Dietary Soy Effects on Mammary Gland Development during the Pubertal Transition in Nonhuman Primates

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Abstract

While epidemiologic studies suggest that soy intake early in life may reduce breast cancer risk, there are also concerns that exposure to soy isoflavones during childhood may alter pubertal development and hormonal profiles. Here, we assessed the effect of a high-soy diet on pubertal breast development, sex hormones, and growth in a nonhuman primate model. Pubertal female cynomolgus monkeys were randomized to receive a diet modeled on a typical North American diet with one of two protein sources for approximately 4.5 years: (i) casein/lactalbumin (CL, n = 12, as control) or (ii) soy protein isolate with a human equivalent dose of 120 mg/d isoflavones (SOY, n = 17), which is comparable to approximately four servings of soy foods. Pubertal exposure to the SOY diet did not alter onset of menarche, indicators of growth and pubertal progression, or circulating estradiol and progesterone concentrations. Greater endometrial area was seen in the SOY group on the first of four postmenarchal ultrasound measurements (P < 0.05). There was a subtle effect of diet on breast differentiation whereby the SOY group showed higher numbers of differentiated large-sized lobular units and a lower proportion with immature ducts following menarche (P < 0.05). Numbers of small lobules and terminal end buds and mammary epithelial cell proliferation did not differ by diet. Expression of progesterone receptor was lower in immature lobules of soy-fed animals (P < 0.05). Our findings suggest that consumption of soy starting before menarche may result in modest effects consistent with a more differentiated breast phenotype in adulthood. Cancer Prev Res; 6(8); 1–11. ©2013 AACR.

Introduction

Environmental exposures to hormonally active compounds during critical windows of development can influence breast cancer risk later in life (1). Soy protein contains a variety of bioactive compounds including isoflavones (IF), which are structurally similar to estrogen, bind to estrogen receptors (ER), and elicit both estrogenic and anti-estrogenic responses depending on dose, tissue location, and estrogen context (2, 3). Soy intake has been widely studied as a potential dietary determinant of breast cancer risk, both as a natural chemopreventive approach and as a potential dietary determinant of breast cancer risk later in life.

Puberty is a developmental process that involves a complex series of interactions between growth factors and sex hormones. Age at menarche, a key milestone of puberty, is a consistent predictor of later breast cancer risk in human observational studies, potentially due to hormonal and developmental factors (7, 8). In females, puberty is a key period for breast morphogenesis and differentiation. Ovarian hormones drive elongation and branching of rudimentary ducts and development of terminal end buds (TEB; ref. 9), which subsequently develop into lobuloalveolar structures. The lobules further undergo gradual maturation wherein the number of terminal ductular and alveolar units per lobule increases, resulting in a concomitantly larger size (10). The degree of lobular differentiation has been inversely related to cancer risk (11, 12), suggesting that modulation of breast developmental patterns may influence later life susceptibility to cancer. We hypothesized that pubertal exposure to soy would enhance mammary gland differentiation, potentially leading to a lower risk breast phenotype.

Previous studies have primarily used rodent models to assess environmental influences on breast development. While these models have contributed greatly to our
understanding of molecular signaling in the breast, there are important developmental and physiologic differences between rodents and primates. Most notably, pubertal breast development in rodents is mainly composed of ductal growth with scant lobular differentiation before pregnancy (13). Macaque monkeys serve as a valuable animal model for women’s health due, in part, to their highly similar reproductive physiology, pubertal stages of development, and patterns of ductal and lobular morphogenesis in the breast (14, 15). Cynomolgus macaques also exhibit a nonseasonal 28- to 30-day menstrual cycle with ovarian hormone and tissue responses comparable to humans (16). The primary aim of the current study was to assess the effect of dietary soy exposure on breast development in cynomolgus macaques across the pubertal transition.

Materials and Methods

Animals and diet treatment

Thirty female cynomolgus macaques (Macaca fascicularis) were imported from the Institut Pertanian Bogor (Bogor, Indonesia) at approximately 1.5 years of age, as confirmed by dentition. Female macaques typically reach puberty at the age of 2 to 3 years. Animals were randomized to social groups of 4 to 5 on the basis of body weight to receive 1 of 2 dietary treatments, one at the beginning of the study (CL, n = 12) or (ii) a diet with isolated soy protein containing IF (provided by Solae, LLC; SOY, n = 18) with the human equivalent of 120 mg/d of IF (in aglycone equivalents), which is ~3–5 times higher than the typical consumption in Asian population (ref. 17; Supplementary Table S1). Macronutrient composition of the diets approximated a typical North American diet with 35% calories from fat. Breast biopsies were taken every 6 months starting at 1 month into treatment, with a total of 9 biopsies per animal spanning the period of puberty breast development. Other measures at the time of biopsy included serum sex hormones, uterine size by ultrasound, bone mineral measures by dual-energy X-ray absorptiometry (DEXA) scans, nipple length, trunk length, and body weight. Animals were swabbed daily for vaginal bleeding throughout the study, and menarche was defined as the initiation of regular monthly vaginal bleeding (16, 18). Following menarche, menstrual cycle stage of the animals (i.e., follicular or luteal) was determined retrospectively based on their menstrual bleeding calendar. Two animals died during the study due to causes unrelated to the dietary treatment, one at the beginning of the study (SOY) and one approaching year 4 of the study (CL); this latter animal did not have data from the final biopsy.

All procedures involving animals were conducted at the Wake Forest School of Medicine (Winston-Salem, NC), which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Procedures were conducted in compliance with state and federal laws and standards of the U.S. Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee.

Serum isoflavonoids

Serum concentrations of the main isoflavones (genistein, daidzein) and isoflavone metabolite (equol) were measured using liquid chromatography electrospray ionization mass spectrometry at the laboratory of Dr. Adrian Franke (University of Hawaii Cancer Center, Honolulu, HI), as described elsewhere (19). The measurements were conducted on 18-hour fasted serum samples collected at the time of biopsy.

DEXA scans

Whole-body DEXA scanning was conducted every 6 months using a Norland XR-46 Bone Densitometer (Norland Corp). Using Norland Host Software, we generated DEXA measures for whole-body bone mineral content (BMC, in grams) and lumbar spine (lumbar vertebrae 2 to 4) bone mineral density (BMD, in grams per cm²; ref. 20). The coefficients of variation were 1.7% for BMC and 2.1% for BMD.

Somatometry

Trunk length, nipple length, endometrial thickness, and uterine and endometrial area were measured as indicators of growth and reproductive maturation. Trunk length was measured from the suprasternal notch to symphysis pubis using a Vernier Caliper (Fischer Scientific). Nipple length was also measured with caliper. Ultrasound of the uterus was conducted using portable ultrasound devices equipped with 5.0-13 MHz linear transducers (SonoSite). Measurements of uterine area, endometrial area, and endometrial thickness were manually conducted on the digitized images using NIH ImageJ (version 1.45q, available at rsbweb.nih.gov/ij/). Postmenarche analysis was adjusted for normal cyclical variation in endometrial thickness across the menstrual cycle, using menstrual cycle days 1 to 9 as the period of anticipated thinnest endometrium, with day 1 counted as the first day of menstrual bleeding (21).

Hormone assays

Serum concentrations of estradiol (E2) and progesterone (P4) were measured by radioimmunoassay (RIA) using commercially available kits and protocols from Siemens Healthcare Diagnostics. Before menarche, serum hormone concentrations were measured in blood samples that were collected at the time of breast biopsy. One year after menarche, blood was collected at 3 consecutive menstrual cycles, twice during each menstrual cycle (at days 9–13 for E2 and days 18–25 for P4). The RIAs were conducted at the Biomarkers Core Laboratory of the Yerkes National Primate Research Center of Emory University (Atlanta, GA) using standard procedures. The normal assay ranges were 2.85 to 546.00 pg/mL for E2 and 0.10–40.00 ng/mL for P4.

Breast biopsy collection

Serial breast biopsies were collected using methods described previously (22). The biopsy samples were wedge-shaped, approximately 200 mg in weight, 2 to 3 cm × 1 cm...
from nipple to the edge of the gland. Each sample was divided; one half was frozen for biomolecular work and the other half was placed on a fiberglass screen to prevent distortion, fixed at 4°C in 4% paraformaldehyde for 24 hours, and then transferred to 70% ethanol. Fixed tissues were then processed for whole-mount staining, histology, and immunohistochemical staining. Hematoxylin and eosin (H&E)-stained slides were evaluated for developmental morphology and pathologic lesions blinded to experimental treatment by a pathologist (C.J. Willson) in consultation with a board-certified veterinary pathologist (J.M. Cline).

Whole-mount mammary gland staining
Whole mounts were stained using 0.27% Toluidine Blue as previously described (15, 23). Whole mounts were photographed in toto, and the digital images were used for morphologic measures.

Mammary gland morphometry
For measurement of mammary gland epithelial area, H&E-stained slides were digitized (Infinity 3 digital camera, Lumenera; Adobe Photoshop version 6.0) and measured with methods described previously (23) and modified as follows for developmental structures. Breast epithelium was subdivided into lobuloalveolar and ductal compartments. Lobules were categorized as immature (type I) or mature (type II and III; ref. 10). Ducts were categorized as either immature (multiple luminal cell layers, columnar cell morphology, rounded myoepithelial cells, with/without a small lumen), transitional (multiple luminal cell layers with less stratification, more elongated morphology, increased lumen size, flattened myoepithelial cell borders), or mature (single luminal cell layer, cuboidal morphology, flattened myoepithelial cells). We did not histologically differentiate between TEBs and immature ducts on H&E images due to their overlapping features (15). Total epithelial area of the biopsy section and area of each lobule and ductal type were determined by manually tracing the structures using a computer-assisted technique with Image Pro-Plus Software (Media Cybernetics Inc.). Epithelial area was expressed as a percentage of the total area examined.

For whole mounts, mammary glandular structures were categorized as TEBs (tear drop–shaped, >100 μm in width), small-sized lobules (area ≤ 100,000 μm²), or large-sized lobules (area > 100,000 μm²), and quantified across the entire tissue using NIH ImageJ. Structural counts were expressed as number of TEBs or lobules per cm².

Quantitative gene expression
Expression of mRNA for markers of mammary gland differentiation (casein alpha s1, CSN1S1; mucin-1, MUC1; E74-like factor 5, ELF5; signal transducer and activator of transcription factor 5A and 5B, STAT5A and STAT5B), proliferation (MKI67), and ER activity (progesterone receptor, PGR) was measured using quantitative real-time reverse-transcriptase PCR (qRT-PCR) based on standard methods described elsewhere (24). Amplification was conducted using the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems). Human or macaque-specific TaqMan primer-probe assays were used to quantify target transcripts (Supplementary Table S2) with normalization to cynomolgus macaque–specific primer-probe sets of housekeeping genes GAPDH and ACTB. Relative gene expression was determined using the ΔCt method calculated by ABI Relative Quantification 7500 Software v2.0.1 (Applied Biosystems). Stock mammary tissues and tumor samples were run in triplicate on each plate as external calibrators.

Immunohistochemistry
We used immunohistochemistry to assess protein expression and localization of markers for proliferation (Ki67) and estrogen activity (PGR) in mammary epithelial cells as previously described (22). Monoclonal antibodies used were anti-Ki67 (Ki67/MB1; Dako) and anti-PGR (NCIPGR; Novoceastra Labs), both with 1:100 dilution. Cell staining was quantified by a computer-assisted technique with grid filter where positively stained cells were scored based on staining intensity (+1, +2, or +3) to obtain a semiquantitative measurement of staining intensity and distribution by H-score calculation (22).

Data analyses
Data that were not normally distributed were transformed by log-logistic or square-root conversions to improve normality of the residuals. Data were back-transformed to original scale for presentation of results; values are presented as least square means (LSM) ± SEM or LSM (LSM = SEM, LSM + SEM) when SEs were asymmetric. We used JMP (version 10.0.0, SAS Institute) to fit a mixed model ANOVA with a random animal effect to model diet and time effects adjusted for body weight and menstrual cycle stage (postmenarche) to estimate and compare differences in serum isoflavonoid levels, hormone levels, somatometric measures, mammary gland differentiation, mammary epithelial cell proliferation, and ER activity markers in the breast, between SOY and CL groups over time. We also used SAS (version 9.2, SAS Institute) to fit a mixed effects logistic regression with a random animal effect to model if a particular duct or lobule type was found at a given time point, and if there were differences by diet group, adjusted for body weight and menstrual cycle stage (postmenarche; ref. 25). Menarche data were analyzed using a survival model and logistic regression to model whether onset occurred by 12 months of treatment adjusted for body weight. All outcomes were compared between monkeys of similar development stage across the pubertal transition, and the analyses were done separately for pre- and postmenarche. We also compared pre- versus postmenarche. Multiple pairwise comparisons were done with Tukey honestly significant difference (HSD) Test. Correlation between differentiation marker expression and large-sized lobule count was analyzed using a pairwise test for the significance of the Pearson product–moment correlation coefficient.
Results

Serum isoflavonoid concentrations

Total serum isoflavonoid concentrations were significantly higher in SOY group than in CL ($P < 0.0001$). The mean concentration in SOY was 119 nmol/L (107.9, 131.2 nmol/L) after overnight fasting, compared with 22 nmol/L (19.8, 25.2 nmol/L) in CL. Equol was the predominant circulating isoflavonoid, accounting for 70% of total isoflavonoids. This finding was similar to our previous work (22).

Pubertal development

Body weight, trunk length, BMC, and BMD increased across the treatment period (time effect, $P < 0.0001$) but did not differ between diet groups. Mean body weights were 1.76 ± 0.08 kg and 1.70 ± 0.07 kg before dietary treatment and 3.51 ± 0.17 kg and 3.79 ± 0.15 kg at the end of the study in the CL and SOY groups, respectively. Trunk length, BMC, and BMD were associated with body weight ($P < 0.0001$). While trunk length increased across both menarchal stages (data not shown), BMC and BMD only significantly increased after menarche (Supplementary Fig. S1).

Timing of menarche varied widely across individuals with no effect of diet (Fig. 1A). By month 12 of treatment, CL showed a nonsignificantly higher proportion of menstruating animals than SOY, and the onset was marginally associated with body weight ($P = 0.07$). By month 18, approximately 70% of animals in each diet group had begun cycling.

Hormone status (Fig. 1B and C) and markers for pubertal progression (Fig. 2) were evaluated. Nipple length increased up to menarche ($P < 0.0001$), which was partially driven by the change in body weight ($P < 0.0001$). Uterine area also increased across premenarche ($P < 0.01$) and was associated with body weight ($P < 0.01$), whereas endometrial area ($P = 0.07$) and thickness ($P = 0.1$) did not significantly change. Importantly, there was no diet effect observed for any of these markers before menarche. Serum hormone concentrations were elevated after menarche ($P < 0.0001$ in both diet groups) but remained unaffected by diet. Uterine area increased across postmenarche ($P < 0.01$) with a significant diet × time interaction ($P < 0.05$) and showed an association with body weight ($P < 0.05$). Endometrial area and thickness were primarily determined by menstrual cycle day ($P < 0.01$ and $P = 0.05$, respectively). There was no diet effect observed on endometrial outcomes when covaried with menstrual cycle day. We found a significant diet × time interaction on endometrial area ($P < 0.05$), with greater area in the SOY group during 1 to 6 months after menarche ($P < 0.05$).

Mammary gland differentiation

Histologic assessment revealed no significant abnormalities in mammary gland morphology throughout the study. Two animals (SOY) had a minimal lymphocytic perilobular infiltrate at one biopsy timepoint. A lobule in one animal (CL) contained abundant globular hypereosinophilic secretory material (eosinophilic secretory change), which was found only on the last biopsy.

Mammary epithelial features showed normal patterns of pubertal breast development (Fig. 3A–C). Premenarchal

Figure 1. Onset of menarche and circulating reproductive hormones in cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL)-based diet. A, the proportion of postmenarchal monkeys across the study period did not differ by diet. B and C, serum concentrations of estradiol (B) and progesterone (C) were higher after menarche, with no dietary effect. Values are LSM for $n = 11–17$ monkeys/group (error bars = SEM). The significant main effect is indicated in each panel. a,b Labeled means without a common letter differ ($P < 0.0005$) by LSM Tukey HSD test. Body weight was included in the model as a covariate.
breast contained predominantly ductal structures, with marginally greater areas of transitional and mature ducts in SOY relative to CL ($P = 0.05$ and $P = 0.06$, respectively; Fig. 3D). Following menarche, the relative area of immature and transitional ducts decreased ($P < 0.001$), whereas that of lobules increased compared with premenarche ($P < 0.0001$). After menarche (Fig. 3F), there was a main effect of diet on immature ducts ($P < 0.05$) with a significant diet × time interaction ($P < 0.05$) whereby the area was greater in CL than in SOY at 1 to 6 months postmenarche ($P < 0.01$). In mixed model logistic regression, the proportion of postmenarchal monkeys that had immature ducts present in the breast was higher in CL relative to SOY (diet effect $P < 0.05$). There was no diet effect on lobular area across the pubertal transition (Fig. 3E and G). Histologically, total epithelial area reached 6% (4.8%, 8.0%) in CL and 10% (8.5%, 11.9%) in SOY by 2 years postmenarche. Similar to other pubertal measures, body weight was associated with the areas of immature ducts, transitional ducts, immature lobules, and mature lobules ($P < 0.05$ for all).

There was no diet effect on the numbers of TEBs and small-sized lobules at either pre- or postmenarche (Fig. 4). Regardless of diet, the number of small-sized lobules after menarche was higher than that before menarche ($P < 0.0001$), and there was an increasing pattern over time with a slight decrease by 1.5 to 2.0 years after menarche. The number of large-sized lobules significantly increased after menarche (time effect, $P < 0.01$), and there was an overall diet effect whereby the number was greater in SOY at this stage (diet effect, $P < 0.05$). This effect was mainly driven by whether or not animals had any large lobules present in the breast (diet effect, $P < 0.01$). Furthermore, the difference was most evident at 1.5 to 2.0 years after menarche when the SOY group showed about 5-fold higher number of large-sized lobules than CL ($P < 0.05$).
We did not find a diet effect on gene expression of differentiation markers before or after menarche (Table 1). There was a significant time effect on mRNA expression for CSN1S1 (P < 0.0001), ELF5 (P < 0.0001), MUC1 (P < 0.01), STAT5A (P < 0.0001), and STAT5B (P < 0.0001). Postmenarchal increases in CSN1S1 and ELF5 were seen in both SOY (P < 0.001) and CL (P < 0.01), whereas STAT5A and STAT5B increased only in SOY (P < 0.01). The mRNA expression of all markers was positively correlated with the number of large-sized lobules (r = 0.35, P < 0.0001 for CSN1S1; r = 0.37, P < 0.0001 for ELF5; r = 0.25, P < 0.01 for MUC1; r = 0.34, P < 0.0001 for STAT5A; r = 0.27, P < 0.001 for STAT5B). When stratified by dietary groups, correlations of CSN1S1 and ELF5 with large-sized lobule number were stronger in the SOY group, and correlations of MUC1, STAT5A, and STAT5B with number of large-sized lobule were only significant in the SOY group. While body weight was significantly associated with lobule count (P < 0.0005 for all lobule types), it was not associated with mRNA expression of differentiation markers.

Epithelial cell proliferation was not affected by soy treatment

Postmenarche MKI67 mRNA expression was 1.7-fold higher than premenarche levels (P < 0.05) but there was no effect of diet (Supplementary Fig. S2). Ki67 protein expression in the ductal and lobular structures of the mammary gland also did not differ by diet, with or without menstrual cycle stage in the model. Before menarche, proliferation of the epithelial cells in the mature lobule compartment marginally increased over time (P = 0.06). After menarche, the proliferation in the immature lobule compartment showed a nonsignificant decreasing pattern with time (P = 0.07). We found a significant association of body weight and Ki67 expression in mature ducts (P < 0.05), immature lobules (P < 0.005), and mature lobules (P < 0.005). This effect was interpreted to reflect the onset of ovarian activity at puberty.

Estrogen activity in the breast decreased following menarche

There was a significant time effect on PGR expression across the pubertal transition (P < 0.05). Regardless of diet...
group, PGR mRNA expression decreased after menarche ($P < 0.0001$; Fig. 5A). While there was no diet effect on PGR mRNA, PGR protein expression in immature lobules postmenarche was lower in the SOY group (diet effect, $P < 0.05$; Fig. 5B). The effect was mainly driven by a higher proportion of monkeys in CL (vs. SOY) that showed positively stained cells in the immature lobule (diet effect, $P < 0.05$). Localized PGR protein expression did not differ by menarche status (Fig. 5C). Body weight was inversely associated with PGR expression in mature ducts ($P < 0.05$), immature lobules ($P < 0.01$), and mature lobules ($P < 0.05$).

Discussion

In this study, we showed that pubertal soy intake did not alter onset of menarche, pubertal progression, circulating reproductive hormones, or mammary epithelial cell proliferation. Soy had a modest effect on postmenarchal breast differentiation as indicated by a higher number of large-sized lobules and fewer immature ducts. The numbers of TEB and small-sized lobules and differentiation markers, however, did not differ significantly by diet. There was lower expression of PGR in immature lobules of the soy-fed animals after menarche, possibly indicating altered estrogen response.

Puberty is the major period for mammary gland morphogenesis in females. In humans, hormonal and growth factor signals induce the rudimentary ductal tree to elongate, branch, and form lobuloalveolar structures (9). In rodents, pubertal breast development is mainly a process of rapid ductal growth; and prominent lobuloalveolar growth only occurs during pregnancy (13, 26). Here, we document for the first time the pattern of mammary gland development across the pubertal transition in the macaque model, showing a high degree of interindividual variation and morphologic similarities to humans. The human TEB is the leading edge of mammary gland growth that gives rise to new branches and alveolar buds that further cluster around a terminal duct, forming nascent lobules (10). Similarly, premenarchal macaque breast consists mainly of TEBs, ductal structures, and immature lobules. After menarche,
fewer TEBs are present and the majority of the epithelial compartment is in the form of large ducts and lobuloalveolar structures. A large increase in macaque mammary lobular differentiation occurs around the time of menarche and more markedly around 1 year after menarche, similar to that reported in humans (10). Lobules type I and II become the predominating unit of the adolescent macaque breast, which is consistent with our prior findings in the adult

Table 1. Gene expression of differentiation markers in the mammary gland of female cynomolgus macaques fed a high-soy (SOY) or casein/lactalbumin (CL)-based diet, as measured by qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stage effect</th>
<th>Diet effect</th>
<th>Diet</th>
<th>Correlation with lobular maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Premenarche</td>
<td>Postmenarche</td>
</tr>
<tr>
<td>CSN1S1</td>
<td>P &lt; 0.0001</td>
<td>P = 0.73</td>
<td>CL</td>
<td>1.00 (0.65, 1.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOY</td>
<td>1.11 (0.77, 1.59)</td>
</tr>
<tr>
<td>ELF5</td>
<td>P &lt; 0.0001</td>
<td>P = 0.22</td>
<td>CL</td>
<td>1.00 (0.74, 1.34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOY</td>
<td>1.16 (0.90, 1.49)</td>
</tr>
<tr>
<td>MUC1</td>
<td>P = 0.01</td>
<td>P = 0.63</td>
<td>CL</td>
<td>1.00 (0.60, 1.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOY</td>
<td>0.75 (0.49, 1.15)</td>
</tr>
<tr>
<td>STAT5A</td>
<td>P = 0.002</td>
<td>P = 0.61</td>
<td>CL</td>
<td>1.00 (0.80, 1.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOY</td>
<td>0.92 (0.77, 1.11)</td>
</tr>
<tr>
<td>STAT5B</td>
<td>P = 0.0003</td>
<td>P = 0.48</td>
<td>CL</td>
<td>1.00 (0.84, 1.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOY</td>
<td>0.96 (0.83, 1.11)</td>
</tr>
</tbody>
</table>

NOTE: Values are presented as fold change of LSM (LSM-SEM, LSM + SEM) from premenarchal CL group; n = 3–11 (CL) and 4–17 (SOY). Letter superscripts indicate difference from premenarchal relative expression with P < 0.01 (a) or P < 0.001 (b) by LSM Tukey HSD test.

Figure 5. PGR expression as a marker for estrogen activity in the mammary tissue of cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL) diet across the pubertal transition. A, mRNA expression of PGR decreased after menarche. B, protein expression of PGR in the immature lobules of the breast was lower in the SOY group after menarche and not affected by diet. Values are presented as LSM and error bars = SEM. *Fold change from CL at 1 to 6 months after menarche. The significant main effect is indicated in each panel. Body weight and cycle stage were included in the model as covariates.

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nulliparous macaque (14). Large-sized lobules, composed of more mature type II and III lobules, also become more abundant and cover the majority of the epithelial compartment. The adult macaque breast is composed of 80% or more stroma, similar to that in nonlactating women (9).

Mammary differentiation is a determinant of breast cancer risk. Less differentiated progenitor-type cells in the terminal ducts are likely founder cells for the majority of ductal carcinomas, the most common type of human breast cancer (12, 26). Mammary stem and progenitor cells are present throughout life and they are targets for transformation. The fate of these cells is mainly regulated during key developmental events such as puberty and pregnancy (27). In human epidemiologic studies, nulliparity is associated with higher breast cancer risk, potentially due to less differentiation of susceptible progenitor cell populations (28–30). Experimentally, in vitro and rodent studies indicate that less differentiated mammary cells and lobules are more proliferative, susceptible targets of carcinogens, and prone to neoplastic transformation (12, 31). Recent evidence also points to epigenetic changes during development that may lead to carry-over effects on cancer risk (32, 33).

Several observational studies have reported that adolescent soy intake may reduce breast cancer risk (34–36). Mechanisms for this effect are unclear, and these studies are generally limited by recall and "healthy person" biases. A prominent hypothesis is that early soy exposure may enhance breast differentiation, potentially through ER agonist effects. Parenteral administration of purified genistein resulted in fewer terminal ducts and increased numbers of alveolar buds in a carcinogen-treated mouse model (37). In rats, early life exposure to dietary soy protein (38) or high-dose genistein (39) reduced the number of TEBs. Our study extends these findings by evaluating developmental effects on the primate breast using soy/IF doses relevant to high dietary human exposures. Our findings suggest that soy intake before menarche may modestly enhance breast differentiation leading to a greater content of mature lobular structures following menarche. The markers CSN1S1, ELF5, STAT5, and MUC1 have important roles in lobular epithelial differentiation (32, 40, 41), and we previously reported that these genes were highly expressed in the mammary gland of pregnant and lactating cynomolgus macaques (42). Here, we confirmed their use as differentiation markers in the macaque breast. The positive correlations between the expression of these markers and lobular differentiation were generally stronger in the soy-fed animals, further supporting the idea that soy may have a mild promotional effect on breast differentiation.

Soy IF share structural similarities to endogenous estrogens, bind to and transactivate ERs, and modulate proliferation of ER-responsive cells in vitro. This evidence has led to the idea that soy may alter ER-mediated activity in the developing breast through agonistic, antagonistic, or other hormone-disrupting effects. Here, we found no evidence of strong ER agonist effects of a high-soy diet on systemic markers such as BMD or mammary gland markers such as epithelial cell proliferation. Interestingly, PGR immunolabeling in immature lobules was lower in soy-fed animals after menarche, suggesting that soy exposure may in some way diminish ER responsiveness in the type I lobule of adolescent breast. This was a modest effect specific to a single compartment, however, and it is unclear how such an alteration in ER activity may relate to differentiation patterns.

Although epidemiologic evidence generally suggests that soy intake may be beneficial for chemoprevention in some populations of women (4, 5), other rodent and cell culture studies have identified soy IF as potential endocrine-disrupting compounds due to ER or hormone-modulating actions. Accordingly, there are concerns that exposure to soy during early life or critical stages of development may be potentially harmful for reproductive development (43). Our study showed that pubertal exposure to relatively high dietary levels of soy IF did not alter reproductive hormone concentrations or time to menarche in the macaque model. The IF dose used in the current study was approximately 60 times greater than those consumed by young girls in Germany (44) and the United States (45); they found delayed breast development with higher daidzein intake (44) or urinary excretion (45). The total amount of circulating IF in our study doubled that in a study on Korean girls which reported an association of high soy IF with accelerated breast development (46). Equol was not assessed in those human studies. Here, we also found no soy effect on nipple length or the prepubertal uterus. We did observe a transiently higher endometrial area in the SOY group at one time point, but no difference in uterine area or endometrial thickness; in the absence of a histologic assessment this finding is difficult to interpret. However, we have in several studies shown a lack of uterotrophic effects of dietary IF at up to 500 mg/woman/d equivalent in adult female macaques (24).

Other than the species differences, several factors may explain the neutral or less-profound effect of IF in our study compared with the previous findings in rodents (43). One factor could be the difference in timing of exposure; in-utero/neonatal may be a more sensitive period for modulation such as via epigenetic modification (47). Another is the delivery method, wherein prior rodent studies often used parenteral administration as opposed to dietary dosing strategy that mimics soy consumption in humans. Many studies also used genistein as purified aglycone which may elicit a different effect than the genistein, daidzein, and glycitein mixture of soy IF consumed within a protein matrix. In addition, equol was the predominating isoflavonoid found in our study. Equol is a natural product of daidzein metabolism by gut flora with distinct biological properties from genistein and daidzein (48). Notably, only approximately 30% of adult non-Asian and nonvegetarian populations have the ability to produce this metabolite (49), and human equol production, when present, is generally less robust than in rodents and macaques. This phenotype could potentially account for the differences between our findings and other studies.
Our results suggest that exposure to a soy diet beginning at puberty does not have overt effects on pubertal growth and development. If anything, pubertal soy exposure may have a subtle effect in enhancing mammary gland differentiation following menarche. Future studies are needed to determine whether such a phenotype may influence breast cancer risk later in life.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C.E. Wood, T.C. Register, J.M. Cline
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F.N. Dewi, C.J. Lees, C.J. Willson, A.A. Franke, J.M. Cline
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