Effects of ethanol exposure on collagen deposition during lobular regression. (A–C) mice fed control (0% ethanol) liquid diets for 4, 9, and 14 d, respectively. (D–F) Mice fed 0.5% ethanol for 4, 9, and 14 d, respectively. (G–I) Mice fed 1% ethanol for 4, 9, and 14 d, respectively. (J–I) Mice fed 2% ethanol containing diets for 4, 9, and 14 d, respectively. Arrows indicate ducts; arrowheads indicate individual alveoli. Aqua stain demonstrates fibrillar collagen deposition. All images show paraffinated sections stained with Masson trichrome; original magnification, 200×.

In addition, the current study describes an orally administered ethanol mouse model to test the effects of ethanol on parous transgenic mouse mammary gland, as a model for type II parity associated breast cancer (breast cancer diagnosed after pregnancy is completed). Despite its effects to protect over a lifetime, parity and its associated period of postlactational involution has recently been recognized as a significant contributor to the aggressive, highly metastatic breast cancers diagnosed 6 to 10 y postpartum; results from both in vitro and in vivo studies strongly support the concept that parity-associated breast cancer is the result of the

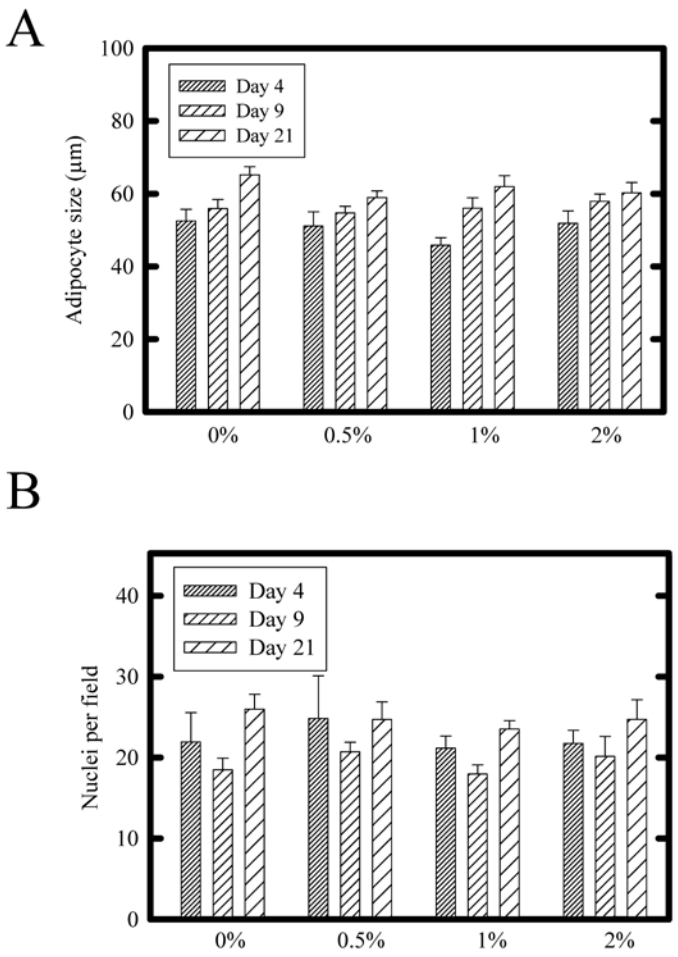

Figure 4. Ethanol exposure does not alter adipose relipidation or inflammation. (A) Ethanol feeding had no effect on the average adipocyte diameter per field between groups. Adipocyte size increased over time (within groups) in the 0% (control) group between days 4 and 21 (P = 0.008) and days 9 and 21 (P = 0.020) and in the 1% ethanol group between days 4 and 9 (P = 0.016) and days 4 and 21 (P = 0.001). (B) Ethanol feeding had no effect on leukocyte infiltration throughout involution, and no significant effect within groups over time. Data are presented as mean ± SEM (n = 6 mice per time point, except for day 4 for the 0.5% ethanol group [n = 5]).

response of epithelial cells to stromal changes initiated during postlactational involution rather than pregnancy itself.[43] To model parity–involution-associated developmental changes that could contribute to breast cancer, these experiments took advantage of a well-established in vivo genetically engineered mouse model that results in overexpression of the wildtype Her-2/neu growth factor receptor in the mammary gland, closely mimicking the etiology and histopathology of many human breast cancers.[7] Tumor incidence is greatly increased by multiple rounds of pregnancy,[3,34] suggesting that this strain is a model for parity-associated breast cancer.

We selected 3 developmental time points during mouse involution for study: day 4, after the point of reversibility; day 9, the time point when regression is newly completed; and day 21, an additional time point at which the gland will have returned to a fully regressed, resting state.[22,54] The day 4 time point is marked by glandular collapse of alveoli, proteolytic breakdown of the basement membrane by matrix metalloproteinases, relipidation of adipocytes, and accumulation of professional macrophages for clearance of residual milk proteins and apoptotic cells.[4,12,37] In rodents, involution is typically complete by 5 to 7 d after weaning, with a majority of the apoptotic cells and milk components cleared and the gland approaching the quiescent state.[4,31,55] We chose day 9 of involution to visualize potential effects of ethanol feeding to slow the involution process. Our day-21 time point (1 wk after completion of 14-d feeding with ethanol), was selected to determine whether ethanol induced any changes in the mammary gland that extended beyond completion of involution. In terms of animal models for type II parity associated breast cancer, postlactational involution in mice and rats represents a rapid developmental window of large-scale mammary remodeling, which may have unprecedented sensitivity to short-term interventions that impact long-term breast cancer progression.[21]

As expected, the time course of involution showed a progressive trend toward a decrease in number of ductules per lobule, an increase in leukocyte infiltration (including mast cells), an increase in adipocyte size as the cells become relipidated, and an increase in collagen deposition. Mast cells showed a trend toward increasing over time, but large variability in abundance per lobular area between mice precluded detecting differences between groups. The mouse mammary gland in general seems volatile in terms of mast cell recruitment, with mast cells being rapidly mobilized in great numbers within 3 d, in response to brown adipocyte necrosis induced by dietary conjugated linoleic acid.[41] In contrast, our previous studies with rat mammary glands during involution (pups were weaned after 21 d of lactation) showed a more moderate mast cell influx, with mast cell numbers per lobular ductule peaking at day 2 of involution and remaining stable thereafter.[38]

Ethanol did not influence the trend toward increasing adipocyte diameter during involution. The adipocytes remain in place during lactogenesis in a lipid-depleted state and rapidly relipidate during involution.[27] In addition, we noted a previously undescribed effect of involution alone on serum lipids and some oxidized species, suggesting that involution is associated with systemic changes in lipid metabolism; alterations in postweaning metabolism (but not lipids) have been described previously in mice.[24] However, none of these indices of inflammation were significantly affected by ethanol feeding.

The lack of effect of ethanol in these studies is not due to inadequate dosing. The diets used in the current study represent 2.85%, 5.7%, or 11.4% of total calories (corresponding to 0.5%, 1%, and 2% ethanol diets, respectively), which was sufficient (at the 1% and 2% levels) to cause vacuoles, which may represent lipid accumulation, in hepatocytes. In comparison, in other mouse studies of ethanol exposure, ethanol represented a range of 6% to 29% of calories.[46] Rat studies that specifically looked at long-term changes in mammary gland structure or susceptibility to chemical carcinogenesis used ethanol at 15% to 30% of caloric content of the diet but did not observe a linear dose response in terms of ethanol exposure and susceptibility to carcinogenesis.[47-50] In contrast, we previously found that the pubertal mouse mammary gland showed increased epithelial density in response to short-term ethanol feeding.[23] Alcohol consumption is similarly associated with increased proliferation in the pubertal rat mammary gland[50] and the human breast.[19,30,56]

In conclusion, the lack of effect of ethanol in these studies suggests that weaning, after natural lactation, represents a time point at which

the mouse mammary gland is refractory to the deleterious effects of ethanol. Ongoing studies will determine whether ethanol feeding for 14 d during weaning causes long-term changes in mammary tumor incidence or progression. Future studies will focus on defining the effects of forced weaning at day 10 of lactation, to determine whether forced weaning (compared with natural weaning) is a more sensitive time for chemopreventive intervention, to define a more appropriate animal model to mimic short-term breast feeding in humans.

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References

1. **American Cancer Society Website.** Internet. What are the key statistics about breast cancer? [Cited date 23 December 2012]. Available at: http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-key-statistics
2. **Allen NE, Beral V, Casabonne D, Kan SW, Reeves GK, Brown A, Green J, Million Women Study Collaborators.** 2009. Moderate alcohol intake and cancer incidence in women. J Natl Cancer Inst **101:**296–305.
3. **Andrechek ER, Hardy WR, Siegel PM, Rudnicki MA, Cardiff RD, Muller WJ.** 2000. Amplification of the *neu/erbB2* oncogene in a mouse model of mammary tumorigenesis. Proc Natl Acad Sci USA **97:**3444–3449.
4. **Atabai K, Sheppard D, Werb Z.** 2007. Roles of the innate immune system in mammary gland remodeling during involution. J Mammary Gland Biol Neoplasia **12:**37–45.
5. **Bills ND, Koury MJ, Clifford AJ, Dessypris EN.** 1992. Ineffective hematopoiesis in folate-deficient mice. Blood **79:**2273–2280.
6. **Boyd NF, Martin LJ, Yaffe MJ, Minkin S.** 2011. Mammographic density and breast cancer risk: current understanding and future prospects. Breast Cancer Res **13:**223–235.
7. **Cardiff RD, Wellings SR.** 1999. The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia **4:**105–122.
8. **Chen WY, Rosner B, Hankinson SE, Colditz GA, Willett WC.** 2011. Moderate alcohol consumption during adult life, drinking patterns, and breast cancer risk. J Am Med Assoc **306:**1884–1890.
9. **Chen Z, Wu AH, Gauderman WJ, Bernstein L, Ma HY, Pike MC, Ursin G.** 2004. Does mammographic density reflect ethnic differences in breast cancer incidence rates? Am J Epidemiol **159:**140–147.
10. **Clarkson RWE, Watson CJ.** 2003. Microarray analysis of the involution switch. J Mammary Gland Biol Neoplasia **8:**309–319.
11. **Collaborative Group on Hormonal Factors and Breast Cancer.** 2002. Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50,302 women with breast cancer and 96,973 women without the disease. Lancet **360:**187–195.
12. **Flanders KC, Wakefield LM.** 2009. Transforming growth factors β and mammary gland involution: Functional roles and implications for cancer progression. J Mammary Gland Biol Neoplasia **14:**131–144.
13. **Gullick WJ, Love SB, Wright C, Barnes DM, Gusterson B, Harris AL, Altman DG.** 1991. c-erbB2 protein overxpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer **63:**434–438.
14. **Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ.** 1992. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA **89:**10578–10582.
15. **Hilakivi-Clarke L, Cabanes A, de Assi S, Wang M, Khan G, Shoemaker WJ, Stevens RG.** 2004. In utero alcohol exposure increases mammary tumorigenesis in rats. Br J Cancer **90:**2225–2231.
16. **Jacoby RO, Fox JG, Davisson M.** 2002. Biology and diseases of mice, p 35–120. In: Fox JG, Anderson LC, Loew FM, Quimby FW. Laboratory animal medicine, 2nd ed. New York (NY): Elsevier.
17. **Jäger R, Herzer U, Schenkel J, Weiher H.** 1997. Overexpression of Bcl2 inhibits alveolar cell apoptosis during involution and accelerates c-*myc*-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene **15:**1787–1795.
18. **Lawrance CC, Lucas EA, Clark SL, Smith BJ, Kuvibidila S.** 2009. Differential effects of isoflurane and CO_2 inhalation on plasma levels of inflammatory markers associated with collagen-induced arthritis in DBA mice. Int Immunopharmacol **9:**807–809.
19. **Longnecker MP.** 1994. Alcoholic beverage consumption in relation to risk of breast cancer: meta-analysis and review. Cancer Causes Control **5:**73–82.
20. **Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Mrusyk A, Tan A-C, Schedin P.** 2011. Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX2. Nat Med **17:**1109–1115.
21. **Lyons TR, Schedin PJ, Borges VF.** 2009. Pregnancy and breast cancer: when they collide. J Mammary Gland Biol Neoplasia **14:**87–98.
22. **Masso-Welch PA, Darcy KM, Stangle-Castor NC, Ip MM.** 2000. A developmental atlas of rat mammary gland histology. J Mammary Gland Biol Neoplasia **5:**165–185.
23. **Masso-Welch PA, Tobias ME, Vasantha Kumar SC, Bodziak M, Mashtare T Jr, Tamburlin J, Koury ST.** 2012. Folate exacerbates the effects of ethanol on peripubertal mouse mammary gland development. Alcohol **46:**285–292.
24. **Mazurek S, Weisse G, Wust G, Schafer-Schwebel A, Eigenbrodt E, Friis RR.** 1999. Energy metabolism in the involuting mammary gland. In Vivo **13:**467–477.
25. **Monks J, Geske FJ, Lehman L, Fadok VA.** 2002. Do inflammatory cells participate in mammary gland involution? J Mammary Gland Biol Neoplasia **7:**163–176.
26. **Neuenschwander S, Schwartz A, Wood TL, Roberts CT Jr, Henninghausen L, LeRoith D.** 1996. Involution of the lactating mammary gland is inhibited by the IGF system in atransgenic mouse model. J Clin Invest **97:**2225–2232.
27. **Neville MC, Medina D, Monks J, Hovey RC.** 1998. The mammary fat pad. J Mammary Gland Biol Neoplasia **3:**109–116.
28. **Newberry RC, Swanson JC.** 2008. Implications of breaking mother–young social bonds. Appl Anim Behav Sci **110:**3–23.
29. **Nguyen AV, Pollard JW.** 2000. Transforming growth factor β3 induces cell death during the first stage of mammary gland involution. Development **127:**3107–3118.
30. **Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW.** 2009. Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell compartmentalization. Cancer Res **69:**5251–5258.
31. **O'Brien J, Schedin P.** 2009. Macrophages in breast cancer: do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer? J Mammary Gland Biol Neoplasia **14:**145–157.
32. **Palmer JR, Boggs DA, Wise LA, Ambrosone CB, Adams-Campbell LL, Rosenberg L.** 2011. Parity and lactation in relation to estrogen receptor negative breast cancer in African–American women. Cancer Epidemiol Biomarkers Prev **20:**1883–1891.
33. **Paterson MC, Dietrich KD, Danyluk J, Paterson AH, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA, Slamon DJ, Fourney RM .** 1991. Correlation between c-*erbB2* amplification and risk of recurrent disease in node-negative cancer. Cancer Res **51:**556–567.
34. **Pattengale PK, Stewart TA, Leder A, Sinn E, Muller W, Tepler I, Schmidt E, Leder P.** 1989. Animal models of human disease: pathology and molecular biology of spontaneous neoplasms occurring in transgenic mice carrying and expressing activated cellular oncogenes. Am J Pathol **135:**39–61.
35. **Polanco TA, Crismate-Gann C, Peuhl KR, Sarkar DK, Cohick WS.** 2010. Fetal alcohol exposure increase mammary tumor sus-

ceptibility and alters tumor phenotype in rats. Alcohol Clin Exp Res **34:**1879–1887.

36. **Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowicz M, Danyluk J, Godolphin W, Sliwkowski M, Akita R, Paterson MC, Slamon DJ.** 1993. Her2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res **53:**4960–4970.
37. **Radisky DC, Hartmann LC.** 2009. Mammary gland involution and breast cancer risk: transgenic models and clinical studies. J Mammary Gland Biol Neoplasia **14:**181–191.
38. **Ramirez RA, Lee A, Schedin P, Russell JS, Masso-Welch PA.** 2012. Alterations in mast cell frequency and relationship to angiogenesis in the rat mammary gland during windows of physiologic tissue remodeling. Dev Dyn **241:**890–900.
39. **Richert MM, Schwertfeger KL, Ryder JW, Anderson SM.** 2000. An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia **5:**227–241.
40. **Rudolph MC, McManaman JL, Hunter L, Phang T, Neville MC.** 2003. Functional development of the mammary gland: use of expression profiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation, and involution. J Mammary Gland Biol Neoplasia **8:**287–307.
41. **Russell JS, Mcgee SO, Ip MM, Kuhlmann D, Masso-Welch PA.** 2007. Conjugated linoleic acid induces mast cell recruitment during mouse mammary gland stromal remodeling. J Nutr **137:**1200–1207.
42. **Sandgren EP, Schroeder JA, Qui TH, Palmiter RD, Brinster RL, Lee DC.** 1995. Inhibition of mammary gland involution is associated with transforming growth factor α but not c-myc-induced tumorigenesis in transgenic mice. Cancer Res **55:**3915–3927.
43. **Schedin P.** 2006. Pregnancy-associated breast cancer and metastasis. Nat Rev Cancer **6:**281–291.
44. **Schwertfeger KL, Richert MM, Anderson SM.** 2001. Mammary gland involution is delayed by activated akt in transgenic mice. Mol Endocrinol **15:**867–881.
45. **Seitz HK, Pelucchi C, Bagnardi V, LaVecchia C.** 2012. Epidemiology and pathophysiology of alcohol-related cancer: update 2012. Alcohol Alcohol **47:**204–212.
46. **Singletary K.** 1997. Ethanol and experimental breast cancer: a review. Alcohol Clin Exp Res **21:**334–339.
47. **Singletary K, Nelshoppen J, Wallig M.** 1995. Enhancement by chronic ethanol intake of N-methyl-N-nitrosourea–induced rat mammary tumorigenesis. Carcinogenesis **16:**959–964.
48. **Singletary KW, McNary MQ.** 1992. Effect of moderate ethanol consumption on mammary gland structural development and DNA synthesis in the female rat. Alcohol **9:**95–101.
49. **Singletary KW, McNary MQ.** 1994. Influence of ethanol intake on mammary gland morphology and cell proliferation in normal and carcinogen-treated rats. Alcohol Clin Exp Res **18:**1261–1266.
50. **Singletary KW, McNary MQ, Odoms AM, Nelshoppen J, Wallig MA.** 1991. Ethanol consumption and DMBA-induced mammary carcinogenesis in rats. Nutr Cancer **16:**13–23.
51. **Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL.** 1987. Human breast cancer: correlation of relapse and survival with amplification of the *HER2/neu* oncogene. Science **235:**177–182.
52. **Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF**. 1989. Studies of the *Her2/neu* protooncogene in human breast and ovarian cancer. Science **244:**707–712.
53. **Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy M-A, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA.** 2004. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14, and STAT3. Breast Cancer Res **6:**R75–R91.
54. **Stein T, Salomonis N, Gusterson BA.** 2007. Mammary gland involution as a multistep process. J Mammary Gland Biol Neoplasia **12:**25–35.
55. **Sutherland KD, Lindeman GJ, Visvander JE.** 2007. The molecular culprits underlying precocious mammary gland involution. J Mammary Gland Biol Neoplasia **12:**15–23.
56. **Tao MH, Marian C, Shields PG, Nie J, McCann SE, Millen A, Ambrosone C, Hutson A, Edge SB, Krishnan SS, Xie B, Winston J, Vito D, Russell M, Nochajski TH, Trevisian M, Freudenheim JL.** 2011. Alcohol consumption in relation to aberrant DNA methylation in breast tumors. Alcohol **45:**689–699.
57. **Tzeng YJ, Gottlob K, Santarelli R, Graessmann A.** 1996. The SV40 T antigen induces premature apoptotic mammary gland involution during late pregnancy in transgenic mice. FEBS Lett **380:**215–218.
58. **Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ.** 2007. Insights from transgenic mouse models of ERBB2-induced breast cancer. Nat Rev Cancer **7:**389–397.
59. **World Cancer Research Fund; American Institute for Cancer Research.** 2007. Cancers. In: Food, nutrition, physical activity and the prevention of cancer: a global perspective. Washington (DC): American Institute for Cancer Research.