Early Soy Exposure via Maternal Diet Regulates Rat Mammary Epithelial Differentiation by Paracrine Signaling from Stromal Adipocytes

Ying Su, Kartik Shankar, and Rosalia C. M. Simmen

Arkansas Children’s Nutrition Center, Department of Physiology and Biophysics, and Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR 72202

Abstract

Diet-mediated changes in transcriptional programs that promote the early differentiation of the mammary gland may lead to reduced breast cancer risk. The disparity in adult breast cancer incidence between Asian women and Western counterparts is attributed partly to high soy food intake. Here, we conducted genome-wide profiling of mammary tissues of weanling rats exposed to soy protein isolate (SPI) or control casein (CAS) via maternal diet to evaluate the contribution of early exposure on mammary gene expression. Of the identified 18 up- and 39 downregulated genes with SPI relative to CAS, a subset was associated with lipid metabolic pathways, consistent with reduced mammary adipocyte size and suggesting stromal adipocyte-specific genomic changes. Female offspring of rats fed SPI tended to have fewer terminal end buds (P = 0.06) and had significantly lower body weight and abdominal fat mass. To demonstrate the functional consequence of SPI-mediated adipocyte metabolic changes on neighboring mammary epithelium, the expression of in vivo regulated genes in 3T3-L1 adipocytes treated with soy isoflavone genistein and effects of the resultant conditioned medium on the differentiation of HC11 mammary epithelial cells were evaluated by quantitative RT-PCR and/or Western immunoblots. In differentiated 3T3-L1, genistein decreased fatty acid synthase and stearoyl-CoA desaturase and increased hydroxysteroid 11-β dehydrogenase 1 expression. CM from genistein-treated adipocytes had higher adiponectin levels and augmented prolactin-induced, glucocorticoid-regulated β-casein levels. These findings suggest that soy-associated components, by targeting mammary adipocytes, alter paracrine signaling to enhance mammary epithelial differentiation, with important implications for the prevention of breast cancer associated with obesity and obesity-related diseases. J. Nutr. 139: 945–951, 2009.

Introduction

Four of the top 10 contributors to mortality in the United States to date are chronic diseases that are influenced by diet, namely heart disease, stroke, type 2 diabetes, and cancer (1). Among women, breast cancer is the leading cause of malignancy, with ~200,000 new cases and 50,000 deaths reported annually in the United States alone (2). Epidemiological and experimental studies strongly implicate diet as a modifiable determinant of breast cancer risk (3–5). Multiple mechanisms potentially underlying the chemopreventive and chemoprotective effects of dietary factors on mammary tumorigenesis have been described (6–8). Nonetheless, the period of vulnerability to carcinogenic agents and during which diet may confer maximal benefits to attenuate adult breast cancer risk remains unclear. The mammary gland is among the most complex tissues in mammalian organisms. The dynamic nature of its cellular compartments, its developmental plasticity (9), and the plethora of local and endocrine-derived factors that regulate its transcriptional programs (10,11) offer considerable challenges for fully elucidating mechanistic aspects of dietary component bioactivities. Thus, an integrated landscape that takes into consideration how dietary factors may influence communication between stromal and epithelial compartments and the cycles of proliferation, differentiation, and apoptosis in specific mammary cell types requires further definition to effectively exploit dietary health benefits.

The disparity in breast cancer risk in women living in Asia and the Western hemisphere has prompted investigations into the mammary tumor protective activities of soy-based diets (12). Work from our laboratory and by other groups has demonstrated that rats lifetime-fed soy protein isolate (SPI)7 or diets

---

1 Supported by the USDA-CRIS-6251-5100002-06S to the Arkansas Children’s Nutrition Center (R.C.M.S.).
2 Y. Su, K. Shankar, and R. C. M. Simmen, no conflicts of interest.
3 Supplemental Tables 1–3 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.
4 To whom correspondence should be addressed. E-mail: simmenrosalia@uams.edu.
5 8022-3166/08 $8.00 © 2009 American Society for Nutrition.
7 First published online March 25, 2009; doi:10.3945/jn.108.103820.
8 Arkansan for Medical Sciences, Little Rock, AR 72202
9 Difference in breast cancer is the leading cause of malignancy, with ~200,000 new cases and 50,000 deaths reported annually in the United States alone (2).
10 Epidemiological and experimental studies strongly implicate diet as a modifiable determinant of breast cancer risk (3–5).
11 Multiple mechanisms potentially underlying the chemopreventive and chemoprotective effects of dietary factors on mammary tumorigenesis have been described (6–8).
12 Nonetheless, the period of vulnerability to carcinogenic agents and during which diet may confer maximal benefits to attenuate adult breast cancer risk remains unclear.
13 The mammary gland is among the most complex tissues in mammalian organisms. The dynamic nature of its cellular compartments, its developmental plasticity (9), and the plethora of local and endocrine-derived factors that regulate its transcriptional programs (10,11) offer considerable challenges for fully elucidating mechanistic aspects of dietary component bioactivities. Thus, an integrated landscape that takes into consideration how dietary factors may influence communication between stromal and epithelial compartments and the cycles of proliferation, differentiation, and apoptosis in specific mammary cell types requires further definition to effectively exploit dietary health benefits.
14 The disparity in breast cancer risk in women living in Asia and the Western hemisphere has prompted investigations into the mammary tumor protective activities of soy-based diets (12). Work from our laboratory and by other groups has demonstrated that rats lifetime-fed soy protein isolate (SPI)7 or diets
15 Supplemental Tables 1–3 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.
16 To whom correspondence should be addressed. E-mail: simmenrosalia@uams.edu.
17 8022-3166/08 $8.00 © 2009 American Society for Nutrition.
rich in isoflavones, specifically genistein, have increased resistance to chemically induced mammary tumor formation (13–15). Although a composite of mechanisms acting at the levels of tumor suppressors, oncogenes, and DNA repair enzymes likely underlie the tumor protective effects of soy- or isoflavone-enriched diets (16–20), the enhanced differentiation status of the mammary gland ductal structures prior to carcinogen insult, as measured by increased expression of differentiation-associated genes and decreased proliferation status of mammary epithelial cells, is considered a key determinant in this process (4,5). Thus, the findings that female rats exposed to genistein after birth but prior to puberty had accelerated differentiation of terminal end buds (TEB) and subsequently, increased latency and reduced incidence and multiplicity of breast tumors (21), suggested that adult breast cancer susceptibility may be attenuated by dietary factors through induction of early proliferation differences in mammary glands. The implications of the latter are noteworthy given the high percentage (25%) of infants in the United States consuming soy infant formula and the increasing popularity of soy food intake among pregnant and breast-feeding mothers and may explain the decreased incidence of breast cancer in Asian-American women who consumed soy-rich diets during adolescence (22).

In addition to their antitumor activities, soy and associated isoflavones are considered to be antilipogenic (23). Decreased body weight, fat mass, and insulin resistance were observed in several genetically modified or diet-induced obese rodent models consuming a soy protein-based diet (24). Similar effects were also reported in obese or overweight human subjects with short-term dietary intake of soy-rich foods, although a consistent outcome remains elusive (24,25). Given that obesity is linked to breast cancer (26,27) and is associated with defects in mammary gland formation and impaired mammary differentiation (28), the relative adiposity of mammary tissues may contribute to the differentiation status of mammary epithelial cells via stromal adipocyte-epithelial interactions.

Our objective in this investigation was to evaluate whether early exposure to soy foods via maternal diet influences the transcriptional program in mammary tissues of prepubertal female rats and determine how these genomic changes may functionally affect the differentiation status of mammary epithelial cells.

**Materials and Methods**

**Rat studies.** Animal experiments were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Time-mated Sprague Dawley rats (Charles Rivers Laboratories) were housed individually in polycarbonate cages under conditions of 24°C, 40% humidity, and a 12-h-light/dark cycle. Rats at gestation d 4 were randomly assigned to semipurified isocaloric diets made according to the AIN-93G formula (29), with corn oil substituting for soybean oil and containing as sole protein source either casein (CAS; New Zealand Milk Products) or SPI (lot no. 241–2; Solae). SPI contained 3.21 g/kg total isoflavones, including 1.87 g/kg genistein-containing compounds and 1.22 g/kg daidzein-containing compounds; these correspond to 1.08 g/kg and 0.69 g/kg aglycone equivalents of genistein and daidzein, respectively, per kg. Rats consumed food and water ad libitum. At delivery, all pups from dams of the same diet groups were pooled and 10 pups (5 per sex) were randomly assigned to each dam for suckling. At weaning [postnatal d (PND) 21], female pups (n = 5) from CAS and SPI dietary groups were killed and the inguinal mammary gland ( gland no. 4) pair of each rat was removed. Portions of the left gland were fixed for paraffin [postnatal d (PND) 21], female pups (sex) were randomly assigned to each dam for suckling. At weaning pups from dams of the same diet groups were pooled and 10 pups (5 per

**Quantitative RT-PCR.** Total RNA (1 μg) from mammary glands and cell extracts (HC11, 3T3-L1) was reverse-transcribed using random hexamers and MultiScribe Reverse Transcriptase in a 2-step RT-PCR (Applied Biosystems). Gene-specific primers (Supplemental Table 1) were designed using PrimerExpress (Applied Biosystems) to yield a single amplicon. Quantitative RT-PCR was performed with the SYBR Green detection system (Applied Biosystems) as previously described (30). mRNA levels were normalized to 18S rRNA (for mammary tissues), Actb (β-actin; for HC11 cells) and Ppia (cyclophilin A; for adipocytes) to control for input RNA. In all cases, expression of normalization genes did not differ in expression between treatment groups.

**Cell cultures, conditioned medium, and hormonal stimulation.** All cells were routinely grown at 37°C in an atmosphere of 5% CO2/95% air. The mouse mammary epithelial HC11 cells (kindly provided by Dr. Jeffrey M. Rosen, Baylor College of Medicine) were grown in medium containing RPMI-1640 (Sigma-Aldrich) supplemented with 10% (v/v) bovine calf serum (Invitrogen), 10 μg/L epidermal growth factor (Invitrogen), 5 mg/L insulin (Sigma-Aldrich), and 50 mg/L gentamicin (Sigma-Aldrich). 3T3-L1 fibroblast cells (American Type Culture Collection) were maintained in growth medium consisting of DMEM (Sigma-Aldrich), 5 mg/L insulin (Sigma-Aldrich), and 50 mg/L gentamicin. The culture medium was replaced with growth medium supplemented with 1 mg/L insulin. After 48 h incubation (d 4), medium was replaced with growth medium and

**RNA isolation, microarray, gene expression data analysis, and hierarchical clustering.** Total RNA was extracted from mammary glands of PND 21 rats (n = 5 each for CAS and SPI diet groups) in TRIzol following the manufacturer’s instructions (Invitrogen), further purified with the RNeasy Mini kit (QIAGEN), and finally by on-column DNA digestion with RNase-Free DNase (QIAGEN). Integrity of total RNA was monitored by absorbance ratios (A260/A280) and by analyses on an Agilent 2100 Bioanalyzer (Agilent Technologies). For each RNA sample, double-stranded cDNA was synthesized (30). The procedures for the generation of biotin-labeled cRNA and processing of GeneChip arrays followed published procedures from our group (30). The microarray chips were scanned using an Agilent GeneArray laser scanner. The microarray data sets are available as accession number GSE12217 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information. The intensity values of different probe sets generated by Affymetrix GeneChip Operating Software were imported into GeneSpring 7.3 software (Silicon Genetics) for data analysis. The CEL files containing the probe level intensities were processed using the Robust Multiarray Analysis algorithm (GeneSpring) for background adjustment, normalization, and log2-transformation of perfect match values. Subsequently, the data were subjected to per-chip and per-gene normalization using GeneSpring normalization algorithms, except for the last step in which each Gene Chip was normalized to the median of the control group instead of the mean. The normalized data were compared between the 2 diet groups. The resulting gene list included genes with a fold-change value $\geq 1.3$ and a P-value $< 0.05$ by Student’s t test. Correlation-based hierarchical clustering between diet groups was performed by the standard correlation for distance measure algorithm (GeneSpring). The output data were displayed as a heat map based on the measured intensity values of the genes in the gene list (above) and is represented as a hierarchical tree with branches to indicate the relationship among the different groups.
medium was changed again at d 6. Differentiation as judged by accumulation of lipid droplets was evident in 95–100% of cells. On d 8, fully differentiated cells were treated with genistein (4,5,7-trihydroxyisoflavone, 5 μmol/L) in dimethylsulfoxide (DMSO) or DMSO alone, in phenol red-free DMEM containing low serum (0.5% charcoal-stripped fetal bovine serum). Cells were collected 48 h later and extracted for RNA and protein. Two to 3 independent experiments were carried out, with each treatment performed in triplicate.

For collection of conditioned medium (CM), 3T3-L1 cells were treated with genistein (5 μmol/L) in DMSO or DMSO alone for 48 h (starting at d 8 of the differentiation protocol). Following treatments, cells were washed with Hank’s balanced salts solution and incubated in low serum (0.5% fetal bovine serum)-containing DMEM. CM collected 24 h later was centrifuged at 1500 g and stored at −80°C prior to use. HCl1 cells, 2 d after reaching confluence, were incubated in undiluted (full-strength) CM with added ovine prolactin (Prl) (1 or 5 mg/L; oPRL-21, AFP-10692C; courtesy of Dr. AF Parlow, National Hormone and Peptide Program, NIH) in the presence or absence of RU486 (1 μmol/L; Sigma). Total incubation time was 72 h, with medium refreshed 48 h after the start of treatments.

**Histological analyses.** For whole-mount analyses, the left inguinal mammary glands from PND 50 rats (n = 6/diet group) were processed as previously described (31). The numbers of TEB in 3 areas (27 mm²/area) per slide per rat, with n = 4 rats evaluated per diet, were analyzed following previously described procedures (31).

To determine adipocyte cell size, sections of the right inguinal mammary glands from PND 21 rats were stained with hematoxylin and eosin. Adipocyte areas were measured in 2–3 random fields (200–300 cells per field) per slide from 3 individual rats per diet group using Axiovision software (Carl Zeiss AG).

Differentiated 3T3-L1 adipocytes grown in 6-well plates were stained with 0.5% Oil Red O (Sigma-Aldrich). Incorporated dye was extracted with DMSO and optical densities of extracts were measured at 492 nm.

**Western blots.** Immunoblot analysis was conducted as previously described (20). Primary antibodies used were 11β-dehydrogenase 1 (11β-Hsd1) (1:500; R&D System), fatty acid synthase (Fasn) (1:500; Abcam), and adiponectin (1:1000; Affinity BioReagents). Immunoreactive proteins were visualized using the Chemiluminescence Reagent Plus system (Perkin Elmer Life Sciences). Blots were stripped with Restore Western Blot stripping buffer (Pierce Biotechnology) prior to reprobing with other antibodies.

**Statistical analysis.** Data are presented as means ± SEM of 2–4 independent experiments as indicated. Significant differences between the means among diet groups was assessed by Student’s t test (between 2 dietary groups or control/genistein-treated 3T3-L1 cells), 1-way ANOVA, and 2-way ANOVA, followed by inspection of all differences between pairs of means by Tukey’s test. P ≤ 0.05 was considered significant, with 0.05 ≤ P < 0.1 as tendency to be significant.

**Results**

**Dietary SPI exposure alters body weight, fat mass, and mammary gland differentiation.** Female rats exposed to dietary SPI had lower body weights at PND 21, 33, and 50, respectively (Fig. 1A) and lower abdominal fat weight at PND 50 (Fig. 1B) compared with the control (CAS) group. At PND 50, rats of the SPI group tended to have lower TEB (P = 0.066; Fig. 1C) and higher mammary β-casein (Csn2) (P = 0.002) and Wap (P = 0.003) transcript levels relative to CAS rats of the same age (Fig. 1D).

**Gene expression profiles of prepubertal rat mammary glands.** To evaluate the early molecular events regulated by dietary SPI exposure in the developing mammary gland, we examined the gene expression profiles of whole mammary glands of weaning (PND 21) rats, using the Affymetrix RAEE230A gene microarray platform. Of the 14,280 unique genes analyzed, 57 transcripts were differentially regulated by dietary SPI relative to CAS. Among these, 18 were induced (by 30–50%) and 39 were repressed (by 25–40%) in the SPI group (Supplemental Tables 2 and 3). The mean expression values for each differentially expressed gene in the SPI relative to the CAS group were calculated and a heat map was generated (Supplemental Fig. 1A). The largest functional category for up- and downregulated genes was metabolism related (26.3%), with other regulated transcripts clustered under signal transduction, growth, extracellular matrix/cell adhesion, and protein folding/turnover signaling pathways. Of the SPI-regulated metabolism-related genes, 12 were associated with lipid metabolic pathways (Supplemental Fig. 1B). Genes related to adipocyte lipogenesis such as those involved in the biosynthesis of fatty acids [Fasn, stearoyl-CoA desaturase 1/2 (Scd1/2); stearoyl-CoA desaturase 1/2], fatty acid desaturase 1/2 (Scd1/2); stearoyl-CoA desaturase 1/2; and acetyl-CoA [ATP citrate lyase (Acly), acetoacetoy-CoA synthase; malic enzyme 1 (Me1)] were downregulated by SPI. Also downregulated by dietary SPI were genes that regulate cholesterol biosynthesis [7-dehydrocholesterol reductase (Dhcr7)] and fatty acid transport (solute carrier family 27, member 3). The

---

**FIGURE 1** Body weights at PND 21, 33, and 50 (A) and abdominal fat weight (B), number of TEB (C), and mammary gland Csn2 and Wap gene expression at PND 50 in CAS and SPI rats. Values in A–C are means ± SEM, n = 10 (A) or 5 (B, C). In D, individual data for 9 rats/group are shown. *Different from CAS, P < 0.05.
Different from CAS, 50- to 2500-μm² fat cell size, resulted in lower mean fat cell size compared with CAS-fed rats. Approximately 80% of adipocytes in the SPI group were in the 50- to 2500-μm² size range, compared to only 5% for the SPI group. By contrast, 60% of the adipocytes in the CAS group had 20% of the adipocytes in the 50- to 1500-μm² size range, with 37% of adipocytes in the 250- to 3500-μm² size range compared with only 5% for the SPI group.

Effects of SPI on mammary adipocyte size. Representative hematoxylin-eosin-stained sections of mammary fat pads from PND 21 rats are shown (Fig. 2B). Dietary exposure to SPI resulted in lower mean fat cell size compared with CAS-fed rats. Approximately 80% of adipocytes in the SPI group were in the 50- to 2500-μm² size range, with the majority falling in the 50- to 1500-μm² group. By contrast, 60% of the adipocytes in the CAS group were in the 50- to 2500-μm² size range, with 37% of those showing larger areas (150–2500 μm²; Fig. 2C). Further, the CAS group had 20% of the adipocytes in the 250- to 3500-μm² size range compared with only 5% for the SPI group.

Genistein regulates lipid metabolism-related genes in mature adipocytes. Given the substantial genistein exposure in utero of fetuses from pregnant dams consuming SPI (32), we determined whether the in vivo effects of dietary SPI on adipocyte lipid metabolism-related genes can be recapitulated by genistein in vitro, using mouse fibroblast 3T3-L1 cells. At a dose (0.5 μmol/L) comparable to that found in sera of PND 50 rats exposed for their lifetime to dietary SPI (33), genistein reduced the transcript levels of Fasn and Scd1 gene and augmented the expression of the Hsd11b1 gene in differentiated 3T3-L1 cells (Fig. 3A), consistent with the observed effects of SPI on the mammary expression of these genes in vivo. The changes in Hsd11b1 (P = 0.02) and Fasn (P = 0.08) gene expression were confirmed for the corresponding proteins by Western blot (Fig. 3B). The transcript levels of nuclear receptor PPARγ2 and of the adipokine leptin were unaffected by genistein (Fig. 3C).

CM from genistein-treated differentiated adipocytes promotes mammary epithelial cell differentiation. Because genistein-induced changes in adipocyte metabolism may alter adipocyte-secreted factors that can promote mammary epithelial cell differentiation by paracrine signaling, HC11 cells incubated in CM harvested from genistein (0.5 μmol/L) or vehicle-treated differentiated 3T3-L1 adipocytes, in the presence of Prl, were evaluated for Csn2 gene expression, the latter as an indicator of differentiation status (Fig. 4A). In 3 independent experiments, the protein concentrations of CM harvested from control (vehicle) and genistein-treated adipocytes did not differ (~0.8–1 g/L). CM from genistein-treated adipocytes had higher levels of immunoreactive adiponectin than CM from control-treated adipocytes at 24 h, which were further increased at 48 h (Fig. 4B). Expression of Csn2 was not detected in HC11 cells treated with either CM in the absence of Prl (data not shown). With added Prl (1 or 5 mg/L), however, epithelial cells treated with genistein-CM had higher Csn2 transcript levels than those treated with control CM (Fig. 4C). 3T3-L1 adipocytes generate the inactive glucocorticoid, 11β-dehydrocorticosterone, during differentiation (34). Induction of glucocorticoid signaling due to higher 11β-Hsd1 levels in genistein-treated adipocytes may contribute to the enhanced differentiation of HC11 cells by genistein-CM. HC11 cells, when pretreated with the glucocorticoid antagonist, RU486 (1 μmol/L), prior to the addition of genistein-CM had a loss of Csn2 gene expression relative to genistein-CM alone in the presence of Prl (Fig. 4D). RU486 in the absence of Prl did not affect Csn2 expression.

Discussion

The present study was undertaken to evaluate whether dietary factors influence early mammary gland development and to identify the biochemical pathways and signals that may underlie these effects. Our results show that exposure of female rats to SPI in utero until weaning via maternal diet significantly altered transcriptional programs associated with lipid metabolism in mammary glands. Using differentiated 3T3-L1 adipocytes in vitro, we further identified the soy bioactive component genistein as a likely candidate contributing to the in vivo effects of dietary SPI. Moreover, using CM harvested from genistein-treated 3T3-L1 cells, we demonstrated that the altered metabolism mediated in adipocytes by genistein has a functional consequence on the transcriptional programs associated with lipid metabolism in mammary epithelial HC11 cells. Together, our results provide a linear pathway whereby soy-associated bioactive components may act directly on mammary adipocytes to enhance the differentiation status of mammary epithelial cells. Given our previous findings that dietary SPI and genistein also influence genome-wide expression profiles of mammary epithelium (30), these data suggest that...
dietary factors inhibit mammary tumorigenesis by their cumulative alterations of transcriptional programs in distinct mammary compartments and underscore the importance of stromal-epithelial interactions in mammary gland development and tumorigenesis (35,36).

The decreased incidence of adult breast cancer in Asian women compared with their Western counterparts, while attributed to high-soy food intake, may be a function also of healthier lifestyles, including decreased consumption of high-energy foods. Recent studies have suggested a role of dietary soy in reducing body weight, fat mass, and plasma lipids in obese human subjects (24), although this remains controversial (25), and of genistein in decreasing adiposity in rodents (37). Consistent with these previous findings, female rats in our study demonstrated lower body weights from weaning to young adult stage. Moreover, rats at PND 50 had reduced accumulation of abdominal fat mass and lower TEB numbers and higher expression of epithelial differentiation-related genes Csn2 and Wap in mammary tissues with dietary SPI, concordant with an inverse association between body weight, fat mass, and mammary gland differentiation and supporting the purported linkage between obesity and related metabolic disorders and breast cancer risk (26,27).

We conducted genome-wide profiling of whole mammary tissues from prepubertal rats to investigate the contribution of altered lipid metabolism by dietary soy on the transcriptional programs of mammary stromal and epithelial compartments. Mammary epithelial cells during this period are characterized by maximal numbers of TEB. Our microarray data showing a disproportionately higher number of downregulated (39) than upregulated (18) genes in whole mammary tissues with early

**FIGURE 3** Expression of lipid metabolism genes (A), protein levels of Fasn and Hsd11b1 (B) and levels of Lep and Pparg2 transcripts (C) in differentiated 3T3-L1 adipocytes that were or were not treated with genistein. Transcript levels were quantified by QPCR and normalized to the control gene, Ppia (A,C). Protein levels of Fasn (molecular weight = 272 kDa) and 11β-Hsd1 (molecular weight = 36 kDa) were analyzed by Western blots. The loading control was an unrelated protein band migrating at ~30 kDa with Fasn antibody (B). Results are representative of 3 (A,C) or 2 (B) independent experiments and are means ± SEM. *Different from control, P < 0.05.

**FIGURE 4** Mammary epithelial HC11 Csn2 expression in CM from control and genistein-treated differentiated 3T3-L1 adipocytes. (A) Graphical representation of cell treatment and subsequent CM collection procedures. (B) Immunoreactive adiponectin (molecular weight = 30 kDa) in CM collected after 24 and 48 h and detected by Western blot. The loading control was an unrelated doublet migrating at ~250 kDa. (C) Transcript levels of Csn2 in cells treated with CM for 72 h in the presence of ovine Prl were normalized to the control gene β-actin and expressed relative to control CM-treated cells in the presence of Prl. (D) Transcript levels of Csn2 in cells incubated in the presence or absence of the glucocorticoid antagonist, RU486. In C and D, results are representative of 2 independent experiments and are means ± SEM. Means without a common letter differ, P < 0.05.
dietary SPI exposure follows a similar trend in mammary epithelial cells of PND 50 rats fed SPI compared with those fed CAS (30). SPI altered the expression of known genes associated with de novo synthesis of fatty acids (Mek, Acly, acetoacetyl-CoA synthase, Fasn), fatty acid desaturation (fatty acid desaturase, Scd1/2), cholesterol production (Dbcr7), and glucocorticoid activation (Hsd11b1) as well as newly identified genes encoding nonsecreted proteins such as adiponutrin, the latter with postulated functions in insulin sensitivity, glucose metabolism, and triglyceride recycling (38). These genomic changes were associated with reduced adipocyte cell size, indicating mammary adipocytes as major targets of SPI bioactive components in vivo. Clinical and animal models have shown that adipocyte size is an important determinant of insulin sensitivity (39,40). Larger adipocytes are more insulin resistant and display distinct gene expression profiles, with higher expression of inflammatory genes and other secreted proteins such as leptin (41). Thus, our data are consistent with the reported effects of soy diets containing isoflavones in decreasing insulin levels and/or increasing insulin sensitivity (42,43). Interestingly, we did not identify lipoprotein lipase to be modulated by SPI, given this enzyme’s key regulation of triglyceride metabolism (44) and the previously demonstrated regulation of its expression by genistein in primary human adipocytes in vitro (45) and in mice in vivo (46). To our knowledge, this is the first report of the antilipogenic transcriptional effects of soy on developing mammary glands. However, because the numbers of genes altered by dietary soy are quite limited, SPI may also influence the secretion of adipocyte factors independent of transcriptional changes.

Based on the reported antiobesogenic activity of the major soy isoflavone genistein (37,46), we tested the hypothesis that genistein mediates in part the inhibition of lipogenesis in mammary adipocytes in vivo. We found that the in vivo effects of SPI on Fasn, Scd1, and Hsd11b1 were recapitulated in genistein-treated 3T3-L1 cells. Further, relative to control CM, CM harvested from genistein-treated differentiated 3T3-L1 adipocytes increased Csn2 transcript levels in nonmalignant HC11 mammary epithelial cells, the latter widely used to evaluate lactogenic hormone-induced differentiation (47) and, most recently, used to demonstrate genistein’s effects on cellular E-cadherin signaling (20). Our findings that in the presence of Prl, the prodifferentiation effect of genistein-treated adipocyte CM was inhibited by the glucocorticoid antagonist RU486 coupled with the observed induction by genistein of Hsd11b1 expression in vivo and in vitro suggest that enhanced adipocyte paracrine signaling mediated by glucocorticoids partly underlies the higher mammary epithelial Csn2 expression. In this regard, glucocorticoids have been shown to synergize with the Prl signaling mediator STAT5a at the level of the Csn2 promoter to induce this gene’s transcription (48).

In the present study, dietary exposure to CAS and SPI occurring via the maternal diet recapitulates the early exposure to soy foods of the female population in Asian countries where the incidence of breast cancer is notably lower (3,12). Because dietary exposure is indirect, the biological effects reported herein likely reflect the actions of components that are able to transfer across the placenta or through milk. Although factor(s) other than genistein may be responsible for the observed effects, the antilipogenic actions of this isoflavone in vivo and in vitro have been reported (37,45,46). In dams consuming genistein, bioactive genistein-aglycone levels in fetal and neonatal serum were lower by only 20% from that found in maternal serum (49), suggesting substantial fetal exposure. Amniotic fluids contain similar levels of genistein-aglycone when corresponding dams consumed either SPI or genistein-supplemented CAS diets (32). Further, although lactational transfer of genistein to pups from dams consuming genistein may be limited (50), the global gene changes noted in mammary tissues of weanling rats may also be a consequence of neonatal responses to fetal exposure. Our findings that in utero–only exposure to genistein-supplemented CAS diet increased tumor latency in female offspring administered the chemical carcinogen NMU (51) is consistent with this possibility.

Our studies have raised a number of intriguing questions for future research efforts. One relates to the potential direct role of the adipokine adiponectin in mammary epithelial differentiation. Serum adiponectin levels are high in human plasma (52) and reported to be inversely correlated with breast cancer risk (53). Given that genistein-CM had higher levels of immunoreactive adiponectin than control CM (our study) and that in liver and skeletal muscle, adiponectin stimulation of fatty acid oxidation leads to a reduction of lipid content in these tissues (54), dietary soy induction of ‘local’ adipocyte-epithelial signaling mediated by adipocyte-secreted adiponectin is likely. Another relevant question relates to the influence of developmental context on the nature of adipocyte-epithelial interactions and how this may relate to the lower breast cancer/tumor incidence seen in humans and animal models with dietary soy intake prior to adolescence/prepuberty (22). Finally, given that obesity is a risk factor for breast cancer in postmenopausal women (26), it would be worthwhile to examine whether soy-rich diets alter similar transcriptional programs in mammary tissues of pre- and postmenopausal obese women.

In conclusion, SPI altered the lipogenic signature of developing mammary glands, suggesting early effects through maternal diets on the stromal adipocyte transcriptome. These genomic changes, which were recapitated by the soy isoflavone genistein in 3T3-L1 cells, were associated with higher mammary epithelial differentiation in vivo and in vitro, suggestive of enhanced adipocyte-epithelial signaling. We suggest that the further understanding of the mechanisms underlying this paracrine signaling during early mammary development could lead to novel targets and effective dietary strategies for the prevention of breast cancer associated with obesity and obesity-related diseases.

Acknowledgments
We thank Dr. Rijin Xiao for assistance with the microarray experiments and Dr. Frank A. Simmen for helpful discussions and critical reading of the manuscript.

Literature Cited


