Expression profiling of rat mammary epithelial cells reveals candidate signaling pathways in dietary protection from mammary tumors

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Su Y, Simmen FA, Xiao R, Simmen RCM. Expression profiling of rat mammary epithelial cells reveals candidate signaling pathways in dietary protection from mammary tumors. Physiol Genomics 30: 8–16, 2007. First published March 6, 2007; doi:10.1152/physiogenomics.00023.2007.—The role of diet in the prevention of breast cancer is widely accepted, yet little is known about how its biological effects mitigate susceptibility to this disease. Soy consumption is associated with reduced breast cancer risk in women, an effect largely attributed to the soy isoflavone genistein (Gen). We previously showed reduced incidence of chemically induced mammary tumors in young adult rats with lifetime dietary intake of soy protein isolate (SPI) than in those fed the control diet containing casein (Cas). To gain insight into signaling pathways underlying dietary tumor protection, we performed genome-wide expression profiling of mammary epithelial cells from young adult rats lifetime fed Cas, SPI, or Cas supplemented with Gen. We identified mammary epithelial genes regulated by SPI (79 total) and Gen (96 total) using Affymetrix rat 230A GeneChip arrays and found minimal overlap in gene expression patterns. We showed that the regulated transcripts functionally clustered in biochemical pathways involving metabolism, immune response, signal transduction, and ion transport. We confirmed the differential expression of Wnt (Wnt5a, Sfrp2) and Notch (Notch2, Hes1) signaling components by SPI and/or Gen using quantitative real-time PCR. Wnt pathway inhibition by Gen was supported by reduced cyclin D1 immunoreactivity in mammary ductal epithelium of Gen relative to Cas and SPI groups, despite comparable levels of membrane-localized E-cadherin and β-catenin. Identification of distinct Gen and SPI responsive genes in mammary epithelial cells may define early events contributing to tumor protection by diet relevant to the prevention of breast and other types of cancer.

mammary gland; genistein; soy proteins; Wnt signaling; tumorigenesis

THE MAMMARY GLAND IS AMONG the most complex tissues in biology. In rodents as well as in human females, mammary gland development occurs primarily postnatally when the ovarian steroid hormones estrogen and progesterone execute overlapping and distinct regulation leading to maturation of this tissue (16). The rudimentary ductal tree with simple branching structures initially observed at birth undergoes rapid growth at puberty and elongates, bifurcates, and finally penetrates the periphery of the stromal fat pad. During pregnancy, more dramatic changes occur, in particular the formation of a complex lobuloalveolar ductal network in preparation for milk synthesis at lactation. At weaning, the mammary gland differentiates, is significantly reduced in size, and reverts to a phenotype with ductal structures of the mature nonpregnant state. The specific programs of cellular proliferation, epithelial and mesenchymal differentiation, and apoptosis in the mammary gland are regulated by diverse molecules, acting in a specific temporal and spatial manner and are themselves subject to multiple regulation (22). In addition to estrogen and progesterone, pituitary hormones and locally acting growth factors, cytokines, and stroma-derived signaling molecules contribute to the developmental cycle of the functional mammary gland (27, 33, 37, 61).

Given the complex nature of its regulation, in part due to significant cross talk among signal transduction pathways for local and endocrine-derived factors, the mammary gland, not surprisingly, is highly subject to deregulation, which can result in the development of carcinoma (53). This tissue also exhibits remarkable plasticity and its developmental program can be altered by positive and negative environmental factors including diet (5, 23). Indeed, important emerging literature based on epidemiological studies support the influence of diet on adult breast cancer risk (1, 15). Because breast cancer is the most frequent malignant disease of women in the Western world, with 200,000 new cases and 50,000 deaths annually (25), further insights into the influence of diet, a highly modifiable risk factor, on mammary cancer initiation and progression may provide strategies for eradicating, or at least, mitigating the incidence of this disease.

Epidemiological and several case-control studies have indicated a negative correlation between breast cancer incidence and intake of soy-rich foods (4, 34, 41, 60). The observations of a primary role for soy products in reducing cancer risk initially derived from reports that Asian females, who consume at least 10–20 times more soy products than U.S. females, have two- to eightfold lower incidence of the disease (55, 62). Using rat models of chemically induced tumorigenesis, we confirmed the effects of soy protein isolate (SPI), which contains the isoflavones genistein (Gen), daidzein, and their respective β-glycosides, in decreasing tumor incidence and increasing tumor latency in young adult females lifetime-fed SPI relative to those fed the control diet casein (Cas) (20, 56, 59). Furthermore, we showed that induction of the expression of the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) (30) in mammary ductal epithelial structures of rats fed SPI or Cas supplemented with the major soy isoflavone Gen may partly underlie the dietary protective effects of soy-rich foods (17). Given the reported context- and dose-dependent estrogen agonist and antagonist activities of Gen (32), the popularity of soy-rich foods or diets enriched or supplemented with Gen as an alternative to postmenopausal hormonal therapy or for additional health benefits (4, 7, 34),...
and the “hidden” presence of soy and soy derivatives in many processed foods, the importance of understanding the effects of soy and its biologically active components on signaling pathways that orchestrate the normal development of the mammary gland is paramount. Of particular interest is if, and if so to what extent, these diets influence the expression levels and genetic pathways of oncogenes, tumor suppressor genes, and genes involved in the maintenance of stem cells and stem cell niche, all of which are important to the evolution of mammary cancer (29, 36, 42, 46).

In the present study, we used mammary epithelial cells (MEC) isolated from young adult female rats of Cas, SPI, and Gen dietary groups in microarray analysis as an unbiased approach to investigate potential biological and molecular pathways altered by Gen similar to or distinct from those of soy proteins. We identify biochemical pathways whose functional components in MEC are altered by each diet. Furthermore, we present evidence for the downregulation of the proto-oncogene Wnt signaling components (28, 39, 42) as likely to be contributory to tumor protective mechanisms of SPI- and Gen-based diets. Our analyses present new putative targets for further understanding dietary control of normal mammary development, which may be relevant to dietary prevention of breast cancer.

MATERIALS AND METHODS

Animal studies. Animals were maintained in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, following procedures approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Time-mated Sprague-Dawley rats purchased from Charles River Laboratories (Wilmington, MA) were housed individually in polycarbonate cages under conditions of 24°C, 40% humidity, and a 12-h light-dark cycle. Rats at gestation day (GD) 4 were randomly assigned to one of three semipurified isocaloric diets made according to the AIN-93G formulation (44), with corn oil substituting for soybean oil and containing as sole protein source either Cas (New Zealand Milk Products, Santa Rosa, CA) or SPI (Solae, St. Louis, MO). These diets are: 1) Cas as sole protein source; 2) SPI as sole protein source and containing Gen (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg) as aglycone equivalents; and 3) Cas as sole protein source to which was added Gen in the aglycone form (Gen, 250 mg/kg feed; Sigma Chemical, St. Louis, MO). Animals were provided 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. Female pups were weaned at postnatal day (PND) 21 to the same diet as their dams and were fed this diet throughout the study. Female pups of all three groups maintained similar body weights and did not differ in the duration of their estrous cycles (data not shown). At PND50, female pups (n = 15 each for Cas and SPI groups; n = 10 for Gen group) were killed, and the abdominal mammary gland (number 4) pairs were removed. A portion of the left mammary gland was fixed for paraffin embedding; the right gland was immediately homogenized in TRIzol (Invitrogen, Carlsbad, CA) and set aside for use in a different study. Mammary gland (number 2) pairs were dissected from the same animals for isolation of MEC (described below). Male pups were used in unrelated studies.

MEC isolation. The third mammary gland pairs from two animals of the same diet group were pooled and processed for epithelial cell isolation, following protocols described by Dr. Jeffrey Rosen’s laboratory (http://www.bcm.edu/rosenlab/protocols/primaryMEC.pdf; Baylor College of Medicine, Houston, TX), as adapted from an initial report by Pullan and Streuli (43). This procedure yielded MEC of ~90% purity, as determined by cell morphology (data not shown) and consistent with those reported by the Rosen group. Individual MEC preparations (n = 7, 7, and 5 for Cas, SPI, and Gen, respectively) were immediately homogenized in TRIzol (Invitrogen, Carlsbad, CA), and homogenates were frozen at −80°C until processed for RNA extraction.

RNA isolation and microarray analysis. Total RNA was extracted from MEC following manufacturer’s instructions (Invitrogen) and further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA), followed by on-column DNA digestion with RNase-Free DNase (Qiagen). Integrity of total RNA was monitored by absorbance ratios (A260/A280) and by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For each RNA sample (n = 3, 4, and 3 for Cas, SPI, and Gen groups, respectively), double-stranded cDNAs were synthesized following previously described protocols from this group (63). Biotin-labeled cRNA was generated by in vitro transcription from the cDNA using the ENZO BioArray High Yield RNA Transcript labeling kit (ENZO, Farmingdale, NY) and fragmented to a size range of 35–200 bp. Each labeled cRNA was hybridized to an Affymetrix rat 230A GeneChip array (Affymetrix, Santa Clara, CA) for 16 h at 45°C. Immediately after the hybridization, all probe arrays were stained with streptavidin-conjugated phycoerythrin conjugate and then with polyclonal antistreptavidin antibody coupled to phycoerythrin. Following automated washings, the DNA chips were scanned using an Agilent GeneArray laser scanner. Signal values for each probe set were processed using Microarray Suite 5.0 (Affymetrix) and adjusted to a common baseline using invariant set normalization. Between different hybridization arrays, each gene was renormalized to itself by creating a synthetic positive control for that gene comprising the median of the gene’s expression values over all samples of an experimental group. Genes were filtered based on their presence or absence in three of three (Cas, Gen groups) or three of four (SPI group) samples, and mean ± SE of gene expression for each treatment group was computed. Genes were selected based on a minimum change of 1.5-fold and a P value of < 0.05 (two-tailed t-test). Data analyses were performed using Microsoft Excel and SpotFire DecisionSite for Functional Genomics (Spotfire, Somerville, MA). Unsupervised nearest neighbor hierarchical clustering was used to validate the gene expression data (Cluster software; Spotfire), and data presentation used the companion software TreeView (http://rana.lbl.gov/EisenSoftware.htm).

Quantitative real-time PCR. Selected genes found to be diet regulated from microarray analysis were validated by quantitative real-time PCR (QPCR). Total RNA (1 μg) from individual MEC preparations was reverse-transcribed using random hexamers and Multi-Scribe Reverse Transcriptase in a two-step RT-PCR reaction (Applied Biosystems, Foster City, CA). Primers (Supplemental Table 1S) were designed using Primer Express (Applied Biosystems) to yield a single amplicon. (The online version of this article contains supplemental material.) Quantitative real-time PCR (QPCR) was performed with the SYBR Green detection system (Applied Biosystems) using an ABI Prism 7000 sequence detector and under thermal cycling conditions of preincubation (50°C, 2 min); DNA polymerase activation (95°C, 1 min); and 40 PCR cycles for 15 s at 95°C, 1 min at 95°C, and 1 min at 60°C. Standard curves were generated by serial dilution of pooled total RNAs prepared from the same MEC used in this study. QPCR was performed using independent MEC RNA sets of 7, 9, and 10 for Cas, SPI, and Gen groups, respectively. For the last two diet groups, the higher numbers of samples reflected the isolation of additional MEC samples from a repeat of the feeding studies, as described above. Each sample was run in duplicate, and mRNA levels were normalized to 18S rRNA to control for input RNA. QPCR data are presented as means ± SE, relative to the control Cas diet (value of 1).

Immunohistochemistry. Mammary glands were fixed overnight in 10% neutral-buffered formalin, dehydrated with a series of descending ethanol concentrations and embedded in paraffin. Antigen retrieval in Citra Plus (Biogenex, San Ramon, CA); incubation with
blocking solution (Casblock, Zymed, San Francisco, CA) to minimize nonspecific binding; and tissue section staining with anti-cyclin D1 (1:250 dilution; M20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin (1:250 dilution; Santa Cruz Biotechnology), and anti-E-cadherin (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) antibodies followed procedures described by the suppliers. After incubation with the appropriate secondary antibodies, the proteins were visualized using the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA). Signals were detected using diaminobenzidine (Dako, Carpinteria, CA) as chromogen, and sections were counterstained with hematoxylin. Immunostaining intensities in ductal epithelium were independently scored by three laboratory personnel who viewed the slides in blinded fashion. Scoring was carried out on four tissue sections representing four individual rats per diet group; a scoring range of 1 (weak), 2 (moderate), and 3 (strong) was used.

Statistical analysis. Statistical analysis was performed using SigmaStat software package version 3.2 (SPSS, Chicago, IL). Statistical significance between diet groups, based on P values ≤0.05, was determined using one-way ANOVA followed by Tukey’s post hoc analysis.

RESULTS

Gene expression profiles of rat MEC of different diet groups. We compared the genomic profiles of MEC preparations isolated from mammary glands of sexually mature (PND50) rats fed SPI or Cas supplemented with Gen with those fed Cas, using the Affymetrix RAE230A gene microarray platform. Isolated MEC, rather than whole mammary glands were evaluated since epithelial cells give rise to and predominantly comprise mammary tumors (49, 51). The microarray data are available as accession number GSE6879 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/). Of the 14,280 unique genes analyzed, 175 transcripts were identified as regulated by either dietary SPI or Gen (Table 1). Of these, 18 were induced and 61 were repressed in the SPI relative to the Cas group. For the Gen group, 53 and 43 genes were identified, respectively, as induced and repressed in comparison with the Cas group. SPI and Gen groups had minimal overlap in gene expression profiles, with only seven induced and three repressed genes in common.

Validation of differential gene expression with dietary exposure. Figure 1A shows hierarchical clustering of transcripts differentially expressed in the SPI and/or Gen diet groups relative to those of the Cas group. The mean expression values for each differentially expressed gene for SPI and Gen diet groups relative to Cas were calculated, and a heat map was generated (Fig. 1B). Clusters I (SPI<CAS), II (GEN<CAS), and III (SPI, GEN<CAS) represent downregulated genes, while clusters IV (SPI, GEN>CAS) and V (GEN>CAS) represent upregulated transcripts. Representative genes in these cluster groups were validated by QPCR. The decreased expression of interleukin-17B (IL17B) and increased expression of transferrin receptor (Tfrc), fatty acid binding protein 3 (Fabp3), and quiescin 6 (Qscn6) respectively were confirmed for the SPI group. We also confirmed the decreased expression of V-Ros and QPCR. Examples were IL17B, which was also downregulated by Gen similar to SPI, and Qscn6, which was also upregulated by SPI similar to Gen, relative to the Cas group.

Functional grouping of differentially expressed genes among diet groups. To gain insights into the biological processes altered by diet that may be relevant to dietary protection against mammary tumorigenesis, we used Gene Ontology together with the NCBI and PubMed databases to cluster the 175 modulated genes into common biochemical pathways. Data presented in Tables 2S–5S demonstrate that diet altered the expression of genes with diverse functions. For the SPI group, the largest functional categories (up- and downregulated) were immunity (11.3%), signal transducer (11.3%), and metabolism and protein synthesis/turnover (16%). For Gen, the major categories were signal transducer (18.2%), metabolism and protein synthesis/turnover (27.3%), and ion transport (9.1%). Other functional groups altered by both or either SPI or Gen include genes encoding structural proteins (e.g., keratin complex 2, collagen type V), transcriptional regulators (e.g., interferon regulatory factor 7, transcription factor 7, T-cell specific), and cell cycle-associated markers (e.g., spermine synthase). In some cases, a disproportionate number of upregulated vs. downregulated genes was associated with one function (e.g., predominantly downregulated immune response genes for SPI). Moreover, for other functions (e.g., signal transduction), there was discordance in the number of up- or downregulated genes with one diet (SPI: decreased>increased genes) relative to the other diet (Gen: decreased=increased genes). Notably, the fold-change in expression for most of the identified genes was 1.5- to 2-fold on average, with few exceptions (e.g., 4-fold for Ca2; 5-fold for Fabp3; 12- to 17-fold for RT1 class I and II immune response genes).

Specific immunity genes altered by diet are presented in Fig. 2. SPI downmodulated most of these genes, predominantly those involved in antigen presentation and processing [RT1 (RT1-Aw2, -Bb, -CE16)]; major histocompatibility complex, class II (Hla-dmb)] and inflammatory response (CD97; interferon regulatory factor 7 (Irf7); pancreatitis-associated protein (Pap); IL17B]. An exception was myelin basic protein (Mbp), which was upregulated by SPI. Gen had more modest repressive effects than SPI (e.g., RT1) and, for a number of genes, opposed (e.g., lactalbumin, Lalba) or lacked (e.g., Pap, Hla-dma, Irf7) the changes elicited by SPI.

Gen elicited more robust changes in metabolic-associated gene expression than did SPI (Fig. 3). With the exceptions of sphingomyelin phosphodiesterase, acid-like 3A (Snpm3a), an enzyme involved in carbohydrate metabolism, and ubiquitin-specific protease 25 (Usp25), an enzyme involved in protein catabolism, GEN induced or inhibited gene expression to a greater extent than did SPI for a number of protein metabolism.
(Coq7, Ube2d3, Slc7a7, Scpep1); lipid metabolism (Sc5d, Thrsp); or carbohydrate metabolism (Gusb)-related genes. Nonetheless, there were also a few genes [e.g., histidine decarboxylase (Hdc); carbonic anhydrase 2 (Ca2); RNA exonuclease 2 homolog (Rexo2)], where SPI and Gen showed comparable levels of regulation.

Wnt and Notch signaling-associated genes. The identification of wingless 5a (Wnt5a), secreted frizzled-related protein 2 (Sfrp2), Hairy and Enhancer of split 1 (Hes1), and notch gene homolog 2 (Notch2) as dietary gene targets in MEC (Supplemental Tables 2S–5S) was also confirmed by QPCR (Fig. 4). Consistent with the microarray data, Gen induced transcript levels for Sfrp2 and decreased those for Wnt5a and Notch2. SPI had no effect on Sfrp2 and increased Hes1 transcript levels as predicted from the microarray data. The expression levels of Notch2 and Wnt5a, while not apparent from microarray data, were also altered by SPI, similar to that observed for Gen.

Because increased Sfrp2 and decreased Wnt5a and Notch2 expression can functionally attenuate Wnt signaling, we evaluated the expression of two downstream Wnt/Notch effectors, namely cyclin D1 and β-catenin by immunohistochemistry, in mammary glands of PND50 rats of the three diet groups (35). β-Catenin has two cellular functions, which are determined by its cellular localization. Membranous or submembranous localization of β-catenin indicates tethering to E-cadherin and maintenance of cell-cell adhesion, the loss of which is a hallmark of tumorigenesis (38). On the other hand, β-catenin accumulation in the cytoplasm/nucleus is indicative of its role
as a transcriptional activator supportive of cell proliferation (31). Cyclin D1 is a key transcriptional target of nuclear β-catenin (54), and its nonnuclear localization in target cells supports inactive β-catenin signaling. Immunoreactive E-cadherin was localized only to sites of cell-cell contact in mammary ductal epithelium (Fig. 5A), and levels did not change among the diet groups. Similarly, ductal epithelial structures stained with anti-β-catenin antibody showed β-catenin only in membranes, with staining intensities comparable among the diet groups (Fig. 5B). With anti-cyclin D1 antibody, immunoreactivity was present exclusively in the cytoplasm of ductal epithelium for all diet groups (Fig. 5C). The staining intensity was higher for Cas and SPI relative to Gen (Fig. 5D). No nuclear immunoreactivity was detected with all antibodies for all diet groups (Fig. 5, A–C).

**Altered genes in common between N-methyl-N-nitrosourea-induced rat mammary tumors and MEC from rats of different diet groups.** Since the chemical carcinogen N-nitrosourea (NMU) induces mammary tumors in rodents that resemble human breast cancers (49), and since our own studies showed protective effects of SPI and Gen from mammary tumors induced by NMU (56, 59), we determined whether identified mammary epithelial genes whose expression was altered by SPI and Gen, relative to Cas in the present study were similarly affected in rat MEC upon NMU-induced tumorigenesis (8). Table 2 lists altered genes identified in common between NMU-induced rat mammary tumors and MEC of rats exposed to dietary SPI or Gen. Of the 11 genes identified, 6 were expressed in opposing manner between SPI and/or Gen dietary groups and NMU tumors, suggesting these as potential gene targets involved in mammary tumor protection by diet. These included Il17-B; Wnt5a; protein kinase inhibitor-beta (Pkb); RT1 class 1b; Mbp; and Thrsp. We also found five genes (Ca2; Fabp3; cadherin 22, cd22; collagen type 5, Col5a3; and osteoprotegerin, Tnfrsf11b) that were regulated in the same direction in normal MEC of SPI and/or Gen dietary groups and mammary tumors relative to normal mammary tissues (Table 2).

**Other tumor-related mammary genes altered with dietary SPI or Gen.** We evaluated the expression of several mammary epithelial genes that were not identified as differentially expressed by microarray analyses in the present study, but whose aberrant expression has been previously associated with mammary tumorigenesis (9, 11, 13, 50). These included estrogen receptor-α (Esr1), estrogen receptor-β (Esr2), progesterone receptor (Pgr), and the tumor suppressor Pten. Relative to the Cas group, higher expression of Esr1 and Esr2 was observed in both SPI and Gen groups, while increased Pgr expression was noted only with SPI (Fig. 6). SPI and Gen numerically increased Pten expression relative to the Cas group, consistent with an increase in protein levels as reported in our previous study (17).

**DISCUSSION**

The present study constitutes an unbiased approach by which to investigate the biochemical pathways and molecular
signals affected by dietary factors in MEC for resistance to chemically induced mammary tumorigenesis. Using isolated epithelial cells from mammary glands of young adult female rats lifetime exposed (from GD4 to PND50) to dietary Cas, SPI, or Gen, we found that: 1) the repertoire of mammary epithelial genes whose expression levels were altered by diet is limited, totaling <1% of the more than 14,000 genes evaluated; 2) the magnitude of the changes in gene expression with diet is modest (an average of ~2-fold), with few exceptions; 3) there are substantial differences in sets of genes altered by SPI and Gen, despite the presence of comparable amounts of Gen present in both diets; and 4) the majority of identified genes altered by the diets (e.g., metabolism, ion transport, immune response) have not been previously characterized in relation to mammary tumorigenic pathways. These results indicate that SPI and Gen alter functionally diverse genes and define a

Table 2. Summary of common genes regulated by diet and NMU

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Diet1,3</th>
<th>NMU2,3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase 2</td>
<td>Ca2</td>
<td>SPI (+), Gen (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Interleukin 17B</td>
<td>Il17b</td>
<td>SPI (−)</td>
<td>(+)</td>
</tr>
<tr>
<td>RT1class Ib, locus Aw2</td>
<td>RT1-Aw2</td>
<td>SPI (−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Fatty acid binding protein 3</td>
<td>Fabp3</td>
<td>SPI (+), Gen (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Wingless-type MMTV integration site 5A</td>
<td>Wnt5a</td>
<td>Gen (−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Protein kinase(CAMP dependent, catalytic) inhibitor beta</td>
<td>Pkib</td>
<td>SPI (−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Cadherin 22</td>
<td>cdh22</td>
<td>SPI (−), Gen (−)</td>
<td>(−)</td>
</tr>
<tr>
<td>Collagen, type V, alpha3</td>
<td>Col5a3</td>
<td>SPI (−)</td>
<td>(−)</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>Mbp</td>
<td>Gen (−)</td>
<td>(−)</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)</td>
<td>Tnfrsf11b</td>
<td>SPI (−)</td>
<td>(−)</td>
</tr>
<tr>
<td>Thyroid hormone responsive protein</td>
<td>Thrsp</td>
<td>Gen (−)</td>
<td>(−)</td>
</tr>
</tbody>
</table>

NMU, N-methyl-N-nitrosourea. 1Relative to Cas. 2Adapted from Ref. 8; relative to normal mammary gland. 3Induced (+); Repressed (−).
subset of these that may serve as potential biomarkers in other target tissues (e.g., prostate, bone, immune cells) for which the health benefits of soy-rich foods and supplemental Gen have been suggested.

In our study we identified 79 and 96 genes, respectively, whose levels of expression were altered by SPI and Gen from an oligonucleotide array containing 14,280 genes. Of these, only 10 were identified as common between the diets by gene array, although with further confirmation by QPCR using mRNAs from both diet groups, the numbers were slightly increased. Interestingly, despite the health benefits of soy-rich foods being largely attributed to Gen, in particular because of Gen’s function as a selective estrogen receptor modulator (47), our results indicate that soy proteins (SPI) elicited unique molecular signatures in MEC distinct from Gen that can be correlated with positive health outcome. Consistent with this observation are our previously reported findings that SPI, but not Gen, demonstrated mammary tumor-protective effects when administered in utero (59) and that the expression of specific genes in whole mammary tissues of PND50 rats differed as a function of dietary SPI or Gen intake (17). The gene expression changes for SPI cluster with those involved in immunity, signal transduction, and metabolism, comprising 38% of the changes observed. For Gen, ~55% of the observed changes in gene expression patterns converged on metabolism, ion transport, and signal transduction pathways. The greater heterogeneity of the biochemical and biological consequences of SPI, compared with the major effects of Gen on limited pathways, is consistent with the heterogenous makeup of SPI. The numerous types of phytoestrogens, lignans, and peptides comprising SPI likely exhibit individual and combinatorial outcomes on pathway signaling and modify (enhance or suppress) Gen actions.

The predominance of immunologically relevant genes downregulated in MEC of rats fed SPI, which was not observed in those of Gen-fed rats, is notable. Two of the most highly downregulated genes are *RT1-Bb* (~18-fold) and *RT1-CE16* (~12-fold), which function in antigen presentation (18, 26). Moreover, *IL17B*, decreased by ~2.5-fold in cells of the SPI group, is a homolog of the prototype cytokine IL-17, which is linked to neutrophil chemotaxis and inflammatory response (52). Although it is difficult to speculate on the functions of these immune-related proteins in mammary tumor protection and to definitively exclude the possibility of contaminating immune cells as source of these immunity genes, our findings predict a diminished immunological environment in MEC with SPI, which might reflect less oxidative stress status. A more detailed study of the consequences of soy-rich foods on the innate immune defense and extent of macrophage and mast cell infiltration of mammmary epithelium will be required to provide relevance to these major gene expression changes.

An interesting finding of the present study is the dietary perturbations of the Wnt and Notch signaling pathways, as demonstrated by decreased expression of Wnt signaling components *sfrp2* and *Wnt5a* and of Notch signaling component *Notch2*, and increased expression of *Hes1*, with SPI and/or Gen diets. Wnt signaling is intimately involved in many developmental processes as well as in tumor development by virtue of its positive regulation of cell proliferation via canonical (with β-catenin) (42) and noncanonical (with ErbB1) (12) pathways. A function for Wnt family members in the negative regulation of stem cell quiescence has also been proposed (45). Sfrp2 is a soluble form of the Wnt receptor frizzled and, by binding Wnts, prevents the activation of the Wnt signaling pathway and the nuclear accumulation of β-catenin (66). Notch2, a member of a family of growth regulatory proteins (6), is found at high levels in cells undergoing metastatic/neoplastic transformations (3) and is involved in the maintenance of undifferentiated epithelial cells (19). In breast cancer cell lines, Hes1 functions as a negative regulator of estrogen-dependent proliferation, with its expression negatively correlated with that of the proliferative marker PCNA (58). Thus, the appropriate directions of the expression changes of *Wnt5a* (decreased), *Sfrp2* (increased), *Notch2* (decreased), and *Hes1* (increased) predict an environment favoring the differentiation of mammary epithelium with dietary intake of SPI and Gen. Given that the differentiation status of the mammary gland at the time of carcinogenic insult is inversely associated with risk of mammary tumor development (24), the defined changes in gene expression preferential for the differentiation of mammary epithelium may contribute to the mammary tumor protective effects of SPI and Gen in our rodent model and may be relevant to the breast cancer preventative effects of soy-rich diets in Asian women.

In addition to the Wnt and Notch signaling components, other growth regulatory genes identified by gene array and confirmed by QPCR as altered by SPI and/or Gen include *Fabp3* (negative regulator of cell proliferation; 65), *Qsn6* (quiescent-induced gene; 14), *Qsn6* (receptor tyrosine kinase; 10), *Tfrc* (requisite for maintenance of tumor cells in a high metabolic state; 64), and *Wap* (associated with mammary gland differentiation state; 48). Furthermore, using the candidate gene approach, we found that the expression of mammary epithelial estrogen receptor *Esr1* and *Esr2* and of progesterone receptor *Pgr* (2) were also influenced by diets. The numerical but nonsignificant increase in tumor suppressor PTEN transcript levels is in keeping with our previously reported findings (17) and those of others (21, 40) that PTEN expression may also be translationally regulated. Collectively, these data suggest that SPI and Gen can alter the expression of disparate genes that may contribute to the maintenance of the
mammary epithelium in a differentiated state resistant to a “second (carcinogenic) hit.” Given the modest effects of these diets on gene expression, the multiplicity of functionally equivalent genes altered by SPI and/or Gen may reflect the need for a “threshold” state that must be attained to distinguish a tumor-resistant from a nonresistant epithelium. Importantly, the participation of multiple pathways in growth regulation may explain the exceedingly large number of mutations in genes found in breast tumors (57).

In an attempt to further understand the functionality of the identified genes altered by SPI and Gen, we compared our gene lists (Supplemental Tables 2S–5S) to those of genes whose expression was altered in NMU-induced rat mammary tumors relative to normal rat mammary glands (8). The identification of Il17B, Wnt5a, Pkl1b, RT1 class I b, Mbp, and Thrsp as genes altered by SPI and/or Gen in the direction consistent with antitumorigenic properties (Table 2) suggests the potential importance of these genes in dietary tumor protection prior to NMU administration. By contrast, the parallel directional changes in the expression of a number of genes altered by SPI and/or Gen with tumorigenesis (Ca2, Fabp3, cdh22, Tnfrsf11b, Col5a3) are difficult to explain in the context of these diets’ inhibition of mammary tumorigenesis; however, this may be related to comparisons being made between isolated MEC gene expression (our study) and those in whole mammary tissues, which would include the contribution of the stromal compartment.

Several interesting questions raised by our study require further evaluation. One relates to the biological rationale for the specific effects of dietary SPI and Gen on the expression levels of Wnt5a (SPI, Gen) and Sfrp2 (Gen), given that their encoded proteins belong to multimeric families with similar functions. A second question relates to the functional relevance of changes in Wnt signaling components, given our data (Fig. 5) showing that in MEC of all three diet groups, Wnt signaling appears to be similarly functionally inactive since its downstream mediator β-catenin is exclusively localized in the membrane, most likely complexed with E-cadherin. A third question relates to the significance and implication of lower immunoreactive cyclin D1 levels for the Gen group, since the immunoreactivity is confined to the cytoplasm, suggesting nonproliferative status. Additional studies to clarify these questions may be useful in understanding growth control in normal MEC.

In summary, we found that dietary SPI and Gen elicited modest, albeit significant, changes in the expression of limited numbers of genes in MEC of young adult rats. These genes functionally clustered into pathways involved in immune response, metabolic control, signal transduction, ion transport, and growth regulation. Dietary Gen generated molecular signatures in MEC that were largely distinct from those of dietary SPI, although for genes associated with growth regulation there was substantial overlap in their molecular profiles. These results provide important insights into signaling pathways that may underlie tumor inhibitory activities of dietary SPI and Gen and that may have relevance to other tissues influenced by these same diets. We suggest that further understanding of the functions of identified genes whose associations with tumor initiation and progression have not been previously explored may lead to novel targets for cancer prevention.

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