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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 (IFs), 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 ~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

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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.

Keywords

puberty; estrogen; estrogen receptor; soy isoflavones; mammary gland; DNA methylation; breast cancer

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 (ERs) are ligand-regulated transcription factors consisting of subtypes α and β (3). Isoflavones (IFs) 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 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). Pre-puberty 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 methodological 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, 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 twenty-nine 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 ~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; provided 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 six 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 immunohistochemistry.

To control for the high inter-individual 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 18–23 months pre-menarche up to 19–24 months post-menarche. 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) using methods described elsewhere (20); results are presented in Supplementary Figure 1.

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, Valencia, CA). Quantitative real-time reverse transcription PCR (qRT-PCR) was used to measure mRNA expression of ERs (ERα, *ESR1*; ERβ, *ESR2*), classical estrogen-induced genes (trefoil factor 1, *TFF1*; growth regulation by estrogen in 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, Foster City,CA), 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 1) and samples were normalized to mean values for housekeeping genes (*GAPDH* and *ACTB*) using cynomolgus macaque-specific primer-probe sets.

Immunohistochemistry

We assessed protein expression and localization of ERs and GATA-3, a transcription factor that regulates ERα-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 pre-menarche, and 7–12 and 19–24 months post-menarche) using a biotin-streptavidin staining method previously described (19). Monoclonal antibodies used were 1:15 anti-ERα (NCL-ER-LH1, Novocastra Labs, Newcastle-upon-Tyne,UK), 1:40 anti-ERβ (Clone 14C8, Thermo Scientific, Rockford,IL), and 1:50 anti-GATA-3 (Clone HG3-31: sc-268, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Cell staining was quantified by a computer-assisted technique with a grid filter; cells were scored based on staining intensity (0,+1,+2,+3) to obtain a semi-quantitative H-score (19). Based on the structures present, H-score data for immature/transitional ducts and mature lobules were limited to pre- or post-menarche 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 immunohistochemistry images for ERs are presented in Supplementary Figure 2.

To evaluate changes in steroidogenic enzyme protein expression, biopsies from two time points at 12–17 months pre- and post-menarche were used for immunohistochemistry. Antibodies and dilutions used were as follows: EST rabbit polyclonal (1:100, Biorbyt, Riverside,UK); HSD17B1 rabbit monoclonal (1:50, Epitomics, Burlingame, CA); HSD17B2 rabbit polyclonal (1:100, Proteintech, Chicago,IL); and STS rabbit polyclonal (1:100, Sigma-Aldrich, St. Louis, MO). Staining was scored qualitatively based on intensity (0,+1,+2,+3) in epithelium and stroma by two board-certified veterinary pathologists (C.J.W., J.M.C.), and descriptive results are presented.

Pyrosequencing

We used breast biopsy samples from three time points: 0–5 months pre-menarche and 7–12 and 19–24 months post-menarche, to assess promoter methylation. Genomic DNA was extracted from the frozen specimens using DNeasy Kit (Qiagen) and treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Cynomolgus macaque-specific pyrosequencing assays (Supplementary Table 2) 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 *ESR1* (promoter B) and *ESR2* (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 25μl 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, Santa Clara,CA). Extracted RNA was assessed for quality and integrity using a Nanodrop ND-2000 UV–VIS spectrophotometer (NanoDrop, Wilmington, DE) and Agilent Bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA). The Ambion WT Expression kit (Life Technologies, Gaithersburg,MD) 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, St. Louis, MO) and the Limma package for R (25). RMA-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 #GEO72940).

For each diet group, Gene Set Enrichment Analysis (GSEA) was performed using pre-ranked method in the GSEA software version 2.0.13 with default parameters (26) on the gene lists generated from pair-wise comparisons by time. Each list of 16,915 genes was ranked based on fold-change, and sets were compared to curated KEGG gene-sets available from molecular signature database (MsigDB) v4.0 (27). Enriched sets with false discovery rate (FDR) <5% were considered significant.

Statistical Analysis

Logarithmic or square-root conversions were used where appropriate to improve normality of the residuals. Data were back-transformed to original scale for presentation as least square means (LSM) + standard error of the mean (SEM) or LSM (LSM-SEM, LSM+SEM) when standard errors were asymmetric. All analyses were done across the pubertal transition, and separately for pre- and post-menarche. For post-menarche, 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; Cary,NC) to fit a mixed model analysis of variance with a random animal effect to model main and interactive effects of diet and time adjusted for body weight and menstrual cycle stage (post-menarche) 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's Honestly Significant Difference Test. Relationships between mRNA and protein or DNA methylation levels were examined by Spearman's rank correlation.

Results

Expression of Estrogen Receptors

Mammary *ESR1* mRNA levels decreased across the pubertal transition ($P<0.05$; Figure 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 *vs.* 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 *vs.* CL at 0–5 months pre-menarche (Figure 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's $\rho=0.50$, $P<0.0001$ for mature ducts; Spearman's $\rho=0.25$, $P<0.05$ for immature lobules). No diet or time effect was observed for ERα expression in transitional ducts or mature lobules. We assessed methylation of CpG sites around the *ESR1* promoter region B (Figure 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 ($P<0.05$) (Figure 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) (Figure 2B). Similar to *ESR1*, promoter methylation of *ESR2* was low (<3%) and did not differ by diet or time (Figure 2C). Only one of 16 CpG sites showed increasing methylation with time ($P<0.01$).

Estrogen Receptor 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) (Figure 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 post-menarche ($P<0.05$ *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 *vs.* the luteal phase (data not shown). Body weight, a surrogate for age, had an inverse association with post-menarchal *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 ($P<0.0001$) (Figure 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's $\rho=-0.31$, $P<0.01$). 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 (Figure 4B) or association between methylation of this region and *GREB1* expression. The CpG sites within the promoter regions for *PGR-A* and *PGR-B* (Figure 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, six showed a significant change with time and one showed a diet x time interaction ($P<0.05$). Methylation of *PGR-A* and *PGR-B* did not correlate with their respective mRNA expression.

Estrogen Receptor 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, while in transitional ducts, there was a diet x time interaction ($P<0.05$) with increased expression prior to menarche only in the SOY group ($P<0.05$). Pre-menarchal GATA-3 expression (Figure 5A) in mature ducts and immature lobules showed a marginal increase with time ($P=0.06$ in both structures) independent of diet. After menarche (Figure 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's $\rho > 0.3$, $P<0.05$) and mRNA (Spearman's $\rho > 0.2$, $P \leq 0.05$).

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 post-menarche 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 Figure 3). Results from this relaxed analysis showed that breast development from 7–12 months to 19–24 months post-menarche 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 (Figure 6, panels A-E). A significant time effect was observed for mRNA expression of several steroidogenic enzymes. For example, *STS* (A) *and SULT1E1* (B) expression decreased over time ($P<0.01$), while *HSD17B1* (C) increased across the pubertal transition, particularly after menarche ($P<0.05$). Expression of aromatase (*CYP19)* was generally low with an increase after menarche (E, $P<0.01$) independent of diet. No significant time or diet effects were observed for expression of *HSD17B2* (D), or for *CYP1A1*, *CYP1B1*, and *CYP3A4* (data not shown).

The results for steroidogenic enzyme immunoreactivity in the mammary gland are summarized in Supplementary Table 3. 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 pre-menarche, 3/15 post-menarche) 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 post-menarche. 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 downregulation 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 to CL-fed animals. Our findings demonstrate that there are high levels of ER signaling in the pubertal breast, and that pubertal soy exposure may have subtle effects on this activity, potentially influencing ER-dependent responses later in life.

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 onwards, 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 pre-menarchal compared to peri-menarchal 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 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/d 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 (post-menarche) and ERβ (pre-menarche). Further, *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 ovariectomized adults (33). The findings may support

the notion that dietary soy exposure initiated at puberty or pre-menarche is beneficial for breast cancer prevention.

Mechanisms for these types of ER-modulating effects beyond simple competitive interactions with E2 are unclear. Different IFs have greater affinity for ERβ (34), which may reduce ERα-mediated transcription (35). Interestingly, soy resulted in lower ERα mRNA after menarche and lower ERβ mRNA before menarche. When considered alongside soy effects on GATA-3, which was higher in pre-menarche and lower in post-menarche, 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 post-menarchal 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, while 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 in 2 of 4 CpG sites with maturity. This increase was subtle (~1%) and the biological relevance of such a modest change is unclear. For *ESR2*, we assessed methylation of the region that corresponds to the promoter 0N 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 ~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 within this region does not appear to regulate *GREB1* transcription. The *TFF1* promoter has high level of methylation, consistent with the fact that the region is low in CpG content. Although methylation may not be the main mechanism regulating transcriptional activity in low CpG promoters, DNA methylation status is important for tissue-specific regulation of certain genes including *TFF1* (39, 45). Dramatic change in methylation is typically attained in genes only under specific circumstances such as cancer and neurodevelopmental disorders, but a study in human embryonic kidney carcinoma cell line derivative showed that mild differences in methylation level may repress gene transcription (46). Here, we found that the change in methylation level around the ERE in the *TFF1* promoter across puberty was relatively mild (~5–10%) but the CpG methylation was inversely correlated with *TFF1* expression. Further investigation is needed to evaluate the biological relevance of changes in *TFF1* promoter methylation across puberty as a biomarker of ERα responsiveness.

GATA-3 is required for luminal differentiation, *ESR1* promoter activity and ER-mediated transcription (47). We showed that in the macaque breast, GATA-3 was expressed in ductal and alveolar luminal epithelial cells but not myoepithelial cells, as in mice (48). Interestingly, GATA-3 expression in the soy group was high around the time of menarche, which could indicate a higher luminal differentiation at this stage as GATA-3 promotes the differentiation of lineage-restricted progenitor cells (23). This finding is consistent with the large increase of lobuloalveolar differentiation found in these animals (18), suggesting a soy promotional effect on breast differentiation through menarche. There was also a trend of lower GATA-3 with soy following menarche, which was consistent with the pattern of ERα and *GREB1* mRNA and a study in ovariectomized mice that showed downregulation of GATA-3 expression through ER modulation induced by exogenous estrogen (49).

The breast is capable of synthesizing and activating estrogen locally by the interaction of various enzymes. Here, we showed that the immunoreactivity profile of these enzymes in the macaque breast is comparable to that in the normal human breast (50), supporting the translational potential of the macaque 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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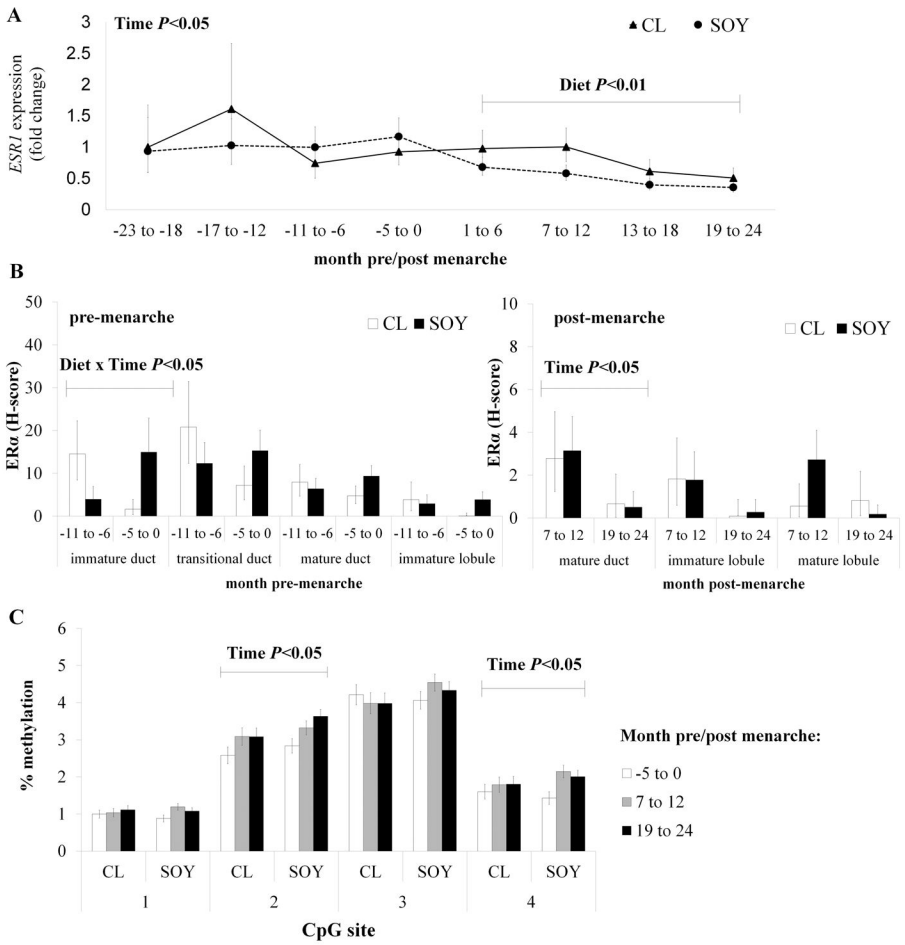

Figure 1.

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 post-menarche (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.

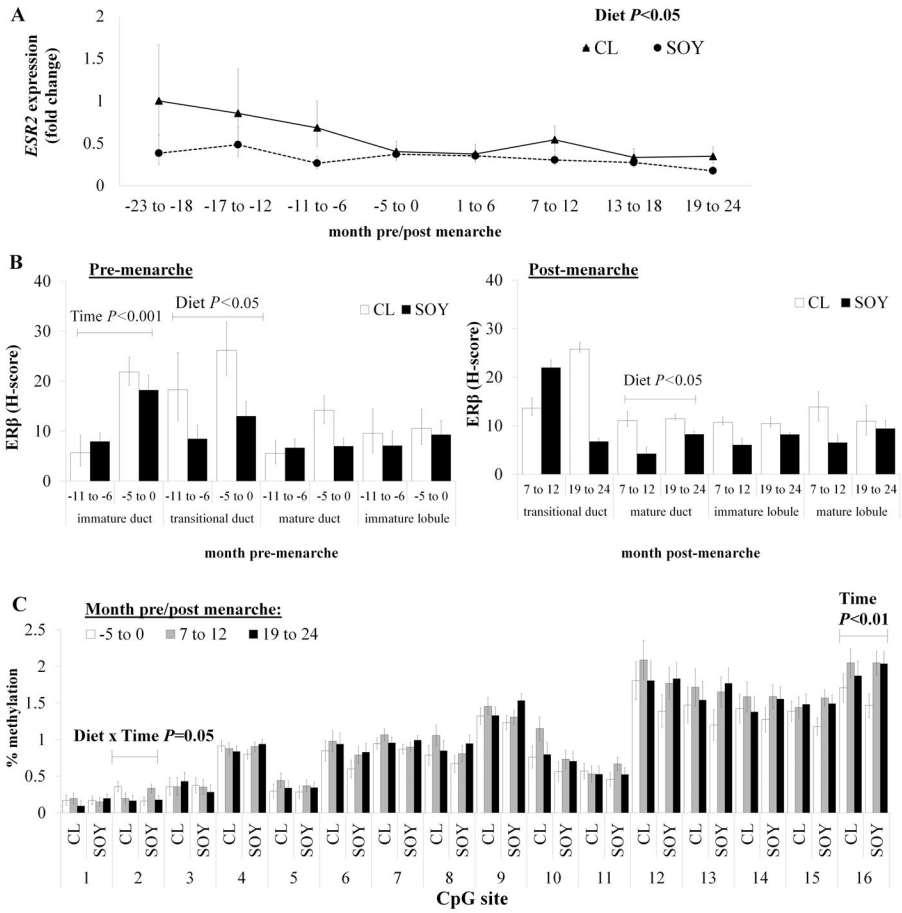

Figure 2.

ERβ expression and promoter methylation in the mammary gland across the pubertal transition. *ESR2* mRNA did not change over time; the overall expression was lower with soy (A). ERβ protein expression was lower in transitional ducts (before menarche) and mature ducts (after menarche) (B). There was no effect of diet or time on methylation of CpG sites in the promoter upstream of *ESR2* (C). Values are LSM for n=11–17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

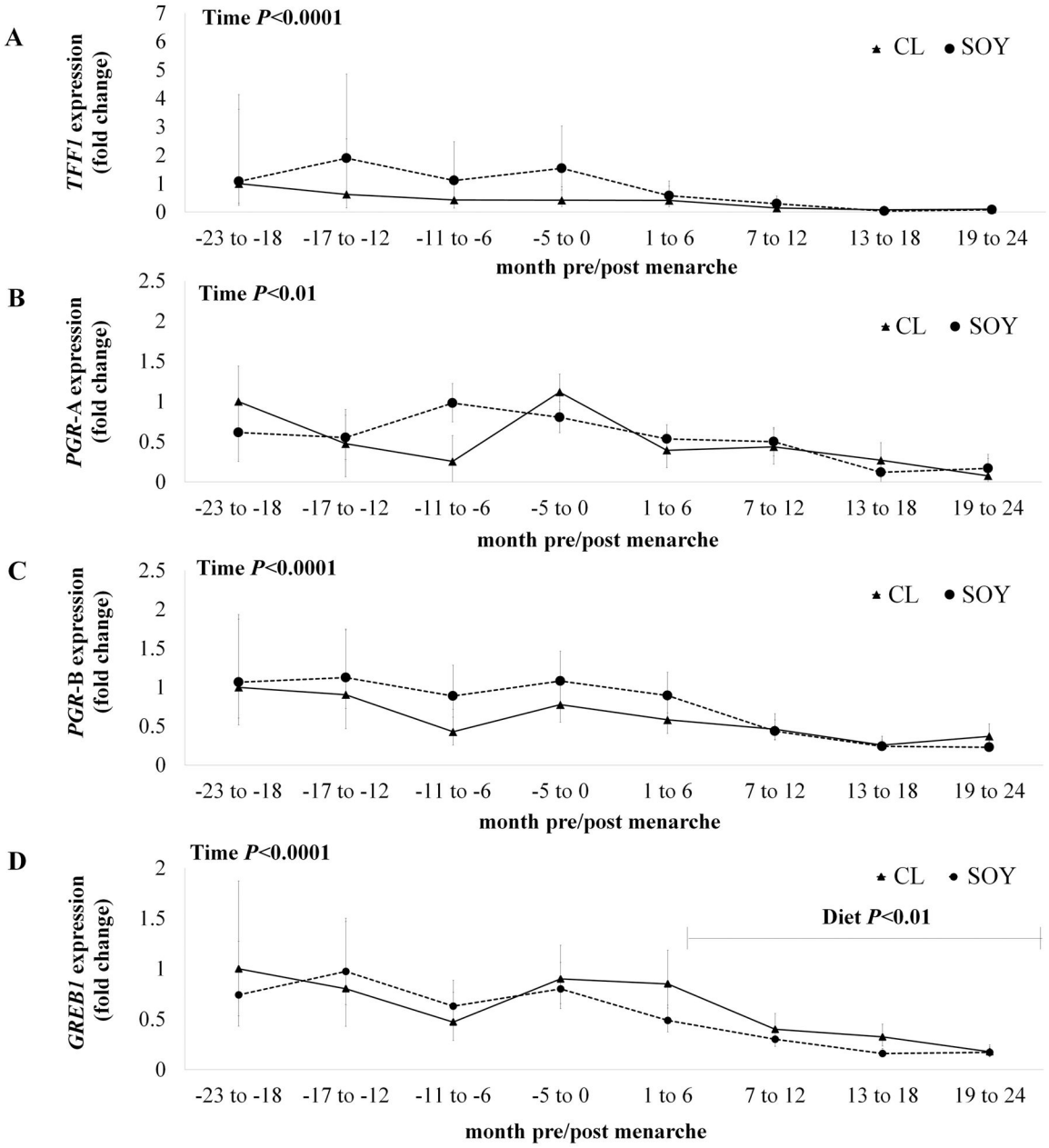

Figure 3.

Expression of ER-regulated markers in the mammary gland during the pubertal transition. mRNA levels for *TFF1* (A) and *PGR* (B,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.

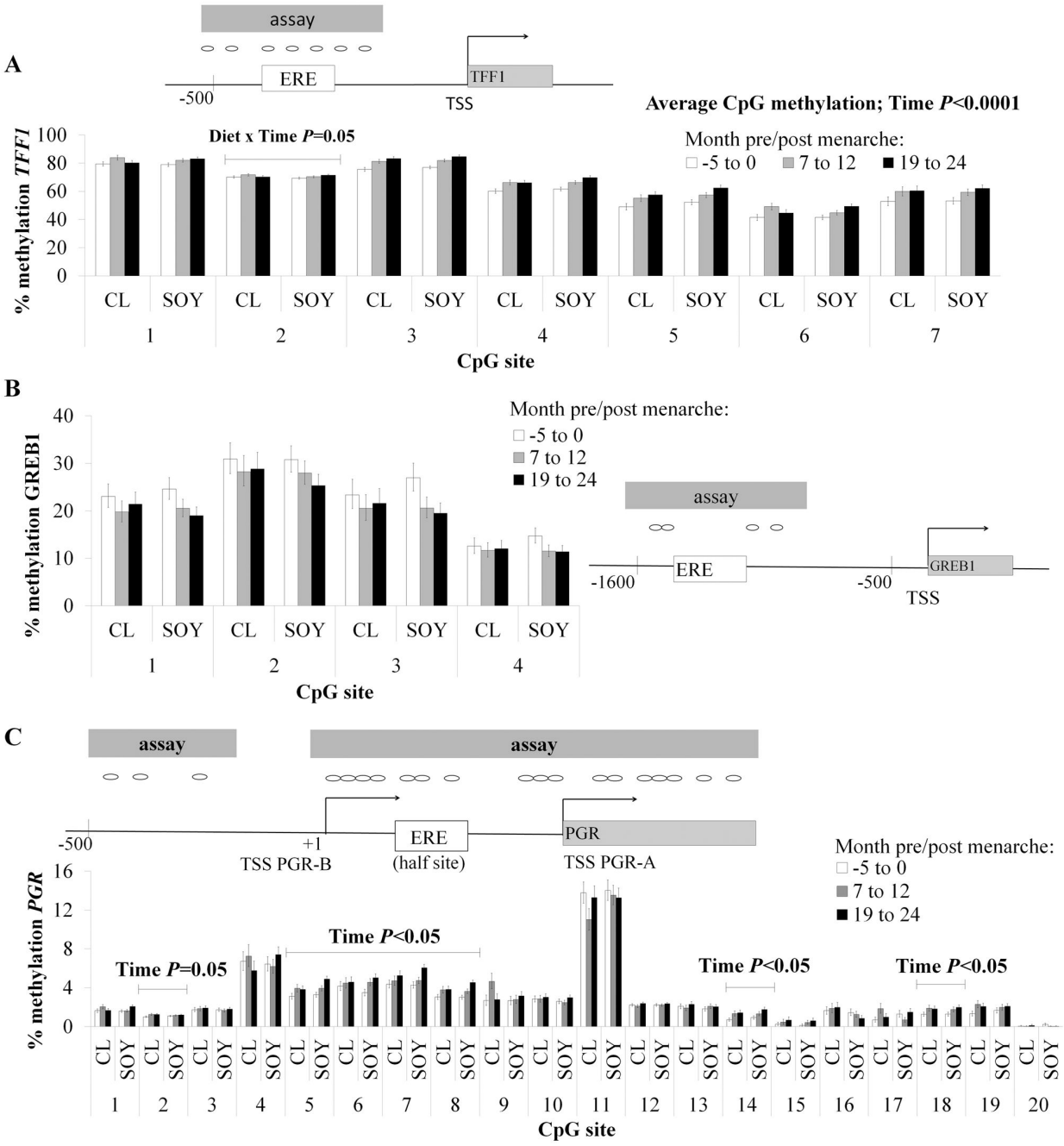

Figure 4.

Promoter methylation of ER-regulated markers. Methylation of 7 CpG sites near the ERE within the *TFF1* promoter increased with time (A). There was no diet or time effect on 4 CpG sites near the ERE proximal to *GREB1* (B). The level of CpG methylation within *PGR* promoters A and B was low with no effect of diet or time (C). Values are LSM for n=11–17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

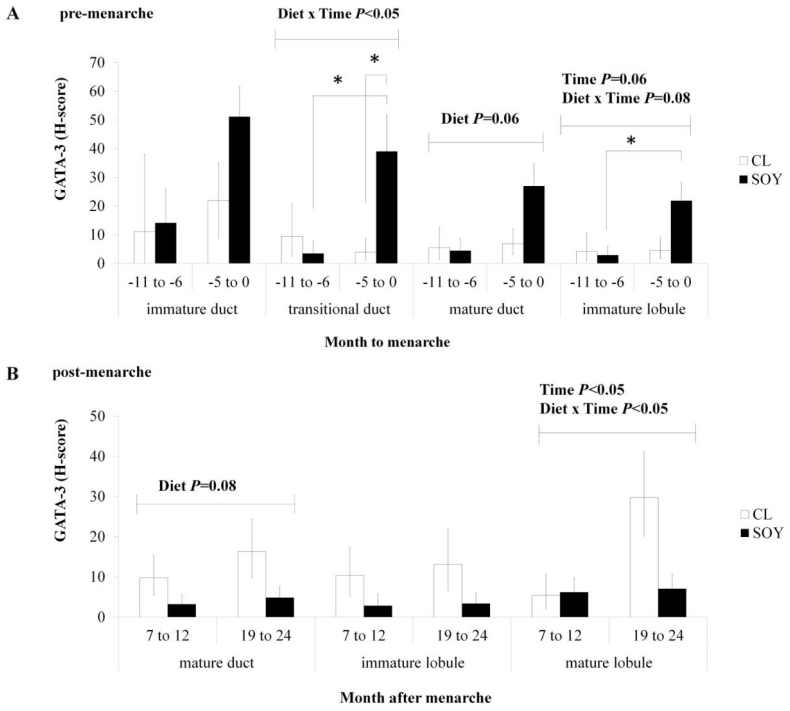

Figure 5.

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).

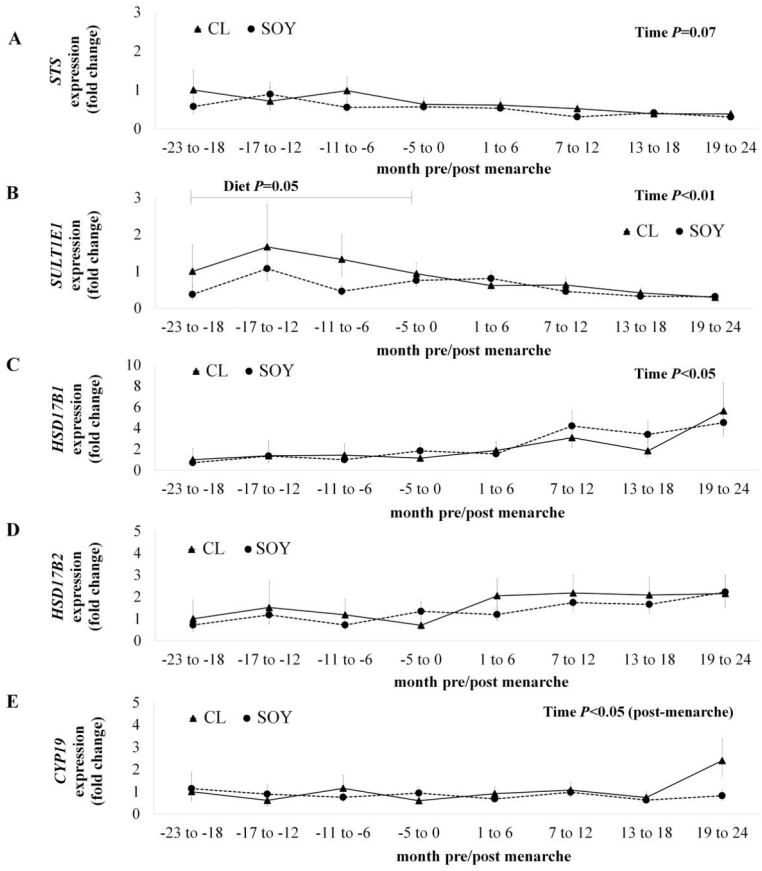

Figure 6.

mRNA levels for genes related to estrogen conjugation (A, B) and synthesis/bioactivation (C–E) were not affected by dietary treatment. *STS* (A) and *SULT1E1* (B) decreased with maturity, whereas *HSD17B1* and *CYP19* increased (C and E), and *HSD17B2* (D) did not differ with time. Values are LSM for n=11–17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.