Effects of Pubertal Exposure to Dietary Soy on Estrogen Receptor Activity in the Breast of Cynomolgus Macaques

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Abstract

Endogenous estrogens influence mammary gland development during puberty and breast cancer risk during adulthood. Early-life exposure to dietary or environmental estrogens may alter estrogen-mediated processes. Soy foods contain phytoestrogenic isoflavones (IF), which have mixed estrogen agonist/antagonist properties. Here, we evaluated mammary gland responses over time in pubertal female cynomolgus macaques fed diets containing either casein/lactalbumin (n = 12) or soy protein containing a human-equivalent dose of 120 mg IF/day (n = 17) for approximately 4.5 years spanning menarche. We assessed estrogen receptor (ER) expression and activity, promoter methylation of ERs and their downstream targets, and markers of estrogen metabolism. Expression of ERα and classical ERα response genes (TFF1, PGR, and GREB1) decreased with maturity, independent of diet. A significant inverse correlation was observed between TFF1 mRNA and methylation of CpG sites within the TFF1 promoter. Soy effects included lower ERβ expression before menarche and lower mRNA for ERα and GREB1 after menarche. Expression of GATA-3, an epithelial differentiation marker that regulates ERα-mediated transcription, was elevated before menarche and decreased after menarche in soy-fed animals. Soy did not significantly alter expression of other ER activity markers, estrogen-metabolizing enzymes, or promoter methylation for ERs or ER-regulated genes. Our results demonstrate greater ER expression and activity during the pubertal transition, supporting the idea that this life stage is a critical window for phenotypic modulation by estrogenic compounds. Pubertal soy exposure decreases mammary ERα expression after menarche and exerts subtle effects on receptor activity and mammary gland differentiation. Cancer Prev Res. 9(5); 385–95. ©2016 AACR.

Introduction

Estrogen signaling plays a central role in the normal development of the mammary gland, and the promotion of breast cancer (1, 2). Estrogen receptors (ER) are ligand-regulated transcription factors consisting of subtypes α and β (3). Isoflavones (IF) are bioactive components of soy foods that bind ERs, producing mixed estrogen agonist-antagonist effects (4). Epidemiologic evidence suggests that soy intake is inversely associated with breast cancer risk, mortality, and recurrence, although the chemopreventive benefits may be limited to specific populations (5, 6). The mechanism for this protective effect is not established. Many in vitro studies suggest that IFs may alter estrogen activity through ER-mediated effects and via modulation of estrogen synthesis and metabolism. IFs can also alter DNA methylation (7, 8), affecting transcription of genes important to breast cancer (9, 10). Whether the epigenetic modulation by IF also involves genes associated with estrogen regulation has not been determined.

The early-life environment can establish trajectories of breast cancer risk extending into adulthood (11). Prepuberty and adolescence may be important windows for nutritional effects on later-life susceptibility to cancer (12), as mammary gland morphogenesis occurs largely during the pubertal transition. Epidemiologic studies suggest that adolescent soy intake may have a preventive effect on breast cancer later in life (13). Rodent studies indicate that IFs may interact with estrogen or ERs to alter breast differentiation, proliferation, and epithelial cell fate (14, 15). It is not known whether these effects occur in the human breast. These gaps in knowledge are due in large part to the methodologic and ethical limitations for evaluating soy effects on the breast of healthy pubertal girls. Here, we used a well-characterized primate model with highly comparable genetic, endocrine, and breast...
development profiles to humans (16, 17) to comprehensively assess dietary soy effects on ER activity and estrogen regulation in the breast across puberty.

Materials and Methods

Diet and animals
All animal procedures were performed 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, in compliance with state and federal laws and standards of the US Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee. This study utilized mammary gland samples from 29 female cynomolgus macaques (Macaca fascicularis) during pubertal development. The experimental design has been described previously (18). Briefly, animals were obtained from the Institut Pertanian Bogor at the approximate age of 1.5 years and randomized by body weight to receive one of two diets for approximately 4.5 years: (i) control diet with casein and lactalbumin as the protein source (CL, n = 12) or (ii) diet with isolated soy protein containing IFs (SOY, n = 17) with the human equivalent of 120 mg/day of IF (expressed as aglycone equivalents; soy protein isolate donated by Solae, LLC). Throughout the study, all animals were swabbed daily for vaginal bleeding; menarche was defined as the initiation of regular monthly vaginal bleeding (18).

Breast biopsy
Serial breast biopsy samples were collected every 6 months spanning the period of pubertal development (18, 19). Each biopsy sample was divided; half was frozen for biomolecular work, and half was fixed, embedded in paraffin, and sectioned for IHC.

To control for the high interindividual variation of puberty onset, all outcomes were compared between monkeys of similar developmental stage across the pubertal transition. Thus, after completion of the experiment, we were able to categorize the biopsy samples into 8 time points relative to the onset of menarche; from 18 to 23 months premenarche up to 19 to 24 months postmenarche. Serum concentrations of total isoflavonoids (genistein, daidzein, and the metabolite equol) were measured at each time point by liquid chromatography electrospray ionization mass spectrometry at the laboratory of Dr. Adrian Franke (University of Hawai‘i Cancer Center; Honolulu, Hawai‘i) using methods described elsewhere (20); results are presented in Supplementary Fig. S1.

Quantitative gene expression
Total RNA was extracted from frozen mammary tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) and purified using the RNeasy Mini Kit (Qiagen). Quantitative real-time reverse transcription PCR (qRT-PCR) was used to measure mRNA expression of ERs (ERα, ERβ; ERβ, ES2), classical estrogen-induced genes (trefoil factor 1, TFF1; growth regulation in the breast cancer 1, GREB1; progesterone receptors A and B, PGR-A, PGR-B), steroidogenic enzymes [steroid sulfatase, STS; aromatase, CYP19; estrogen sulfotransferase (EST) family 1E, SULT1E1; hydroxysteroid (17β) dehydrogenase 1 and 2, HSD17B1 and HSD17B2] and enzymes for estrogen catabolism (CYP1A1, CYP1B1, and CYP3A4) using methods described previously (21). qRT-PCR reactions were performed on the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems), and relative expression was determined using the ΔCt method calculated by ABI Relative Quantification 7500 Software v2.0.1 (Applied Biosystems). Human or macaque-specific TaqMan primer-probe assays were used as targets (Supplementary Table S1) and samples were normalized to mean values for housekeeping genes (GAPDH and ACTB) using cynomolgus macaque-specific primer-probe sets.

IHC
We assessed protein expression and localization of ERs and GATA-3, a transcription factor that regulates Erre-mediated transcription in the breast (22) and serves as a marker for luminal cell differentiation (23), in mammary gland epithelium in tissue sections from a subset of samples (time points 0–11 months premenarche and 7–12 and 19–24 months postmenarche) using a biotin–streptavidin staining method described previously (19). mAbs used were 1:15 anti-ERs (NCL-ER-LH1, Novocastra Labs), 1:40 anti-ERβ (Clone 14C8, Thermo Fisher Scientific), and 1:50 anti-GATA-3 (Clone HG3-31: sc-268, Santa Cruz Biotechnology). Cell staining was quantified by a computer-assisted technique with a grid filter; cells were scored on the basis of staining intensity (0,+1,+2,+3) to obtain a semiquantitative H-score (19). On the basis of the structures present, H-score data for immature/transitional ducts and mature lobules were limited to premenarche or postmenarche time points, respectively, whereas H-score data for mature ducts and immature lobules were obtained for all time points. Morphologic criteria for immature, transitional, and mature mammary gland structures are described elsewhere (18). Representative IHC images for ERs are presented in Supplementary Fig. S2.

To evaluate changes in steroidogenic enzyme protein expression, biopsies from two time points at 12–17 months premenarche and postmenarche were used for IHC. Antibodies and dilutions used were as follows: EST rabbit polyclonal (1:100, Bioreby); HSD17B1 rabbit monoclonal (1:50, Epitomics); HSD17B2 rabbit polyclonal (1:100, Proteintech); and STS rabbit polyclonal (1:100, Sigma-Aldrich). Staining was scored qualitatively based on intensity (0,+1,+2,+3) in epithelium and stroma by two board-certified veterinary pathologists (C.J. Willson, J.M. Cline), and descriptive results are presented.

Pyrosequencing
We used breast biopsy samples from three time points: 0–5 months premenarche and 7–12 and 19–24 months postmenarche, to assess promoter methylation. Genomic DNA was extracted from the frozen specimens using DNaseasy Kit (Qiagen) and treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research). Cynomolgus macaque–specific pyrosequencing assays (Supplementary Table S2) were designed using PyroMark Assay Design software (Qiagen) for CpG sites around/near the estrogen responsive element (ERE) of TFF1, GREB1, and PGR (half-site ERE), and CpG islands in the promoter regions of ERα (promoter B) and ERβ2 [up to 300 bp upstream of transcriptional start site (TSS), covering the region that corresponds to promoter 0N in human ERβ (24)]. Bisulfite-converted DNA (40 ng) was amplified by PCR in a 25 μl reaction using the PyroMark PCR Kit (Qiagen). Pyrosequencing was performed on Qiagen PyroMark Q96 MD Pyrosequencer with Pyro Q-CpG software at the Duke Epigenetics Research Laboratory.
Duke University Medical Center. The values shown represent the mean methylation for the CpG sites contained within the analyzed sequence.

Expression microarray

Breast gene expression profiles were obtained from four animals/group at two time points (7–12 and 19–24 months after menarche) utilizing the Affymetrix GeneAtlas System (Affymetrix). Extracted RNA was assessed for quality and integrity using a Nanodrop ND-2000 UV–VIS spectrophotometer (Nanodrop) and Agilent Bioanalyzer-2100 (Agilent Technologies). The Ambion WT Expression Kit (Life Technologies) was used to generate sense-strand cDNA, and fragmentation and labeling of the cDNA was done using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). Samples were hybridized to Rhesus Gene 1.1 ST WT Array Strips. Data analysis and quality control were performed using Partek Genomics Suite software (Partek) and the Limma package for R (25). RNA-normalized data were analyzed for difference in expression over time using a paired t test; expression was also compared between diet groups using empirical Bayesian analysis at the two different time points. The microarray data are available in the Gene Expression Omnibus repository at the NCBI (accession no. GEO:72940).

For each diet group, gene set enrichment analysis (GSEA) was performed using preranked method in the GSEA software version 2.0.13 with default parameters (26) on the gene lists generated from pairwise comparisons by time. Each list of 16,915 genes was ranked on the basis of fold change, and sets were compared with curated KEGG gene sets available from molecular signature database (MsigDB) v4.0 (27). Enriched sets with FDR 5% were considered significant.

Statistical analysis

Logarithmic or square-root conversions were used where appropriate to improve normality of the residuals. Data were backtransformed to original scale for presentation as least squares means (LSM) ± SEM or LSM (LSM – SEM, LSM + SEM) when SEs were asymmetric. All analyses were done across the pubertal transition, and separately for pre- and postmenarche. For postmenarche, the menstrual cycle stage of the animals (follicular or luteal) during each biopsy was determined retrospectively based on the menstrual bleeding calendar for each animal and used as a covariate. We used JMP (version 10.0.0, SAS Institute) to fit a mixed model ANOVA with a random animal effect to model main and interactive effects of diet and time adjusted for body weight and menstrual cycle stage (postmenarche) to estimate and compare differences in relative mRNA expression, protein expression, and methylation ratios in SOY and CL groups over time. Multiple pairwise comparisons were done with Tukey Honestly Significant Difference (HSD) test. Relationships between mRNA and protein or DNA methylation levels were examined by Spearman rank correlation.

Results

Expression of ERs

Mammary ESR1 mRNA levels decreased across the pubertal transition (P < 0.05; Fig. 1A), and were inversely associated with body weight (P < 0.001; data not shown). ESR1 expression was lower in the SOY group only after menarche (P < 0.01 for diet effect), and higher in the follicular versus luteal phase independent of diet (P < 0.05; data not shown). Before menarche, ERα protein in immature ducts showed a diet x time interaction (P < 0.05) in which expression was higher in SOY versus CL at 0–5 months premenarche (Fig. 1B). Following menarche, ERα expression in mature ducts (P < 0.05) and immature lobules (P = 0.07) decreased with time independent of diet, showing positive correlation with mRNA expression (Spearman ρ = 0.50, P < 0.0001 for mature ducts; Spearman ρ = 0.25, P < 0.05 for immature lobules). No diet or time effect was observed for ERβ expression in transitional ducts or mature lobules. We assessed methylation of CpG sites around the ESR1 promoter region B (Fig. 1C) and found no diet effect. Two CpG sites showed modest but significant increases in methylation over time (P < 0.05). Regardless of treatment, methylation levels were low at all assessed CpG sites across pubertal development (<5%).

For ERβ, we observed a main effect of diet but not time on ESR2 expression across the pubertal transition (P < 0.05; Fig. 2A). Before menarche, there was marginally lower ESR2 expression in the SOY group (P = 0.08). After menarche, no diet or time effects were observed. ERβ protein expression was lower following SOY treatment in transitional ducts before menarche and mature ducts after menarche (P < 0.05 for both; Fig. 2B). Similar to ESR1, promoter methylation of ESR2 was low (<3%) and did not differ by diet or time (Fig. 2C). Only 1 of 16 CpG sites showed increasing methylation with time (P < 0.01).

ER activity markers

Relative expression of ER-regulated gene markers TFF1, PGR, and GREB1 decreased across the pubertal transition (P < 0.01 for time effect in all genes; Fig. 3A–D). GREB1 was marginally decreased after menarche (P = 0.08) and showed a significant diet effect in which expression was lower in the SOY group postmenarche (P < 0.05 vs. CL). Menstrual cycle stage had a significant effect on TFF1 (P < 0.01), GREB1 (P < 0.05), and PGR-B (P < 0.0001); these markers were higher in the follicular phase versus the luteal phase (data not shown). Body weight, a surrogate for age, had an inverse association with postmenarchal TFF1 (P < 0.05), PGR-A (P < 0.05), PGR-B (P < 0.01), and GREB1 (P < 0.05).

A significant time effect on TFF1 promoter methylation was observed (P < 0.0001; Fig. 4A). Seven CpG sites flanking the promoter ERE showed increased methylation levels with development (by ~5%–7%), and there was an inverse correlation between TFF1 methylation and mRNA expression (Spearman ρ = −0.31, P < 0.01). We assessed CpG methylation around an ERE located at 1.6 kb upstream of the GREB1 TSS but did not find a diet or time effect (Fig. 4B) or association between methylation of this region and GREB1 expression. The CpG sites within the promoter regions for PGR-A and PGR-B (Fig. 4C) showed low levels of methylation across the pubertal transition (<10% and <5% for PGR-A and PGR-B, respectively) independent of dietary treatment. PGR-A showed a modest increase in methylation level with maturity (P < 0.05 for time effect) and body weight (P < 0.05; data not shown). Among the 17 CpG sites assessed within the PGR-A promoter, 6 showed a significant change with time and 1 showed a diet x time interaction (P < 0.05). Methylation of PGR-A and PGR-B did not correlate with their respective mRNA expression.

ER regulation and luminal cell differentiation

GATA-3 expression was localized to the luminal epithelium of mammary ductal and lobular structures. In immature ducts,
GATA-3 did not differ by diet, whereas in transitional ducts, there was a diet x time interaction ($P < 0.05$) with increased expression prior to menarche only in the SOY group ($P < 0.05$). Premenarchal GATA-3 expression (Fig. 5A) in mature ducts and immature lobules showed a marginal increase with time ($P = 0.06$ in both structures) independent of diet. After menarche (Fig. 5B), GATA-3 expression in immature lobules was lower in SOY ($P < 0.05$); however, this effect disappeared with adjustment for menstrual cycle stage. For mature lobules, there was a main effect of time ($P < 0.05$) and a diet x time interaction ($P < 0.05$) in which increased GATA-3 expression was only observed in the CL group. Except in the transitional duct, GATA-3 expression was significantly correlated with expression of ERα protein (Spearman $\rho > 0.3$, $P < 0.05$) and mRNA (Spearman $\rho > 0.2$, $P < 0.05$).

Figure 1.
ERα expression and promoter methylation in the mammary gland across the pubertal transition. ESR1 mRNA level decreased with maturity, with a significant soy effect after menarche (A). ERα protein expression did not differ by diet but was lower postmenarche (B). Methylation of CpG sites within promoter B of ESR1 did not differ by diet (C). Values are LSM for $n = 11$–17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.
Global gene expression

We did not find a significant difference in the transcriptional profiles between the two diet groups and across 7–12 months and 19–24 months postmenarche using data globally adjusted for multiple comparisons. We used the unadjusted P values to identify genes that changed over time in each diet group, and genes that differed by diet at each time point (Supplementary Fig. S3). Results from this relaxed analysis showed that breast development from 7–12 months to 19–24 months postmenarche across the two dietary groups involved different sets of genes associated with distinct KEGG pathways.

Estrogen-metabolizing enzymes

We previously reported no difference in serum estradiol (E2) concentration between SOY and CL groups (18). Here, we also found no diet effect on mammary mRNA expression of genes involved in estrogen conjugation, synthesis, bioactivation, and catabolism (Fig. 6A–E). A significant time effect was observed for...
mRNA expression of several steroidogenic enzymes. For example, STS (Fig. 6A) and SULT1E1 (Fig. 6B) expression decreased over time ($P < 0.01$), whereas HSD17B1 (Fig. 6C) increased across the pubertal transition, particularly after menarche ($P < 0.05$). Expression of aromatase (CYP19) was generally low with an increase after menarche (Fig. 6E; $P < 0.01$) independent of diet. No significant time or diet effects were observed for expression of HSD17B2 (Fig. 6D), or for CYP1A1, CYP1B1, and CYP3A4 (data not shown).

Figure 3. Expression of ER-regulated markers in the mammary gland during the pubertal transition. mRNA levels for TFF1 (A) and PGR (B and C) decreased with maturity but did not differ by diet. GREB1 (D) was lower in the soy group after menarche. Values are LSM for $n = 11$–17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

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The results for steroidogenic enzyme immunoreactivity in the mammary gland are summarized in Supplementary Table S3. Briefly, EST showed cytoplasmic expression in stromal cells which was moderate intensity before menarche and weaker intensity after menarche. A subset of animals (8/15 premenarche and 3/15 postmenarche) displayed weak to moderate cytoplasmic staining in ductal and lobular epithelial cells for STS. HSD17B1 expression was cytoplasmic and most intense in the myoepithelial cells and stroma immediately surrounding lobules and ducts. Generally <20% of epithelial cells in either lobules or ducts showed weak cytoplasmic immunoreactivity for HSD17B1; there was no appreciable trend in the expression between pre- and postmenarche. Similarly, no developmental pattern was observed in HSD17B2 expression between the two time points; the staining was cytoplasmic and intense in stromal cells, and weak in <10% of epithelial cells.
Discussion

Diet during adolescence can alter developmental signaling networks and influence later-life susceptibility to cancer. Here we evaluated the effects of a high-soy diet with phytoestrogenic IFs on ER expression and ER-dependent activity in the breast during pubertal development. Soy treatment resulted in a modest down-regulation of ERα transcription after menarche, which appeared to be independent of ER promoter methylation. This change occurred alongside a decrease in expression of GREB1, which is a classic estrogen-induced marker, suggesting a mild buffering effect of soy on ER activity for select targets after menarche. Expression of the GATA-3 differentiation marker, which regulates ERα-mediated transcription, tended to be higher before menarche and lower following menarche in soy-fed compared with CL-fed monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

Endogenous estrogens are key regulators of mammary gland morphogenesis and important risk factors for breast cancer during adulthood (1). In humans (28) and macaques (18), a large increase in mammary lobular differentiation can be seen around the time of menarche. In human breast, ERs are present from the fetal stage onward, although little is known about the dynamics of this expression over time (29). An autopsy study showed that ERα mRNA level was higher in the breast of premenarchal compared with perimenarchal girls (30); we previously reported a similar finding in monkeys (21). The current report is the first to longitudinally illustrate the developmental profile of ER expression and activity markers in the breast across puberty in the same subjects. We found decreasing ERα but not ERβ across the menarchal transition. Classic ERα-regulated markers TFF1, PGR, and GREB1 also decreased over time, which supports the idea that estrogen responsiveness in the breast is highest during early puberty and decreases with adulthood. Our findings suggest that adolescence may be a critical period of susceptibility to hormonal disruption by environmental factors.
estrogens through both direct ER interactions and alterations of endogenous estrogen metabolism.

Structural similarities between soy IFs and E2 allow IFs to bind to ERs but with weaker affinity. In previous studies in both premenopausal and postmenopausal monkeys, dietary doses of IFs (≤129 mg/day human equivalent dose) did not elicit clear estrogenic effects, while having modest selective ER-inhibitory effects when given with exogenous estrogen (31, 32). Here, we showed that pubertal soy intake resulted in lower mRNA expression of ERα (premenarche) and ERβ (premenarche). Furthermore, GREB1 expression was also lower in the soy group after menarche, whereas other ER-regulated markers (i.e., TFF1 and PGR) were not altered. These findings suggest that adolescent soy intake may produce a subtle decrease in estrogen responsiveness that carries forward into adulthood. Consistent with this idea, exposure of pre/peripubertal rats to an IF-rich diet reduces estrogen-induced proliferative responses in the mammary gland of ovarieectomized adults (33). The findings may support the notion that dietary soy exposure initiated at puberty or premenarche is beneficial for breast cancer prevention.

Mechanisms for these types of ER-modulating effects beyond simple competitive interactions with E2 are unclear. Different IFs (34), which may reduce ERα-mediated transcription (35). Interestingly, soy resulted in lower ERα mRNA after menarche and lower ERα mRNA before menarche. When considered alongside soy effects on GATA-3, which was higher in premenarche and lower in postmenarche, this pattern supports the idea that a higher ratio of ERα:ERβ activity early in puberty may facilitate greater mammary gland differentiation. Our results indicate that the postmenarchal breast tissue of soy-fed and casein-fed animals expressed different set of genes and pathways, which could reflect induced differences in mammary gland differentiation. This finding, however, should be interpreted with caution.

Recent evidence suggests that early-life exposure to exogenous estrogens may increase future breast cancer risk (36). Epigenetic changes such as DNA methylation may have an important role in mediating this type of latent effect (37). Promoter methylation is generally associated with transcriptional silencing although there is a limited understanding of methylation-dependent regulation of ERs and ER-regulated genes, particularly in normal breast development. We examined the relationship between early-life soy exposure, DNA methylation, and ER-mediated responses. The soy effect on ERα mRNA after menarche did not appear to be mediated by altered methylation within the CpG sites examined. We did observe several interesting methylation patterns based on pubertal development. The ESR1 gene has multiple promoters (38); we assessed CpG sites within promoter B, which is a CpG-rich region. CpG island promoters are mainly unmethylated regardless of expression status of the gene, whereas low CpG promoters are methylated during active or inactive states (39). In breast tumors, however, promoter B is often methylated (40). Here we found a generally low methylation level within this region across pubertal development with a significant increase

Figure 5.
GATA-3 protein in the pubertal mammary gland. Soy increased GATA-3 before menarche (A), but decreased it after menarche (B). Values are LSM for n = 11–17 monkeys/group (bars = SEM). Significant main effect and interactions are indicated in each panel. Asterisks (*) indicate pairwise differences (P < 0.05, Tukey HSD).
in 2 of 4 CpG sites with maturity. This increase was subtle (~1%) and the biologic relevance of such a modest change is unclear. For ESR2, we assessed methylation of the region that corresponds to the promoter ON described in humans; this region is hypermethylated in most breast cancer cell lines (24). Similar to normal human breast epithelial cells, the macaque breast showed low methylation in this region. The promoter for PGR is also CpG-rich with a low overall methylation level. PGR expression in cancer is epigenetically regulated via methylation of the alternative promoters A and B (41), but little is known regarding the regulation in normal breast. We found that specific CpG sites within PGR promoter A also had subtle increases in methylation level after menarche regardless of diet. It is interesting to point out the tight regulation that maintains methylation level at each CpG site, as shown by sequential CpG sites having different levels of methylation. Whether small methylation changes in certain sites of the...
promoter could contribute to the decrease in ESR1 and PGR mRNA with maturity is unclear. It has been shown that the state of only 2 CpG sites within the promoter region of oxytocin receptor (OXTR) is crucial for the overall effect of promoter methylation on OXTR expression (42). Overall, there was a tight constraint on shifting methylation at the regions examined; it is possible that these regions are more vulnerable to shift at other developmental periods. For example, prenatal exposure to genistein has been shown to enhance DNA methylation and counter the hypomethylating effect of bisphenol A (7, 43).

There was no change in the methylation of ERE within GREB1 promoter despite the differential gene expression with diet and time. GREB1 has three consensus EREs spanning approximately 20 kb upstream of the TSS that are functional transcription enhancers. The region assessed in this study is the closest to the TSS (1.6 kb upstream), which has basal promoter activity, strongest ER recruitment, and repressed activity in the presence of ER antagonism (44). Our results show that the methylation status of CpG sites in the human embryonic kidney carcinoma cell line derivative showed sensitivity to soy in the pubertal breast model for studying agents that may alter estrogen metabolism. No effects of soy IF exposure on steroidogenic enzyme expression in the pubertal breast were observed.

Estrogen signaling during adolescence is a key driver of mammary gland development. We show for the first time that ERα expression and response markers in the breast are higher early in the pubertal transition, supporting the idea that this life stage represents a critical period for phenotypic modulation by ER-modulating compounds. Our findings suggest that adolescent intake of dietary soy may modestly enhance breast differentiation early in puberty and dampen estrogen responsiveness in the breast later in adulthood. Both of these phenotypes would be anticipated to lower breast cancer risk. Additional experimental evidence is needed to confirm these findings and to examine whether any such shifts in ER activity may reduce risk of breast cancer development later in life.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: F.N. Dewi, 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. Willson, T.C. Register, C.J. Lees, S.K. Murphy, J.M. Cline

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