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Psychosocial Stress Exposure Disrupts Mammary Gland Development

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Abstract

Exposure to psychosocial stressors and ensuing stress physiology have been associated with spontaneous invasive mammary tumors in the Sprague-Dawley rat model of human breast cancer. Mammary gland (MG) development is a time when physiologic and environmental exposures influence breast cancer risk. However, the effect of psychosocial stress exposure on MG development remains unknown. Here, in the first comprehensive longitudinal study of MG development in nulliparous female rats (from puberty through young adulthood; 8–25 wks of age), we quantify the spatial gradient of differentiation within the MG of socially stressed (isolated) and control (grouped) rats. We then demonstrate that social isolation increased stress reactivity to everyday stressors, resulting in downregulation of glucocorticoid receptor (GR) expression in the MG epithelium. Surprisingly, given that chemical carcinogens increase MG cancer risk by preventing normal terminal end bud (TEB) differentiation, chronic isolation stress did not alter TEBs. Instead, isolation blunted MG growth and alveolobular differentiation and reduced epithelial cell proliferation in these structures. Social isolation also enhanced corpora luteal progesterone at all ages but reduced estrogenization only in early adulthood, a pattern that precludes modulated ovarian function as a sufficient mechanism for the effects of isolation on MG development. This longitudinal study of natural variation provides an integrated view of MG development and the importance of increased GR activation in nulliparous ductal growth and alveolobular differentiation. Thus, social isolation and its physiological sequelae disrupt MG growth and differentiation and suggest a contribution of stress exposure during puberty and young

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COMPLIANCE WITH ETHICAL STANDARDS

National Institutes of Health and University of Chicago Animal Care Guidelines were followed for the use of animals in all studies.

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adulthood to the previously observed increase in invasive MG cancer observed in chronically socially-isolated adult Sprague-Dawley rats.

Keywords

mammary gland; puberty; young adulthood; stress; terminal end buds; alveolobular development

INTRODUCTION

Social isolation is a psychosocial stressor for rats, a social species. Exposure to social isolation beginning in puberty is associated with earlier incidence of large malignant mammary tumors in Sprague-Dawley rats [1]. Because mammary cancer risk is increased when the mammary gland (MG) fails to fully differentiate [2–5], we hypothesized that rats exposed to social isolation during MG development would have less differentiated MGs. Indeed, isolated rats have less developed nipples than their grouped counterparts, an external indicator of delayed MG development [6, 7].

Sexual differentiation and growth of the MG begins as the ovary matures between four and eight weeks of age [8]. Ductal extension is led by the penetration of terminal end buds (TEBs) into the mammary fat pad [9–11]. TEBs differentiating into alveolar buds (ABs) between 6 and 8 weeks of age are more susceptible to DNA-damage from carcinogens than other ductal structures, and thus have been the primary focus of studies of environmental causes of mammary cancer [12–14]. Exposure to carcinogens sustains TEB number, enlarges them and induces malignant transformation of the ductal epithelium likely through impaired DNA-repair and/or epigenetic alterations [15, 16]. Moreover, young adult women exposed to carcinogens or radiation when they have terminal ductal lobular units (homologous to TEBs), are at significantly higher risk of developing breast cancer later in life [4, 17]. Therefore, we hypothesized that chronic social isolation, a psychosocial stressor, during TEB transformation might also have sustained TEB number by 8 weeks of age relative to their group housed counter parts and thereby increase mammary cancer risk.

In addition, rodent experiments and human epidemiological studies indicate that reducing the later stages of MG differentiation also increases breast cancer risk [3, 18–22]. As the ducts reach the end of the mammary fat pad, the TEBs cavitate, creating terminal ducts (TDs). Some of the ducts differentiate into alveolar buds (AB) and then lobules (L), which displace the mammary fat and take up progressively more space in the developing MG [23]. While ductal extension is complete in 25-week old rats [24, 25], its time course has not been established nor has that of mammary tree differentiation, although nipples do continue to enlarge from 16 to 17 wks of age [6]. Rather, the literature focuses primarily on early puberty (6–10 wks; [22, 25–32]).

Alveolobular differentiation is dynamic and asynchronous, with only a subset of the mammary tree differentiating during any given ovarian cycle [8, 33]. Thereby, it is possible that exposure to repeated ovarian cycles will continue to drive MG differentiation well into adulthood. Differentiation is at its greatest during the luteal phase of the estrous cycle when luteal progesterone is high followed by regression during estrus [31, 34]. Estrous-cycle

dependent MG proliferation is still ongoing in 36 wk old virgins [27]. Final differentiation occurs with full-term pregnancy and lactation. A MG that remains relatively undifferentiated is at greater risk for developing most types of breast cancer [3, 18–20], and it is before the first pregnancy, during puberty and young adulthood, that women are the most vulnerable to environmental exposures [4]. Indeed, women in the US and world-wide are increasing the delay in childbearing, lengthening this sensitive period [35]. Therefore, we hypothesized that social isolation might also limit the degree of ductal extension and alveolobular differentiation that occurs not only in early puberty, but throughout young adulthood.

MG development is under the control of ovarian steroids [36, 37], which may mediate the effect of social isolation on MG development. Estrogen is the primary driver of ductal extension and its proliferative effects are at least in part mediated by paracrine activity via adipocytes and other MG cells [38]. Estrogen signaling also upregulates progesterone receptor (PR) expression in the developing MG, enabling the mammary epithelial cell response to circulating progesterone [39]. Progesterone signaling occurs through both direct and paracrine mechanisms and is involved in ductal branching and alveolobular development (reviewed in [40]). Variation in estrogen and progesterone throughout the ovarian cycle results in alternating proliferation (driven by the peaking of these hormones during the luteal phase) and apoptosis (caused by their nadir during follicular phase) [31, 34], suggesting that less differentiation in the isolates may result from delayed ovarian function. However, both socially isolated rats and women undergoing psychosocial stress have accelerated ovarian development in early puberty, which could accelerate, not delay MG development [6, 41–43]. We evaluated the effects of social isolation on ovarian function from early puberty throughout late puberty and early adulthood to determine whether ovarian function could mediate the effects of isolation on the developing MG.

Social isolation is a mild unremitting psychosocial stressor that increases anxiety and induces a hypervigilant state [1]. Rats exposed to social isolation acquire heightened glucocorticoid (GC) reactivity to everyday stressors compared to grouped counterparts, who have a social buffer ameliorating their stress response [1, 44]. Our lab and others have demonstrated that GCs inhibit estrogen receptor-induced proliferation in the mammary epithelium [45, 46]. Indeed, administering GC decreases the total volume of mammary epithelium in a dose dependent manner [47]. Although the rat model of socially isolated housing is not directly applicable to humans, it is a model for psychosocial situations that heighten GC reactivity, particularly to everyday stressors. Dysphoric adolescent women and young adults exhibit similar heightened GC reactivity if they have limited social supports, low self-esteem, and/or low social economic status [48–51]. Similar to our experimental model of social isolation, women exposed to these unremitting stressors have a higher risk of developing aggressive breast cancers, such as the triple-negative subtype [52–54]. Using a longitudinal study of natural MG development to identify endocrine mechanisms, by which social isolation affects MG development and in turn increases breast cancer risk, will provide direction for cellular studies and underscore the importance of prevention strategies during late puberty and early adulthood, an understudied developmental period in both rodents and humans.

In this longitudinal study of natural development, we determined the effect of social isolation on rat MG development by examining MG architecture and histology at four week intervals throughout puberty and early adulthood. Morphology of the MG including ductal extension and structures (ducts, TEBs, ABs and Ls) were assessed using whole mounts. We also used histologic measurements to quantify the degree of alveolobular development after ductal extension and the composition of the MG. Suppressed development was further quantified with Ki67 staining to assess the level of epithelial cell proliferation in each structure of the developing mammary gland.

Serum GC levels before, during, and after an acute restraint stressor were also measured to assess GC reactivity to daily stressors associated with routine animal husbandry [1, 56], along with behavioral assessment of hypervigilance and evidence of adrenal fatigue. A non-invasive ovarian hormone bioassay (vaginal cytology) was used to determine relative systemic exposure to estrogen and progesterone throughout MG development [55]. GR, ER and PR expression in the MGs of isolated and grouped rats were assessed with immunohistochemical staining.

MATERIALS AND METHODS

Sprague-Dawley Rats

Female Sprague-Dawley IGS rats were bred at Charles River Laboratories, Inc. (Portage, MI and Kingston, NY), weaned into all-female groups at three weeks of age and shipped in a series of cohorts to our laboratory at four weeks of age. Upon arrival, they were housed in groups, and at 4.5 weeks of age transferred to either single or uncrowded grouped housing of five rats, balanced for vigilant temperament as measured during an exploration stressor (described below;[57]). Uncrowded caging provided single and grouped rats the same area per animal ($20 \times 24 \times 18$ cm and $46 \times 61 \times 36$ cm). All were housed in solid stainless steel cages hung on shared racks in the same colony room ($21 \pm 1^\circ\text{C}$), with wire covering the front and floor over shared bedding (Harlan Teklad Aspen Sani-Chips, 7090A) pans. Thereby, rats could see, hear and smell each other, but could interact socially only in the group housing cages. Rats were housed under a reversed L:D cycle (14:10, dark onset 08:00 h) so that all experimental manipulations were performed during rats' behavioral day. Food (Harlan Teklad Rodent Diet, 8604) and water were provided *ad libitum*. Rats were routinely handled during cage changes and health inspections. National Institutes of Health and University of Chicago Animal Care Guidelines were followed for all studies.

Experimental Design

MGs were harvested during puberty (8 and 13 weeks of age) and early adulthood (17, 21 and 25 weeks of age). Ovarian cyclicity was quantified in the two weeks prior to sacrifice and behavioral stress reactivity one week prior. Rats were sacrificed in proestrus/estrus to minimize ovarian cycle variability in mammary morphology [31, 58]. All ovarian and stress measures were standardized at 5–6 hours into their behavioral day, when GC rhythm is at its nadir. The total ovarian weight and total adrenal weight were adjusted for body weight (mg/100g body weight).

MG Whole Mount Preparation and Analyses

Upon sacrifice, the right inguinal MG quadrant was removed, spread onto a large microscope slide (2"x3" or 3"x4") and fixed with Carnoy's fixative. The MG was then stained with carmine alum and the lipid removed using xylene [59]. An image of the cover slipped MG whole mount slide was created with a 3600 dpi flatbed scanner (optimal for double blind anatomical measurements on the digital images with ImageJ software).

Ductal extension was measured from the top of the lymph node (LN) to the most distal ductal structure (TEB or TD) at the leading edge of the MG ductal tree (Fig. 1a). Because we measured ductal extension in a MG whole mount, we standardized gland size to body size. Although there was a range in body weight among all rats, there were no significant differences in body weight between rats in the two social conditions. Distal MG ductal area was the area between a horizontal line transecting the LN and the traced perimeter of the mammary whole mount (zones B and C; [25]). A minimum of 9 rats in each social condition were used for statistical comparison of MG ductal extension and ductal area.

Spatial differences in MG development were measured within four equal sectors of distal ductal area of the MG: adjacent to the nipple (S1), middle of MG along the dorsal fat ridge (S2), middle of MG thin ventral fat pad (S3), and at the distal end, furthest from the nipple (S4). The prevalence of all MG ductal structures, including TEBs ($103 \pm 16.7 \mu\text{m}$), TDs, alveolar buds (AB), and lobules, were quantified in the outer 1 mm annulus (perimeter) of each section [10, 33]. As a context for interpreting these results, we made similar MG whole mount measurement of 84 wk old nulliparous rats.

MG Ductal Structures (TEB, TD, AB and Lobules), Fat, and Stroma

The development of ducts, alveolobular units, stroma and adipose tissue was quantified in H&E stained tissue from the left inguinal MG, standardized immediately rostral to the LN (in Zone B, [25]). The tissue was fixed, paraffin embedded, sectioned, and H&E stained. We performed whole slide scanning (CRi Panoramic Scan Whole Slide Scanner). The slide was parsed into 1000×1000 pixel images. The tissue structures on each parsed image were digitally separated using color deconvolution to measure the different MG components [60]. This approach enables multiple measures per individual, reducing the number of rats needed to statistically compare MG structures.

Pink (eosin) stained tissue is representative of stroma and blood vessels, purple (hematoxylin) stained tissue is ductal structures (DS) and unstained white is adipose tissue. Only ductal structure-associated stroma was included in the analysis. Excluded stroma included muscle and other non-adipocyte stroma that did not directly support the growing ductal structures.

MG Epithelial Cell Proliferation

Epithelial cell proliferation was quantified with an anti-Ki67 protein stain (ThermoScientific, Cat#RM-9106-s, Clone: SP6, Rabbit monoclonal antibody), which in the rat MG correlates highly with other markers of cell proliferation (BrdU and PCNA; [27]). Slices adjacent to those for H&E staining of MG structures were stained on Leica Bond RX

Automatic Stainer using the protocol “Envision x rabbit Long”. After antigen retrieval treatment (epitope retrieval solution I, AR9961, Leica Biosystems) for 20 minutes, anti-Ki67 antibody (1:200) was applied on tissue sections for 60-minute incubation and the antigen-antibody binding was detected with Envision+ anti-rabbit HRP (DAKO, K4003). The tissue sections were counterstained with hematoxylin and coverslipped.

Mammary epithelial structures were identified, and the adjacent lymph node served as a positive control for each slide. Cellular proliferation was measured as a percentage of Ki67 protein marker stain. Across each slide, the fraction of Ki67 positive cells was determined separately for MG ducts, TEBs, alveolar buds (AB; < 6 acini) and lobules (L; > 8 acini) [24, 25, 28, 31, 33] by the pathologist (R.L.), blind to age and housing condition. Note that terminal ducts are rare in Zone B, and cannot be distinguished in cross section as they can be in a whole mount [15].

Steroid Receptors

Antibodies for GC, estrogen, and progesterone nuclear receptors (GR, ER-alpha, and PR) were: GR-XP (Cell Signaling 3660), ER-alpha (Novocastra clone 6F11), and PR (ThermoFisher RM-9102). Following standard protocols, the slides were stained for GR and ER using the Leica Bond RX automatic stainer. Epitope retrieval solution II (Leica Biosystems, AR9640) was used for 20 minutes. Anti-GR antibody (1:1500) was applied for 25 minutes and anti-ERalpha antibody (1:160) was applied for one hour. Antigen-antibody binding was detected with Bond polymer refine detection (Leica Biosystems, DS9800). For PR, the tissue sections were deparaffinized and rehydrated using xylene and serial dilutions of EtOH to distilled water. They were incubated in antigen retrieval buffer (DAKO, S1699) and heated in a steamer over 97°C for 20 minutes. Anti-PR (1:50) antibody was applied on tissue section for one hour at room temperature in a humidity chamber. Following TBS washing, the antigen-antibody binding was detected with Envision+ anti-rabbit system (DAKO K4003) and DAB+ chromogen (DAKO, K3468). For all three stains, tissue sections were briefly immersed in hematoxylin for nuclear counterstaining and were coverslipped.

A pathologist (R.L.) estimated the % ER+ and %PR+, blind to social and age conditions, and found no differences in either variable. His estimates were successfully validated by scanning slides from 21 weeks of age (CRi Panoramic Scan Whole Slide Scanner) and scoring the %ER+ and %PR+ in the luminal and basal ductal epithelial cells of five grouped and five isolated rats. In contrast, blind estimates of %GR+ revealed lower levels in isolates at 21 and 25 weeks of age, a difference also validated by scoring the scanned slides from 21 weeks of age.

Corticosterone Stress Reactivity

Serum concentrations of baseline, reactive and recovery corticosterone (in response to a mild stressor) were performed in a subset of 17-week-old rats (n=9) using a mild restraint test (restraint tube, Harvard Apparatus)[1]. One handler removed rats, one at a time, from the housing room, alternating housing conditions, and carried them into an adjacent procedure room where two handlers performed the restraint stress procedure. Baseline blood samples were obtained by tail nick and subsequent samples collected by gently disturbing the clot.

Blood was collected into microcentrifuge tubes at baseline, after 30 min (16:30 h) of imposed stress and during recovery, 1 (17:00 h) and 2 h (18:00h). In between sample collection, animals were placed into clean recovery cages in a room adjacent to the procedure room. Samples were kept on ice during blood collection and stored overnight in at 4°C until centrifuge and serum extraction the next day. Serum samples were frozen at -30°C until corticosterone ELISA (IBL America B79175) assay. Samples were run in duplicate on the same assay (intra-assay coefficient of variance 9.4%; inter-assay variance 8% [1, 61]). Serum corticosterone reactivity and recovery was standardized to baseline levels to control for individual differences in baseline corticosterone and slight variation in time of day of sample collection.

Behavioral Response to an Exploration Stressor

Exploration in an unfamiliar environment is a classic stressor for rodents. We measured stress behavior twice during the lifespan with an exploration stressor at 4.5 weeks (3'x3') and again 2 weeks before sacrifice (4'x4') [1, 62]. The exploration arena was made of opaque Plexiglas walls, with a home base enclosure in a corner scented with familiar odors (handful of soiled bedding from the subject rat's bedding pan) and, in the center, a small overturned ceramic bowl. The rat was brought from its home cage in the adjacent colony room and gently placed into the home base. Vigilance was measured as the latency to emerge from home base within five minutes. The same handler was used throughout, and the open field base was wiped down with Cavi-wipes between animals to remove stress odors.

Ovarian Steroid Exposure

Estrogen and progesterone exposure during successive ovarian cycles was assessed with daily vaginal saline lavage, a well-established bioassay of ovarian function enabling noninvasive repeated measures of cycles [55]. Samples were collected and analyzed at 2 to 5 hours into the behavioral day. We quantified the changing proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes as reliable indicators of ovarian cycle phase [55]. Duration of estrogen exposure was quantified by the percent of the 2-week epoch with only nucleated or cornified vaginal epithelial cells, a well-established bioassay of estrogenization [55]. Luteal progesterone exposure was estimated by the percent of epochs with primarily leukocytes during metestrus.

Statistical Analysis

Statistical analyses were conducted with *R* (NS = not significant ($P > 0.05$, two-tailed tests), all means $\pm$ SEM)[63]. Arcsine transformed arcsine [sqrt (p)] percentages met parametric distribution requirements. To account for genetic or significant variation among this outbred nonsyngeneic Charles River rat strain, we included cohort as a variable in the multivariate analyses.

RESULTS

Mammary Ductal Extension rather than TEB Number is Altered by Chronic Social Isolation

Terminal end buds.—Social isolation had not altered TEB number in the mammary tree perimeter by 8 weeks of age, when carcinogens exert their malignant effects [15] (Figs. 1a

and b; 27 ± 7 TEBs in grouped versus 28 ± 7 TEBs in isolated rats, $P = 0.68$, Student's t -test). By this age, the TEBs have led the mammary ductal tree from the nipple, past the lymph node (LN) and into the mammary fat pad, beginning to wrap around the flanks of the rat (Supp. Fig. 1). By 13 weeks of age, TEB number had dropped precipitously at the leading edge of the ductal tree in both social conditions, and remained low as the ductal tree continued to extend. Social isolation had no effect on TEB number at any age (social condition: $F(1, 71) = 0.2$, $P = 0.68$, age: $F(3, 71) = 127.6$, $P < 0.0001$, interaction: $F(1, 71) = 0.9$, $P = 0.46$).

Social isolation also did not disrupt the average size of TEBs at 8 weeks of age, when they are most abundant ($107.9 \pm 1.2 \mu\text{m}$ in grouped versus $106.2 \pm 1.5 \mu\text{m}$ in isolated rats, $P = 0.366$). Because TEB size is correlated with its proliferative index [11], this finding indicates a similar TEB proliferative index in the two housing conditions. Moreover, TEB density was not increased in the isolated rats (10 ± 1 TEBs per mm^2 in both grouped and isolated rats. $P = 0.70$, Student's t -test).

Ductal extension.—Ductal extension continued throughout puberty and young adulthood, reaching a maximum at 17 weeks of age (Fig. 1c; Supp. Fig. 1). Throughout late puberty and young adulthood, ductal extension in social isolates was significantly shorter than in rats living in groups (Fig. 1c, Supp. Fig. 1; age: $F(3, 72) = 9.96$, $P < 0.0001$, social condition: $F(1, 72) = 7.1$, $P = 0.01$, interaction: $F(3, 72) = 0.6$, $P = 0.6$, cohort $F(1, 72) = 17.7$, $P < 0.0001$).

MG Distal Ductal Area.—As ductal extension increased throughout puberty and young adulthood (until 17 weeks), the ductal tree expanded (distal ductal area) in both social conditions (Fig. 1d; Supp. Fig. 1). Social isolates had significantly smaller distal ductal area compared to grouped rats at all ages (8–21 weeks; social condition: $F(1, 90) = 4.9$, $P = 0.03$, age: $F(3, 90) = 127.8$, $P < 0.0001$, interaction: $F(3, 100) = 1.2$, $P = 0.30$). The observed decrease in ductal extension and distal ductal area in social isolates did not manifest until 13–17 weeks (see SEMs in Fig. 1c–d).

Spatiotemporal Differences in Ductal Structures

Removing fat from whole mounts revealed structural details of mammary tree differentiation. Ductal differentiation into alveolobular units had begun close to the nipple (Sector 1 (S1)) as early as 8 weeks of age when TEBs were still extending through the distal fat pad (Sector 4 (S4), Fig. 1a, Fig. 2a; Supp. Fig. 1). Therefore, the proximal portion of the mammary ductal tree (S1) had more time to differentiate than did the distal portion (S4). This created a spatial gradient of differentiation, with the most differentiated MG in S1 near the nipple, followed by adjacent S2, S3 opposite the nipple and finally S4, the most distal sector (Fig. 2a). During 13–25 weeks of age, differentiation progressed in all sectors. Nonetheless, in the most distal sector (S4), most rats still had predominantly undifferentiated TDs in the perimeter, either accompanied by TEBs, with the capacity for continued development, or ABs; only 10% had predominantly ABs, and none developed lobules. Close to the nipple, 90% of rats had TDs with ABs and 10% had lobules. The middle sectors were intermediate in differentiation, sustaining the spatial gradient despite all being exposed to

the same hormonal environment. Indeed, growth and differentiation in these nulliparous animals continued through 84 weeks of age, retaining the spatial gradient of predominantly undifferentiated TDs with TEBs (S1 0% to S4 20% of rats) versus lobules as the primary structures (S1 50% to S4 33% of rats).

Overall, location in the MG (S1-S4) had a bigger effect on variation in MG differentiation than did chronological age. Moreover, the effect of age was different in the various sectors. In the least mature sector (S4), development was characterized by the loss of TEBs as the primary or secondary structures, whereas in the most mature sector (S1), ABs and lobules were surpassed by TDs as the most prevalent structures. (Fig. 2. MG Sector $F(3, 337) = 6.0$, $P = 0.00001$, age: $F(4, 113) = 1.4$, $P = 0.17$, interaction: $F(12, 337) = 2.7$, $P = 0.002$). To standardize MG tissue collection for analysis of MG composition, a strip was taken at the intersection of the four sectors, just distal to the LN.

Chronic Social Isolation Alters Central MG Composition: Ducts, Stroma, and Fat

Ductal Differentiation.—Analysis of H&E stained central MG tissue, just rostral to the LN, was consistent with the developmental pattern seen in peripheral ductal structures of MG whole mounts. After maximal ductal extension (17 weeks), the proportion of the MG consisting of ductal structures (ducts, TDs, alveoli and lobules; see Fig. 3) continued to increase through 25 weeks, reflecting continued arborization and differentiation of the MG tree. However, socially isolated rats had significantly smaller and less elaborated ductal structures (e.g. fewer alveoli and lobules) compared to their grouped counterparts (Fig. 3a–c; social condition: $F(1, 23) = 5.3$, $P = 0.03$, age: $F(2, 23) = 5.6$, $P = 0.01$, interaction: $F(2, 24) = 1.2$, $P = 0.10$). In isolated rats, alveolobular maturation stopped by 21 weeks, whereas it continued through 25 weeks in the grouped rats, evidencing stunted maturation of ductal structures in isolated rats.

Stroma.—Stroma continued to develop only in group housed rats (17–25 weeks), not in the isolates (Fig. 3c, social condition: $F(1, 23) = 4.0$, $P = 0.06$, age: $F(2, 23) = 3.8$, $P = 0.04$, interaction: $F(2, 23) = 4.0$, $P = 0.03$). Overall, more surrounding stroma was associated with larger ductal structures in both grouped and isolated rats (Supp. Fig. 3, $R = 0.70$, $P < 0.001$), consistent with previous reports that stromal growth follows ductal structure differentiation and its invasion into the fat pad [64, 65]. The stunted stromal development in isolates indicates impairment of this process.

Fat.—In isolated rats, the proportion of MG adipose tissue was sustained through 17–25 weeks of age, whereas it decreased in grouped rats, particularly in early adulthood (Fig. 3c, social condition: $F(1, 23) = 5.6$, $P = 0.03$, age: $F(2, 23) = 5.4$, $P = 0.01$, interaction: $F(2, 24) = 4.5$, $P = 0.02$). In grouped rats, the fat occupied a smaller proportion of the MG as it was displaced by the expanding ductal and stromal structures. Therefore, the fat to duct ratio was much higher in the isolates as fat was maintained with only a minimal expansion of ducts or stroma. Thereby, in the isolates, fully 80% of the MG was comprised of fat throughout early adulthood, versus 65% in grouped rats.

Epithelial Cell Proliferation Is Reduced in MG Structures of Isolated Young Adult Rats

Relative proliferation assessed by Ki67 expression was greater in alveolar buds (ABs) and lobules (Ls) than in ducts (ducts = 5%, AB = 10% and L = 10% Ki67 staining; $F(2,27) = 26.3$, $P = 0.0000$). While TEBs were rare near the lymph node of the MG, 80% of TEB cells were proliferative as expected [33]. Both luminal epithelial and myoepithelial cells stained positive for Ki67. Because ductal structures (TEBs, ducts, ABs and lobules) proliferate asynchronously with each successive ovarian cycle, there was significant variation in staining level within each type of MG structure (see Fig. 4a). Nonetheless, by 21 and 25 weeks, isolates had significantly less MG epithelial cell proliferation than rats in social groups, especially in ABs and Ls (Fig. 4b; interaction between housing and MG structure $F(2,27) = 4.7$, $P = 0.02$; housing $F(1,27) = 3.9$, $P = 0.07$). Ki67 staining was decreased in both luminal epithelial and myoepithelial cells. Age during young adulthood did not affect epithelial cell proliferation (neither as a main effect nor in interaction with housing or structure; all F s < 0.9 , all P s > 0.42).

Increased Vigilance and Glucocorticoid Stress Reactivity in Chronically Isolated Rats

During the stressor of exploring a novel environment, isolated rats, compared to those in social groups, exhibited greater vigilance throughout puberty and early adulthood. When placed in the home base of a novel exploration arena, isolated rats remained for prolonged times (Fig. 5a; 13–25 weeks of age, latency to emerge = 128 ± 17 secs vs 55 ± 10 secs, Log rank MantleCox test $X^2 = 14.18$ $P < 0.001$). Longer latency to emerge is indicative of greater vigilance, a behavioral trait that is associated with increased GC reactivity and also with mammary cancer risk among rats living in groups as well as social isolation [6, 44, 57, 66].

We next determined whether isolates indeed had more robust GC stress reactivity by imposing a restraint test on isolated and grouped rats at 17 weeks of age. After the stressor ended, the serum corticosterone levels of isolated rats continued to rise while the grouped rats immediately began to recover (Fig. 5b; 127.8 ± 32.5 ng/mL versus 44.3 ± 16.7 ng/mL, $P = 0.05$, Student's t -test). Total exposure to corticosterone following the restraint stress challenge was twice as high in isolated versus grouped rats (8351.1 ± 1108.7 ng/mL/90 min versus 4118.3 ± 1585.0 ng/mL/120 min, $P = 0.08$, Student's t -test).

Finally, we looked for evidence that exposure to repeated everyday stressors produced adrenal fatigue [67]. Throughout 13–25 weeks, the isolated rats had lower total adrenal gland weights (mg/100g body weight), which is consistent with adrenal fatigue (Fig. 5c; social condition: $F(1, 59) = 6.88$, $P = 0.01$, age: $F(3, 59) = 3.91$, $P = 0.01$, interaction: $F(3,59) = 0.72$, $P = 0.54$, cohort $F(4, 59) = 0.36$, $P = 0.84$). Moreover, isolated rats had lower baseline corticosterone than their grouped counterparts (32.5 ± 8.5 ng/mL versus 65.3 ± 5.8 ng/mL, $P = 0.0006$, Student's t -test) consistent with adrenal fatigue from repeated stressors [58].

Ovarian Steroid Exposure is Altered by Chronic Social Isolation

Estrogenization.—At 13 and 17 weeks of age, isolation was not associated with lower estrogenization (Fig. 6a; social condition: $F(1, 44) = 0.7$, $P = 0.40$, age: $F(1, 44) = 1.3$, $P = 0.27$, interaction: $F(1, 44) = 1.0$, $P = 0.33$). Estrogenization is quantified by nucleated and

cornified epithelial cells in vaginal cytology without leukocytes [55]. Nonetheless, they did have lower estrogenization in early adulthood when isolated rats had detectable stunted alveolobular and stromal differentiation (Fig. 6a, 21 and 25 weeks of age; social condition $F(1, 26) = 4.91$ $P = 0.04$, age: $F(1, 26) = 0.03$ $P = 0.87$, interaction: $F(1, 26) = 1.5$ $P = 0.24$). Moreover, the strong positive association between estrogenization and proliferation (Ki67) seen in group housed rats ($r = 0.78$, $P = 0.008$) was not found among isolates at 21 and 25 weeks ($r = 0.37$, $P = 0.30$).

Luteal Progesterone Exposure.—Between 13 and 25 weeks of age, isolated rats sustained more mature ovarian cycles than grouped rats and were more likely to be exposed to corpora luteal progesterone after ovulation (Fig. 6b; social condition: $F(1, 79) = 11.4$, $P = 0.001$, age: $F(3, 79) = 0.5$, $P = 0.65$, interaction: $F(3, 79) = 0.6$, $P = 0.59$). Luteal progesterone is indicated by predominance of leukocytes in vaginal cytology on estrus through diestrus [55].

Ovarian Weight.—The ovarian weights of grouped and isolated rats were not significantly different (Fig. 7c; mg/100g body weight; social condition: $F(1, 30) = 2.2$, $P = 0.15$, age: $F(1, 30) = 3.5$, $P = 0.07$, interaction: $F(1, 30) = 1.0$, $P = 0.32$, cohort $F(2, 30) = 4.1$, $P = 0.03$).

Expression of Glucocorticoid (but not Estrogen or Progesterone) Receptor becomes Downregulated in Mammary Epithelium of Chronically Isolated Rats

Glucocorticoid Receptor (GR).—Ductal cells (including both the luminal epithelial cells and the basal myoepithelial cells), stromal cells and mammary fat cells expressed GR throughout late puberty and early adulthood (13–25 weeks; Fig. 6c). Although isolates and groups had a similar developmental pattern in ductal GR expression, the isolate's developmental pattern was accelerated, creating differences by 21 weeks (Fig. 6c). Specifically, in grouped rats, GR was highly expressed in all ductal, alveolar and lobular epithelial cells until 25 weeks of age, whereupon GR expression dropped, becoming only heterogeneously positive in the developing alveoli and lobules. In contrast, isolates demonstrated this drop in GR expression intensity 4 weeks sooner, at 21 weeks (grouped $89.8\% \pm 2$ GR+ versus isolated $77.4\% \pm 3$ GR+, $P = 0.04$, Mann Whitney U). Thus, isolates had an accelerated drop in GR expression density while their MG architecture was delayed.

Estrogen Receptor (ER).—Ductal cells (including both the inner layer of luminal ductal cells and the outer myoepithelial cells) expressed ER- α throughout late puberty and early adulthood (13–25 weeks), with maximal expression at younger ages and less expression at older ages (Fig. 6a). ER was also expressed in mammary fat, and stroma. At 21 weeks, there was no significant difference in ER expression between rats of either social condition ($67.0\% \pm 2$ ER+ versus $63.3\% \pm 1$ ER+, $P = 0.69$, Mann Whitney U).

Progesterone Receptor (PR).—PR was primarily expressed in ductal tissue and only weakly expressed in stromal cells at the oldest age (25 weeks, Fig. 6b). PR was not observed in the adipose tissue. PR expression in the ductal cells was heterogeneous throughout development, typically in luminal cells, but some PR+ myoepithelial cells were observed. The strength and pattern of PR expression in the ducts remained unchanged between 13 and

25 weeks of age and unlike GR expression, did not differ between grouped and isolated rats at 21 weeks of age (27.5% PR+ $\pm$ 3 versus 28.5% $\pm$ 3 PR+, P = 0.83, Mann Whitney U).

DISCUSSION

We have previously demonstrated that chronically socially isolated Sprague-Dawley rats have a higher risk for spontaneously developing invasive MG cancer compared to their group-housed counterparts [1]. Here we show that isolation reduced mammary epithelial cell proliferation in addition to stunting MG architectural differentiation (Figs. 1, 3, 4 and 7; [6]), a well-known developmental risk factor for many types of breast cancer [3, 18–21]. It also reduced the size of the mammary ductal tree by blunting ductal extension and thereby shrinking the overall area of the mammary ductal tree (Fig. 1 and 3; [6]). An underlying mechanism appears to be decreased proliferation in both luminal epithelial cells and myoepithelial cells during young adulthood, particularly in alveolar buds and lobules, which also confirms earlier observations that adult MG epithelial cell proliferation is ongoing (36 weeks of age) [27]. It is noteworthy that isolation did not sustain the number of TEBs by reducing their differentiation into ABs, which extensive work has previously shown can mediate the malignant effects of chemical carcinogens [10, 15]. The action of carcinogens is greatest in early puberty, before the ovary and adrenal gland fully mature [10, 68, 69], and a period on which carcinogen-based studies have keenly focused as a key window of carcinogen exposure that increases later cancer risk. However, our results demonstrate that social isolation does not affect TEBs, but rather impairs proliferation of MG epithelial cells and both the growth and differentiation of the nulliparous MG architecture after TEBs have cavitated. Thus, our results provide evidence that assessing MG structures in addition to TEBs are necessary to fully understand how environmental stressors can impact MG development, and highlight a newly appreciated process for increasing the risk of spontaneous MG cancers [4, 21, 70].

Our longitudinal study extends data that MG development continues after TEBs cavitate in early puberty [10, 11, 15, 25] with multiple measures of MG size and spatiotemporal development in whole mounts and detailed histological analysis of MG structures, proliferation and steroid receptors. It clarifies two fundamental phases of MG development that progress during late puberty and young adulthood: growth (ductal extension and expansion of the ductal tree through undifferentiated structures) and differentiation of the ductal tree morphology from ABs to lobules. Indeed, to our knowledge, this is the first study to quantify differentiation of the MG compartments and ductal structures throughout young adulthood in nulliparous rats (25 weeks), while being exposed to the hormones of the ovarian cycle. Previous work has focused primarily on early puberty (55 days of age (e.g. 7.8 weeks) and not quantified the full complement of MG structures from puberty through young adulthood [6, 15, 22, 24, 26–29, 31, 71–73]. Thus, our work is a model for breast differentiation from exposure to repeated ovarian cycles and subsequent cancer risk among the growing number of women who delay pregnancy beyond their teen years (a 42% decline) and until early adulthood (currently 57.9% of first births are 25 years of age or older [35]).

While individual differences exist in the morphologic make-up of each sector of the MG, there is a common spatial gradient in mammary ductal development that is sustained throughout adulthood (Fig. 2). During late puberty, in the most distal part of the ductal tree (S4), TEBs are continuing to extend the ductal tree, while closer to the nipple (S1) maximal ductal extension has been reached and alveolobular development has already begun. Even in young adulthood, TDs are the predominant structure in the distal sector (S4), while closer to the nipple (S1) the differentiated ABs and lobules are primary or secondary, having had the longest opportunity to differentiate. Clinically, breast cancer is less likely to arise close to the nipple, suggesting that a similar spatial pattern may be seen among spontaneous rat MG tumors.

By 13 weeks of age (late puberty) the adrenal and ovarian axes are maturing and regular ovarian cycles become more common [6, 68, 69, 74]. The ductal tree has extended further, and TEB number has significantly dropped. While TEBs are largely considered to be the drivers of ductal extension, TDs are the most abundant structures in the distal ductal areas (S24), and growth of the ductal tree continues through 17 weeks. Therefore, TDs likely retain some proliferative capacity and drive ductal growth under the instructive signals from the ovary, as ovarian cycles regularize.

By 17 weeks of age (early adulthood) maximal ductal extension and MG area have been reached and a shift occurs from primarily ductal growth to ductal differentiation. During this period, both the ovarian and adrenal axes are fully mature [6, 69, 74, 75]. During the subsequent ovarian cycles, now with regular exposure to progesterone from the corpus luteum, the ductal tree differentiates into ABs and then lobules. MG differentiation continues through 25 weeks of age, although TDs are still the predominant structures, particularly in the distal areas (S2-S4; Fig. 2). Much of the extant literature on physiological, cellular, and genetic mechanisms of MG development uses rats that are just 8 weeks of age, still in early puberty. Yet, the MG continues to develop through late puberty into early adulthood while maturation of the ovarian and adrenal axes creates a changing hormonal milieu. Given that human breast development also continues into young adulthood, future mechanistic studies of MG development can use this rat model, and capitalize on the striking spatial gradient that corresponds to stages of MG development.

Social isolation slows growth of the mammary ductal tree, primarily during late puberty, and also impairs its differentiation, primarily during early adulthood. Because both MG growth and differentiation are promoted by progesterone and estrogen [9–11, 34, 37, 76–83], one would have expected that isolated rats would have less luteal progesterone and less estrogenized ovarian cycles if ovarian function were sufficient to mediate these MG effects. However, the opposite was observed; isolates had more, not less, luteal progesterone (confirming prior results [6, 84, 85]) and did not manifest less estrogenization until after completion of ductal extension (Fig 6b; 13–17 weeks). Moreover, estrogen signaling increases PR expression in the developing ductal epithelium [86]. Despite the decrease in circulating estrogen in isolates, MG epithelial PR expression remained the same between rats of both social conditions (Fig. 7b). Thus, although progesterone and estrogen clearly are necessary for MG growth and differentiation, they alone cannot explain the difference between isolated and grouped rats in MG development.

Glucocorticoids are a more plausible candidate for mediating the effects of social isolation on stunted MG growth and differentiation, and likely do so through multiple mechanisms. Elevated GC reactivity resulting from social isolation is detectable as early as 13 weeks of age, sustained throughout adulthood and is associated with adrenal fatigue, evidenced by smaller adrenals (Fig. 5b; [1, 6]). We report for the first time that GR is expressed in the epithelium of all rat MG ductal structures (Fig. 7c) and isolates, with greater GC reactivity, show less growth and differentiation in all ductal structures throughout late puberty and early adulthood. Likewise, in early puberty (4–8 weeks) female rats fed corticosterone-supplemented diet also have decreased mammary area and ductal branching [47].

Increased epithelial cell GR activation provides a possible mechanism by which social isolation decreases estrogen-dependent mammary growth and differentiation. For example, the association between estrogenization and epithelial cell proliferation seen in group housed rats was obliterated in the isolated animals after being exposed to chronic stress from puberty until early adulthood (21–25 weeks of age). This is the age by when estrogenization in the isolates had dropped, which increases circulating Follicle Stimulating Hormone (FSH; [87]). FSH signaling results in GR phosphorylation of Ser211 in hepatocytes, potentiating its activity [88]. In the MG, our lab and others have shown that GR activation inhibits estrogen-induced proliferation in the MG epithelium by remodeling chromatin, resulting in altered ER association with DNA regulatory regions [45, 46]. In addition, as GR activity is potentiated, GR expression can be downregulated, as we observed in the mammary ductal tree of isolated rats at 21 weeks of age. After the decrease in GR expression there was no further alveolobular differentiation in the isolates (Fig. 1; 21–25 weeks), whereas it continued through 25 weeks in the grouped rats.

GCs may also contribute to the defect in alveolobular differentiation. Differentiation requires cycles of augmented proliferation followed apoptosis to clear the developing lumen and form alveoli (reviewed in [65]), which occur in a subset of epithelial cells during the luteal and ovulatory phases respectively [31]. *In vitro*, GC also have an antiapoptotic effect on MG epithelial cells [89, 90]. It is plausible that the antiapoptotic effect of GR activation in the MG of the isolated rats prevent the proper clearing of the alveolar lumen [89].

Finally, the decrease in GR expression could prevent further alveolobular differentiation, potentially via down regulation of multiple genes encoding growth factors [22]. Indeed, deletion of the GR DNA-binding domain impairs the ductal development of the mammary gland in virgin female mice [90]. Together, these data underscore the need for further study of aberrant GR activation and MG development, particularly nulliparous alveolobular differentiation, because to date it has remained largely unknown [91].

The decrease in alveolobular differentiation in the isolates was associated with a decrease in stromal area and a relative increase in mammary adipose tissue (Fig. 3c). Stromal area correlated highly with ductal structure area in all rats (Supp. Fig. 3); it may be the impaired alveolobular development that drove the decrease in stroma. However, it is unclear whether the increase in mammary adipose tissue is solely the result of a decrease in the proportion of ductal structures or an additional increase in fat deposition. The latter is supported by previous data that increased exposure to GCs, such as that observed in the isolates, promotes

expansion of fat depots including in the MG [92, 93]. Moreover, previous studies from our lab demonstrate that there are significant changes in the mammary adipose secretome in response to social isolation that can increase cancer cell proliferation and survival [92]. How these adipocyte secreted factors may influence MG epithelial cell proliferation and MG architectural development is the subject of future research.

Recent data suggest that women at high-risk for developing breast cancer have less differentiated ductal trees and have a relative increase in mammary progenitor cells [94]. This association between decreased ductal differentiation and increased mammary cancer risk is independent of the association between mammographic MG density and breast cancer risk, which is mediated by stroma [95]. We hypothesize that the decrease in ductal differentiation observed in our studies may in part be a result of the inability of mammary ductal progenitor cells to produce differentiated daughter cells. Indeed, the relationship between MG composition early in life and later adult mammographic density may not be applicable to our social isolation model [95,96].

The public health implications of this work are broad. Our longitudinal study of natural MG development in nulliparous rats connects decreased differentiation of the mammary ductal tree with glucocorticoid stress reactivity heightened by social isolation. We demonstrate that psychosocial stress can significantly alter MG development spanning puberty and early adulthood. The developing MG is susceptible to environmental insult for a longer portion of the lifespan than previously appreciated [4]. Moreover, we provide evidence that variation in the ovarian cycles observed in the isolates, including increased luteal progesterone and later decreased estrogenization, is not sufficient to mediate decreased MG development by social isolation. We propose that decreased ductal differentiation and accelerated luteal function in the isolated female rats are both a result of elevated GC signaling and contribute to decreased alveolobular development. Understanding the exact mechanisms by which stressor and GC exposure decrease ductal growth and differentiation, potentially mediating increased mammary cancer risk, is likely to reveal important mechanisms in normal and abnormal breast development and biology, particularly as more women delay pregnancy and lactation well into young adulthood [35].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effect of social environment on mammary gland (MG) development of terminal end buds, ductal extension, and distal ductal area (measured in MG whole mounts).

a, a representative digital image of a MG from a group-housed Sprague-Dawley rat (8 weeks of age) stained with carmine alum, indicating the fat pad, lymph node (LN) terminal end buds (TEBs), and nipple. Scale bar = 5 mm. **b**, **c**, **d**. Effects of social condition on MG structures from puberty to early adulthood (8, 13, 17, and 21 weeks of age), measuring (**b**) TEB number at the periphery, (**c**) maximal ductal extension, and (**d**) distal ductal area; mean $\pm$ SEM; nine rats per age and social condition. * $P < 0.05$, 3-factor ANOVA (housing, age, and cohort).

Fig. 2. Differentiation of ductal structures in four different sectors of the distal rat mammary gland (MG) (S1 nearest the nipple to S4 most distal).

A representative digital image of a MG from a group-housed Sprague-Dawley rat (8 weeks of age) stained with carmine alum. White dashed lines depict maximal ductal extension, the perimeter and four equal sectors (S1-S4) within the distal ductal area. Scale bar = 5 mm. TEB (terminal end bud), TD (terminal duct), AB (alveolar bud), L (lobule). The most prevalent structure is listed first, followed by the secondary structure: Individual variation is indicated by the % of rats at each age that had a specific pattern of primary and secondary structures. The spatial gradient in MG differentiation was greater than differences across chronological age.

Fig. 3. Characterization of mammary gland (MG) composition.

Representative H&E stained cross-sections rostral to the lymph node of the left inguinal MG, at 17, 21, and 25 weeks of age (**a**) Ductal Structures (D), fat (F), and stroma (S), 5X magnification, scale bar = 500 μ m and (**b**) 20X magnification, scale bar = 100 μ m. **c**, Effect of social condition on % MG area occupied by ducts, stroma, and fat (mean $\pm$ SEM, four rats per age and social condition; 2-factor ANOVA (social condition and age), * interaction $P < 0.05$).

Fig. 4. Epithelial cell proliferation in ducts, alveolar buds (ABs) and lobules (L) of the mammary gland (MG).

(a) Ki67 staining (brown) in representative cross-sections rostral to the lymph node of the left inguinal MG in early adulthood (21–25 weeks of age). Magnification 10X, scale bar = 200µm. (b) Effect of social condition on %Ki67 in three mammary structures: ducts, ABs and Ls; 3-factor repeated measures ANOVA (social condition x structure interaction $P=0.02$).

Fig. 5. Vigilance testing, glucocorticoid stress reactivity, and adrenal weights.

a, percentage of rats emerging from home base in an open-field test (13–25 weeks of age).

Log rank Mantle-Cox test, $***P < 0.001$ **b**, corticosterone stress reactivity and recovery during and after a passive restraint stressor (Lined bar; 17 weeks; 30 minutes post restraint

$*P < 0.05$ Student's *t*-test). **c**, total adrenal gland weight (mg/100g body weight; mean $\pm$

SEM. $**P < 0.01$, 3-factor ANOVA (housing, age, and cohort).

Fig. 6. Ovarian steroid exposure (measured by vaginal cytology and ovarian weight).

a, number of days of luteal progesterone exposure per 14-day epoch. **b**, number of days of high estrogenization per 14-day epoch. **c**, average ovarian weight (mg/100g body weight).

Mean $\pm$ SEM; nine rats per age and social condition. * $P < 0.02$, *** $P < 0.001$ 3-factor ANOVA (housing, age, and cohort).

Fig. 7. Characterization estrogen, progesterone, and glucocorticoid receptors.

Representative cross-sections rostral to the lymph node of the left inguinal MG at 13, 17, 21, and 25 weeks of age stained for (a) estrogen receptor (red), (b) progesterone receptor (brown), and (c) glucocorticoid receptor (brown). Arrows indicate positive receptor expression staining; for a-c, 20X magnification, scale bar = 100 μm. d, high resolution images of each receptor (ER, PR, and GR) expression at 21 weeks of age, scale bar = 50 μm. For all slides a-d, Hematoxylin (blue) was used for a counterstain.