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Asymmetric development of the male mouse mammary gland and its response to a prenatal or postnatal estrogen challenge

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Abstract

The CD-1 mouse mammary gland is sexually dimorphic, with males lacking nipples. Recent studies have revealed that the underlying epithelium in the male mammary gland is sensitive to estrogenic environmental chemicals. In ongoing investigations, we observed asymmetric morphology in the left and right male mouse mammary glands. Here, we quantified these asymmetries in the embryonic, prepubertal, pubertal and adult male mammary gland. We found that the right gland was typically larger with more branching points compared to the left gland. We next evaluated the response of the left and right glands to 17 α -ethinyl estradiol (EE2) after perinatal or peripubertal exposures. We found that the right gland was more responsive to EE2 than the left at both periods of exposure. These results reveal novel aspects of male mammary gland biology and suggest that future studies should control for laterality in the evaluation of hazards associated with exposures to estrogenic chemicals.

Keywords

endocrine disruptor; xenoestrogen; left-right asymmetry; terminal end bud; estrogen receptor; proliferation; whole mount; gynecomastia

1. Introduction

In the mouse, mammary gland development begins at approximately embryonic day (E)11 and proceeds similarly for both males and females through E13 [1]. At approximately E14, a surge of testosterone is produced by the testes of male embryos, inducing an androgen receptor-dependent condensation of the mammary mesenchyme around the stalk of the mammary epithelial anlage. This causes the epithelium to detach from the overlying epidermis, preventing the formation of a nipple [2]. In several mouse strains, the remaining epithelial tree is completely destroyed [3] whereas in other strains (including the CD-1 mouse), a small epithelial rudiment remains in the fat pad and can be detected in the male throughout life [4].

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In recent years, the male rodent mammary gland has been shown to be affected by environmental chemicals with endocrine disrupting properties. Male mice and rats retain nipples when exposed to anti-androgenic chemicals during critical windows of development [5, 6]. Nipple retention has thus become a non-invasive, robust, and easily accessed endpoint that is indicative of anti-androgenic chemical exposure [7]. Estrogenic chemicals like bisphenol A (BPA) have also been shown to induce nipple retention in male rats, although only after high dose exposures (50 mg/kg/day) [8]. In contrast, morphological changes in the male mouse mammary gland (e.g., larger, more developed ductal trees) have been observed after doses 200,000x lower (250 ng BPA/kg/day) when exposures occur during prenatal development, suggesting that male mammary gland morphology is a highly sensitive endpoint that can be disrupted by low dose estrogenic endocrine disruptors [4]. The male mouse mammary gland may therefore be a suitable ‘sentinel’ organ for the evaluation of estrogenic chemicals [9].

In order for this model to be more widely used in the screening of chemicals, or in regulatory decision-making, more information about its basic biology, development, and hormonal responsiveness is needed. When conducting studies of environmental chemicals including bisphenol S (BPS) [10], we observed what appeared to be consistent left-right (LR) asymmetric development of the male mouse mammary gland (Kolla and Vandenberg, unpublished observations). LR asymmetry is a highly conserved feature of vertebrates, which orient their hearts and visceral organs with reliable biases in placement and morphology [11, 12]. Over several decades of research, ‘cryptic asymmetries’ (e.g., asymmetries that appear unrelated to organ function, or are only revealed under certain circumstances) have been uncovered in humans and other vertebrates. For example, LR biases have been observed in the location of disease and infection in bilateral organs such as the kidney [13], the incidence of unilateral polydactyly [14], and the sidedness of birth defects such as cleft palate [15]. LR biases have also been found for the site of cancers in bilateral organs including the ovaries, lung, testes and breast (reviewed in [16]).

Although unilateral gynecomastia, e.g., the development of breast tissue in boys and men that occurs in only one breast, has been reported [17, 18], there is little information available about whether consistent LR biases occur. Similarly, breast development in girls can be asymmetric, with unilateral development that can persist for up to 2 years before the second breast develops [19]; again, LR biases have not been routinely reported.

The effects of estrogens on different tissues of the body are quite variable; this variability can be explained by the presence/absence of estrogen receptor (ER) α , ER β and other estrogen-responsive receptors, the presence of cell- and tissue-specific coregulators, and the overall number of receptors [20]. Humans are exposed to estrogens from a wide variety of sources including numerous endogenous hormones (estrone, estriol, 17 β -estradiol) that vary in concentration depending on the individual’s physiological state [21]. Exposures also arise from use of pharmaceutical estrogens including 17 α -ethinyl estradiol (EE2), the common estrogenic component of contraceptive pills [22] and estrogens used in hormone replacement therapies, as well as xenoestrogens present in foods, food packaging, personal care products, detergents, pesticides, and other environmental sources [23, 24]. Not only do *exposures* vary significantly between individuals [25, 26], it is also likely that *responses to estrogens* vary

between individuals as well. For example, breast epithelial cells collected from high-risk donors display greater variability in response to xenoestrogens compared to commonly used breast cancer cell lines or immortalized primary breast cells [27]. Yet, the variability of hormone-induced responses in paired organs such as the breast *within individuals* has received little attention. Studies suggesting that breast cancers are 10–25% more likely to occur in the left breast [28, 29], are consistent with within-individual variability in response to cancer promoting events including estrogen exposures.

Here, we have characterized and quantified LR asymmetries in the mouse mammary gland and asymmetric biases in the response of the left and right glands to EE2, a prototypical estrogen that is often included in experiments as a positive control for estrogenicity [30, 31]. The results of this study reveal fundamental aspects of mammary gland biology and suggest that caution is needed in studies evaluating these mammary glands if laterality is not controlled during sample collection.

2. Materials and Methods

2.1 Animals

Pregnant female mice were individually housed (until parturition) in polysulfone cages with food (ProLab IsoDiet) and water (in glass bottles) provided ad libitum. The animals were maintained in temperature and light controlled (12h light, 12h dark, lights on at 0800 h) conditions at the University of Massachusetts, Amherst Animal Facilities. For most experiments, CD-1 mice (Charles River Laboratories, Wilmington, MA) were used; Balb:c mice (Jackson Laboratories) were also evaluated for some endpoints as indicated in the text. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

2.2 Chemical administration

Mice were randomly allocated to groups using statistical software to equalize body weight across treatments at the beginning of the dosing period. For the animals perinatally treated with estrogen, dams were orally dosed with either 1 µg EE2/kg body weight/day or vehicle (Tocopherol Stripped Corn Oil) from pregnancy day 9 through lactational day 20. This period of time was selected to entirely encompass the embryonic and perinatal windows of sensitivity to estrogens in the mammary gland. For the animals postnatally treated with estrogen (during the peripubertal period), male pups were orally dosed with 1 µg EE2/kg body weight/day or vehicle (Tocopherol Stripped Corn Oil) from postnatal day (PND) 21 – PND30. EE2 was diluted in corn oil at a concentration to allow administration of 1 µl oil for every 1 g of body weight.

2.3 Necropsies

All mice were euthanized via CO₂ inhalation followed by decapitation to ensure death. For the evaluation of cryptic asymmetries, necropsies were performed on male and female offspring at PND24 and PND32–35, and male offspring at postnatal week 9–13. For the evaluation of animals prenatally or postnatally treated with estrogen, necropsies were performed on male offspring at puberty (PND31–35). The left and right fourth inguinal

mammary glands were isolated using standard dissection methods. Each mammary gland was spread on a positively charged slide and fixed in neutral buffered formalin for whole mount processing.

For evaluation of the fetal mammary gland, pregnant mice were euthanized by CO₂ inhalation followed by decapitation on pregnancy day 16. Whole fetuses were collected, decapitated, and drop-fixed in neutral buffered formalin for histological analyses. Sex of each fetus was confirmed by measurement of anogenital distance and the presence of nipples.

2.4 Whole mount processing

Whole mounted mammary tissues were fixed overnight in neutral buffered formalin at room temperature, washed with phosphate buffered saline (PBS) and stored in 70% ethanol at 4°C. Whole mounts were dehydrated through a series of alcohols, cleared of fat with toluene, rehydrated through a series of alcohols, and stained overnight with carmine alum. They were then dehydrated through a series of alcohols, washed with xylenes, and placed in heat-sealed bags with methyl salicylate for preservation.

2.5 Whole Mount Morphometrics

Whole mounted mammary glands were imaged using a Zeiss AxioImager dissection microscope, Zeiss high-resolution color camera and ZEN software. Parameters for morphometric analysis included the total area subtended by ducts (ductal area), the total number of branching points, the number of terminal end buds (TEBs, bulb shaped structures at least 0.03 mm²), and area of TEBs.

2.6 Paraffin embedding and sectioning

Following evaluation of whole mounted mammary glands, a subset of glands was removed from the heat-sealed bags and the mammary epithelial trees were dissected from the whole mount using a scalpel. These excised pieces were washed in xylene and embedded with paraffin under vacuum. Similarly, whole embryos were fixed overnight, washed in PBS, and stored in 70% ethanol at 4°C. Samples were processed through a series of alcohols and embedded with paraffin under vacuum in a supine position. All embryos were aligned so that both left and right mammary glands could be observed. Five micrometer sections were collected using a rotary microtome (Fisher Scientific) and mounted on positively charged Superfrost slides (Fisher Scientific).

2.7 Basic histological evaluations

Tissue sections were deparaffinized through a series of xylenes, rehydrated through a series of alcohols, and then stained with hematoxylin and eosin. After staining, samples were dehydrated through a series of alcohols and xylenes and coverslipped with permanent mounting media. Stained mammary glands were imaged and analyzed using a Zeiss Observer.Z1 inverted microscope with 20x and 40x objectives and Zeiss high-resolution color camera. In fetal mammary glands, ductal area was collected by measuring the area subtended by epithelial cells in each section containing mammary tissue.

2.8 Immunohistochemistry

5 µm sections were evaluated with immunohistochemistry for Ki67, a marker of proliferation and ERα using standard protocols [32]. Commercial antibodies were used including rabbit anti-ERα (EMD Millipore, Cat# 06–935, Temecula, CA, diluted 1:1000) and rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1, diluted 1:1000). Secondary antibody (goat anti-rabbit, Abcam, Cat# ab64256) was followed by streptavidin peroxidase complex (Abcam, Cat# ab64269) and diaminobenzidine chromogen (Abcam, Cat# ab64238) to visualize reactions. Samples were counterstained with hematoxylin and coverslipped with permanent mounting media. Images were collected using a Zeiss Observer.Z1 inverted microscope with a 40x objective and Zeiss high-resolution color camera. Expression of each marker was determined by counting either mesenchymal or epithelial cells in each sample as indicated in the text.

2.9 Statistics

For evaluations of LR asymmetry in male CD-1 mice, sample sizes were: PND24, n=26; puberty, n=24; adult, n=24. In female CD-1 mice: PND24, n=15; puberty, n=20. In Balb:c males: PND24, n=7; puberty, n=7; adult, n=11. In fetuses: males, n=4; females, n=5. In experiments with prenatal EE treatment, n=12 per treatment. In experiments with postnatal EE treatment, n=10 per treatment.

SPSS v24 (IBM) was used for all statistical analyses. All data were collected by experimenters blind to treatment group. For the analysis of LR asymmetry in untreated males and females, an unpaired T-test was used to compare normally distributed parameters in the left and right mammary glands. For non-normally distributed data, a Mann-Whitney non-parametric test was used to compare left and right mammary glands. Chi Square tests were used to compare the right:left ratios (to evaluate which side of the tree was larger and compare it to a 50:50 random comparison). For the analysis of mammary gland parameters in animals exposed perinatally or postnatally to either vehicle or ethinyl estradiol, pair-wise comparisons using Student T-tests were made (left vs right, vehicle vs EE2-treated). Test results were considered significant at $p < 0.05$. All data exhibited in the graphs represent mean $\pm$ SEM.

3. Results

3.1 LR asymmetry is observed in CD-1 mouse mammary glands

Prior studies of female C57Bl6 and FVB/N mice have revealed no asymmetric morphologies of the mammary gland [33–35]. Yet, in our ongoing studies with CD-1 mice, we noted distinct LR biases in the size and overall development of the male mammary gland. To determine if these observations were reproducible, we first quantified several growth parameters in whole mounted mammary glands (Figure 1A) collected from CD-1 male mice at different stages of postnatal development: just prior to the onset of puberty (PND24), at the height of puberty (PND32–35), and in adulthood (9–13 weeks of age). Our analyses revealed mammary glands that were consistently larger on the right side, with significantly more branching points in the right glands compared to the left at PND24 and in adults, but not at puberty (Figure 1B). Further, when each mouse was considered as an individual, the

ratio of mammary ductal area was consistently biased, with larger glands on the right side observed more often than would be predicted by chance (Figure 1C).

We also evaluated proliferation in the male mammary epithelium at PND24, after differences in morphology were detected in the left and right mammary glands. Ki67 expression was almost twice as high in the right mammary epithelium compared to the left, consistent with the larger gland on the right side (Figure 1D,E).

To determine if these asymmetries were limited to males, we next evaluated growth parameters in the left and right mammary glands from female CD-1 mice at the onset of puberty (PND24) and at the height of puberty (PND32–35) (Figure 2A). Adult glands were not evaluated due to their relative complexity and the different morphometric tools needed to quantify changes in structures. At both ages examined, the right gland of female CD-1 mice was consistently larger than the left gland, although these differences were not statistically significant (Figure 2B). At puberty, the left gland had significantly more TEBs compared to the right gland, indicative of a more advanced stage of development (Figure 2B).

3.2 LR asymmetry is also observed in mammary glands from Balb:c male mice

Because prior studies had failed to identify consistent morphological asymmetries in two inbred strains of mice (C57Bl6 and FVB/N), we next evaluated male mammary gland morphology in Balb:c mice, an inbred strain commonly used in the study of hormonally active compounds [36–39]. Overall, the ductal trees were smaller in Balb:c males compared to CD-1 males, and there was no obvious expansion of ductal area in the Balb:c males at puberty (Figure 3A). Consistent with what was observed in CD-1 males, the ductal tree on the right side was typically larger than on the left side (Figure 3A). There were significantly more branching points in the right glands compared to the left at puberty and in adults (Figure 3B). These results suggest that LR asymmetric mammary gland development in male mice is not limited to a single strain.

3.3 Evaluating the fetal origin of LR asymmetry in the CD-1 mammary gland

To determine if the LR asymmetry observed in the CD-1 male mammary gland is established during fetal development, we conducted morphometric evaluations of male glands collected at embryonic day 16 (E16). We observed modest increases in the size of the epithelial anlagen on the right side compared to the left, consistent with the growth parameters measured in males postnatally, but this increase was not statistically significant (Figure 4A, B). We also evaluated the expression of Ki67, a marker of proliferation, in males at E16. Ki67 was rarely observed in either the developing mesenchyme and or the mammary epithelium in males at E16; no differences in Ki67 expression were observed on the left and right sides (Figure 4C).

These same evaluations were conducted in female fetuses at E16. Again, the epithelium on the right side was consistently larger than the left side (approximately 60% larger, Figure 4A,B) but this difference was not statistically significant. Ki67 expression was observed in a small proportion of mammary epithelial cells as well as in approximately 3–4% of cells in the mammary mesenchyme of females (Figure 4C). However, there were no LR biases observed for this parameter.

3.4 Prenatal or postnatal EE2 treatment alters ductal area in CD-1 male mammary glands

To determine if there is an asymmetric response of the mammary gland to developmental estrogen exposures, pregnant mice were exposed to vehicle (oil) or a low dose of ethinyl estradiol (1 µg EE2/kg/day) from pregnancy day 9 through lactational day 20. Male offspring were evaluated at puberty, a period when LR asymmetries were not apparent in untreated males. Remarkably, the left mammary gland was unaffected in males prenatally exposed to EE2, whereas the right mammary gland was larger and more developed (Figure 5A,B).

A second set of male CD-1 mice were exposed to EE2 for a 10-day postnatal period (from PND21 – PND30) overlapping with the onset of puberty. 24 hours after the last EE2 administration, the left and right mammary glands were evaluated. Postnatal EE2 treatment did not affect the size or development of the left mammary gland whereas the right mammary gland was larger and more developed after EE2 treatment (Figure 5A,C). Collectively, these results suggest a heightened sensitivity to estrogens on the right, but not the left, side; this increased sensitivity was present in both perinatally and postnatally (peripubertal) treated male mice.

3.5 LR biases in estrogen-sensitivity are not dependent on ER α expression

To determine if the LR biases in response to EE2 treatment could be due to differences in the expression of ER α on the left and right sides, we used immunohistochemistry methods to quantify the expression of this receptor at E16 and during the prepubertal period (PND24). At E16, as expected based on prior studies [40, 41], ER α was not expressed in the mammary epithelium of either males or females but was expressed in the mammary mesenchyme of both sexes (Figure 6A, and data not shown). LR biases in expression were not observed for either males or females (Figure 6B). At PND24, ER α was expressed in the male mammary epithelium, but again there was no apparent LR asymmetry (Figure 6A,C), suggesting that differences in ER α expression are not responsible for the asymmetric responses to estrogens observed in the left and right glands.

4. Discussion

This study revealed two cryptic asymmetries in the male mouse mammary gland. The first LR asymmetry we observed was the consistent bias in mammary gland size and development, with the right side larger than the left. Similarly, there was a consistent bias in the expression of Ki67, a marker of proliferation, in males at PND24. The second LR asymmetry we observed was a bias in growth in response to either perinatal or peripubertal estrogen treatment, where the right side was more responsive than the left. Somewhat different asymmetries were observed in females; although the right gland was typically larger, the left gland had more TEBs. This may suggest that the female's left gland has a heightened response to estrogens at puberty; additional studies are needed to evaluate this hypothesis.

Several studies in humans suggest that breast cancers are 10–25% more likely to occur on the left side [28, 29]. Some researchers have proposed that the larger size of the left

mammary gland is sufficient to explain the increased incidence of carcinoma [42, 43] (e.g., the presence of greater volumes of epithelial tissue on the left). One study reported that left-sided biases in breast cancer occur in pre-menopausal but not post-menopausal patients, suggesting that estrogen may play an important role in lateralization of this disease [42]. Other mechanisms have been proposed for this left-dominance, including a role for asymmetric sleeping and nursing behaviors, differences in blood flow between the left and right breasts, or asymmetric circulation of toxins following oral chemical exposures [44].

Only a small number of prior studies have examined LR asymmetry in the mouse mammary gland; these studies have focused on female C57Bl6 and FVB/N mice [33-35]. Wildtype female FVB/N mice displayed no asymmetries in a range of growth parameters (ductal area, density, ductal complexity, TEBs). Yet, MMTV-Neu^{Tg/Tg} mice, which model HER2/Neu amplification common in some human breast cancers (created on the FVB/N strain), do display asymmetric morphologies, with right glands that are larger than the left and left glands with more TEBs than the right [35]. These results are consistent with the morphologies we observed in CD-1 females. Interestingly, over time, the left glands of MMTV-Neu mice develop more pronounced abnormalities, consistent with a heightened sensitivity to neoplasia on the left side [34]. In studies of C57Bl6 mice, no asymmetries were reported in wildtype females. However, in mice heterozygous for the retinoid X receptor (RXR) α , asymmetry was observed in about half of the females, with the left gland smaller than the right, similar to what we have observed in wildtype CD-1 mice [33]. Future studies should compare RXR α expression across mouse strains with and without LR asymmetric mammary development.

Is the LR asymmetry we observed in the CD-1 male mouse mammary gland established *in utero*? We examined male and female fetuses at E16 and found consistently larger mammary epithelia on the right side, although these biases were not statistically significant. There were no biases in ER α expression at E16, and also no biases in proliferation. Cells in the early embryonic mammary gland migrate into the anlage from the overlying ectoderm [45]. Thus, differences in expression of embryonic patterning genes such as the secreted molecule nodal or the transcription factor Pitx2, which are typically expressed on the left side, could contribute to asymmetric development of the mammary glands [46]. These LR asymmetric signaling molecules are expected to be conserved in both males and females, particularly because orientation of the LR axis occurs several days prior to sexual differentiation [16].

Sexual dimorphism of the mammary gland is more extreme in rodents than in humans. Only a few days after initiation of mammary gland development, the production of testosterone in the male mouse testes induces a detachment of the epithelium from the epidermis [3], ultimately preventing the overlying epidermis from forming a nipple [47]. Numerous studies have evaluated chemicals that can interfere with these processes. Most have focused on phthalates, pesticides, and other chemicals (and chemical mixtures) with anti-androgenic properties, which can induce nipple retention in males exposed during prenatal development [48-50]. Fewer studies have examined the underlying epithelium in male rodents. One study in Sprague-Dawley rats found that males exposed to either EE2 or genistein during development had ductal hyperplasias, budding alveoli, and a histological appearance that was more similar to females in adulthood [51]; these authors concluded that “mammary

gland hyperplasia in the male rat is one of the most sensitive markers of estrogenic endocrine disruption.” Another study in CD-1 mice found that males exposed to BPA during perinatal development have dose-dependent increases in ductal area and branching points in adulthood [4]. Here, we show that morphology of the male mammary gland is altered by either perinatal or peripubertal exposure to EE2, with asymmetric responses to this estrogen. Collectively, these studies show that, in spite of the limited studies evaluating epithelial morphology, the male mammary gland is a highly sensitive organ that is responsive to estrogenic environmental chemicals.

Failure to consider LR asymmetry in the collection of bilateral tissues could influence the conclusions reached in both human and animal studies. In the case of the mammary gland, mixing evaluations of the left and right glands would increase variability, potentially preventing the detection of effects on this sensitive tissue. This may be the case for other tissues as well; additional studies are needed to carefully evaluate the presence of cryptic LR asymmetries in other paired organs, as well as their responses to hormonal stimulation.

5. Conclusions

Diseases of the male mammary gland are relatively rare; this may be one reason why the biology of the male mammary gland has not received significant attention from the scientific community. The absence of nipples in males of most mice and rat strains has also likely influenced lack of interest in this tissue, except when environmental chemicals have been shown to induce nipple retention. This study and others demonstrate that the underlying epithelium in the male mouse is sensitive to low doses of estrogenic chemicals. We have also shown that at the time we evaluated the glands (e.g., the height of puberty), the right was more responsive than the left to either perinatal or peripubertal estrogen treatments. This indicates that future studies should use caution if researchers select only one gland, or if the sidedness of the gland is not controlled, as LR asymmetric development of the male mammary gland could influence any conclusions that are drawn.

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Abbreviations:

BPA	bisphenol A
BPS	bisphenol S
E	embryonic day
EE2	17 α -ethinyl estradiol
ER	estrogen receptor

LR	left-right
PBS	phosphate buffered saline
PND	postnatal day
PR	progesterone receptor
TEB	terminal end bud

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Figure 1. LR asymmetry in the male CD-1 mouse mammary gland throughout postnatal life. A) Representative whole mounted mammary glands from males at the onset of puberty (PND24), at the height of puberty (PND32-35), or in adulthood (9-13 weeks of age). At PND24 and adulthood, clear asymmetries were observed, with larger glands found on the right side. At puberty, these differences were not observed. Scale bar = 0.5 mm. B) Quantification of ductal area and number of branching points in the left and right mammary glands of males at the three ages indicated. *, $p < 0.05$, Fisher's T-test. C) The right:left ratio was calculated for each individual male at the three ages indicated. Ratios greater than 1 indicate a larger right gland; ratios less than 1 indicate a larger left gland. Blue dots indicate males with both left and right glands present; red dots indicate males with no visible ductal tree on one side of the body. To calculate ratios in males missing a ductal tree, a value of 0.001 mm^2 was inputted for the missing tree; this value is $\frac{1}{2}$ the size of the smallest measurable tree. δ , $p < 0.05$, Chi Square. D) Immunohistochemistry for Ki67. A representative example of Ki67 expression in the left and right ductal epithelium is shown.

Scale bar = 100 μ m, arrows indicate positive cells. E) Quantification of Ki67 in the male mammary gland at PND24. #, $p < 0.05$, Mann-Whitney test.

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Figure 2. LR asymmetry in the female CD-1 mouse mammary gland at the onset and height of puberty.

A) Representative whole mounted mammary glands from females at the onset of puberty (PND24) and at the height of puberty (PND32-35). Scale bar = 2 mm. B) Quantification of ductal area and number of TEBs in the left and right mammary glands of females at the two ages indicated. *, $p < 0.05$, Fisher's T-test.

Figure 3. LR asymmetry in the male Balb:c mouse mammary gland throughout postnatal life.

A) Representative whole mounted mammary glands from males prior to puberty (PND24, at the height of puberty (PND32-35), or in adulthood (9-13 weeks of age). Glands were more difficult to distinguish in Balb:c males compared to CD-1 males; they are outlined in each panel by a dotted box. Asymmetries were observed at puberty and in adulthood, but not evident prior to puberty. Scale bar = 0.5 mm. B) Quantification of ductal area and number of branching points in the left and right mammary glands of males at the three ages indicated. #, $p < 0.01$, Mann-Whitney test.

Figure 4. Morphology of the left and right mammary glands at E16.

A) Histological sections of the left and right mammary glands from male and female embryos. Scale bar = 100 μ m. B) Total epithelial area on the left and right sides of male and female embryos. The right glands were consistently larger. C) Ki67 expression in the male and female mammary gland at E16 revealed no LR biases. Positive cells indicated by arrows. Scale bar = 50 μ m.

Figure 5. Perinatal or peripubertal EE2 exposures alter morphology of the right, but not the left, male mammary gland.

A) Example whole mounts from the left and right glands, in animals exposed to vehicle (oil) or 1 μ g EE2/kg/day during either the perinatal or peripubertal periods. LN = lymph node, scale bar = 1mm. B) Quantification of whole mount morphologies in mice exposed to oil or EE2 during the perinatal period. *, $p < 0.05$, Fisher's T-test. C) Quantification of whole mount morphologies in mice exposed to oil or EE2 during the peripubertal period. *, $p < 0.05$, Fisher's T-test.

Figure 6. The increased sensitivity of the right mammary gland to EE2 is not due to greater expression of ERα.

A) Expression of ERα in a female mammary gland at E16 and a male mammary gland at PND24. Positive cells in the fetal mesenchyme are indicated by arrows; positive cells in the PND24 epithelium are indicated by arrowheads. Scale bar = 50 μm. B) Quantification of ERα expression in the left and right mammary gland mesenchyme revealed no LR asymmetries in male or female fetuses at E16. C) No LR asymmetries in expression of ERα were observed in males at PND24.