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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/physiolgenomics.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 dedifferentiates, 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 malignancy 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),

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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 3*) 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 (A_{260}/A_{280}) 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 MultiScribe 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

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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 expression compared 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 1*A* 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. 1*B*). *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 Ur2 sarcoma virus oncogene homolog 1 (*Ros1*) and activin receptor IIA (*Acvr2a*) and the increased expression of *Fabp3*, *Qscn6*, *Tfrc*, and whey acidic protein (*Wap*) transcripts by dietary Gen. Interestingly, several of the Gen only- and SPI only-modulated transcripts, as detected by microarray analyses, were found to be co-regulated by both diet groups using QPCR (Fig. 1). 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.

Table 1. *Dietary effects on mammary epithelial gene expression*

Diet	Up	%*	Down	%	Total	%
SPI	18	0.13	61	0.43	79	0.55
Gen	53	0.37	43	0.30	96	0.69

"Up" or "Down" is the number of genes up- or downregulated relative to control [casein (Cas)] diet. *From a total of 14,280 genes analyzed (Affymetrix rat RAE230A GeneChip). SPI, soy protein isolate; Gen, genistein.

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 *RTI* 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., *RTI*) and, for a number of genes, opposed (e.g., lactalbumin, *Lalba*) or lacked (e.g., *Pap*, *Hla-dma*, *Ifr7*) 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 (*Smpdl3a*), 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

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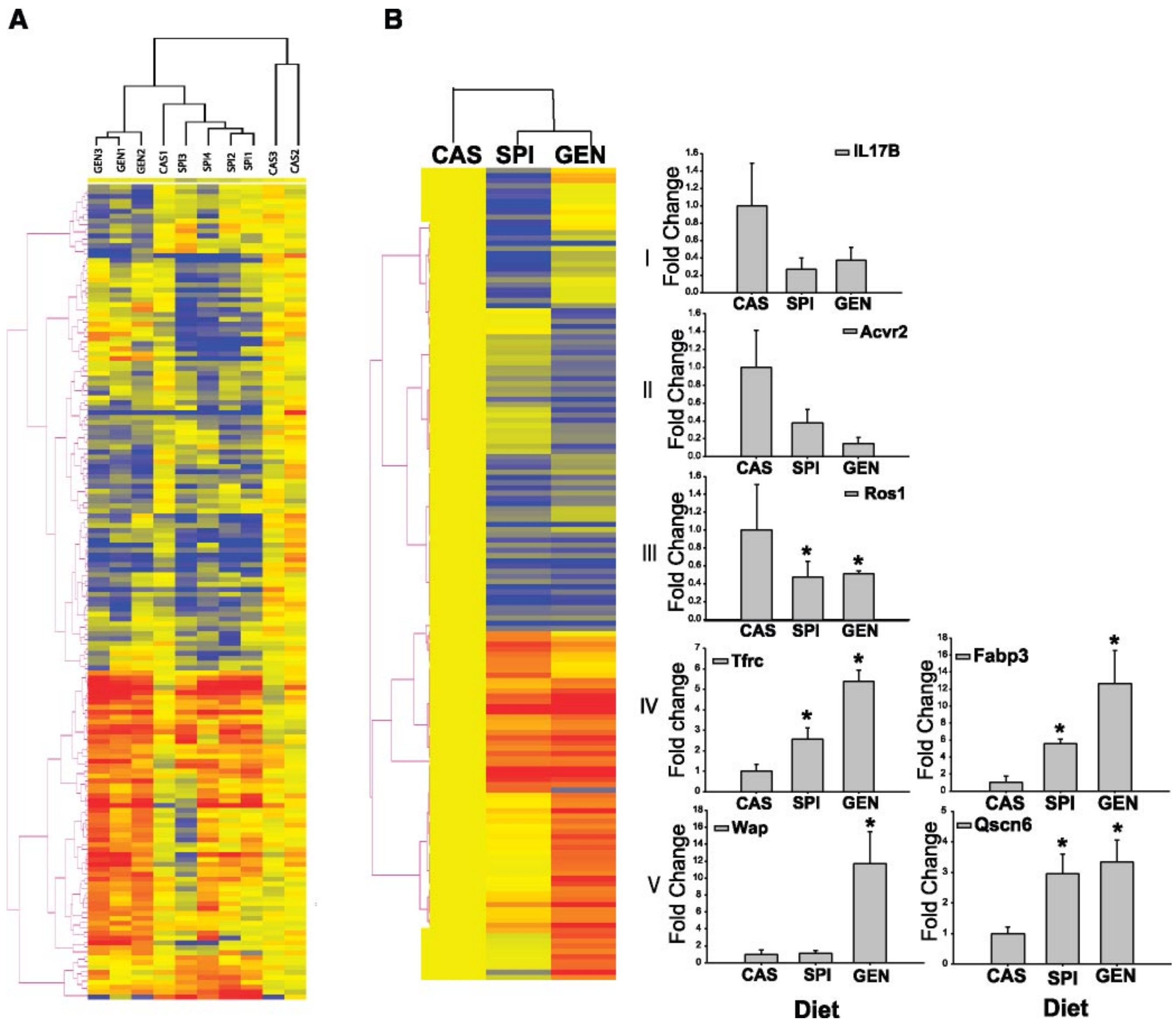

Fig. 1. Transcriptome alterations in rat mammary epithelial cells as a function of dietary exposure. *A*: hierarchical clustering of differentially expressed mammary epithelial genes regulated by dietary soy protein isolate (SPI) and genistein (Gen), relative to the control diet casein (Cas). A total of 3, 4, and 3 individual chip arrays for Cas, SPI, and Gen diet groups, respectively, were analyzed. *B*: heat map of the mean expression values for each differentially expressed gene in SPI and Gen groups relative to that of the Cas group. Genes were grouped based on cluster analyses. *Clusters I* (SPI<Cas), *II* (Gen<Cas), and *III* (SPI, Gen<Cas) represent downregulated genes (blue), while *clusters IV* (SPI, Gen>Cas) and *V* (Gen>Cas) represent upregulated (red) transcripts. *$P < 0.05$ relative to CAS group.

(*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

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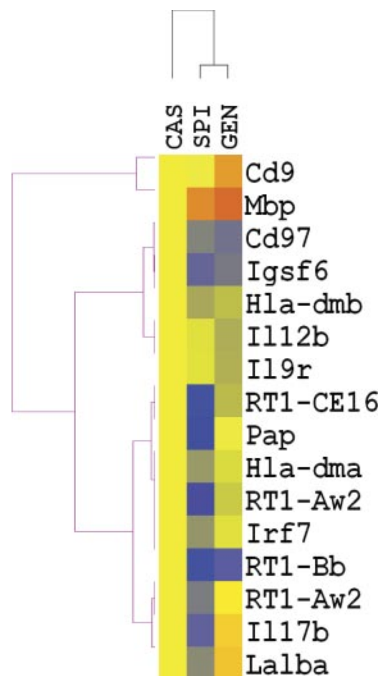

Fig. 2. Heat map showing mean expression profiles for immunity-related genes altered by SPI and Gen in mammary epithelial cells. Gene names are listed in Supplemental Tables 2S–5S. Blue are downregulated, while red are upregulated genes, relative to Cas.

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. 5*A*), 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. 5*B*). With anti-cyclin D1 antibody, immunoreactivity was present exclusively in the cytoplasm of ductal epithelium for all diet groups (Fig. 5*C*). The staining intensity was higher for Cas and SPI relative to Gen (Fig. 5*D*). No

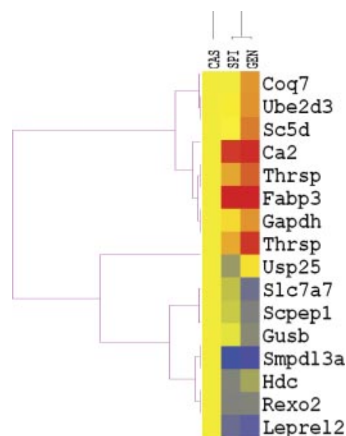

Fig. 3. Heat map showing mean expression profiles for metabolic-related genes altered by SPI and Gen in mammary epithelial cells. Gene names are listed in Supplemental Tables 2S–5S. Blue are downregulated, while red are upregulated genes, relative to Cas.

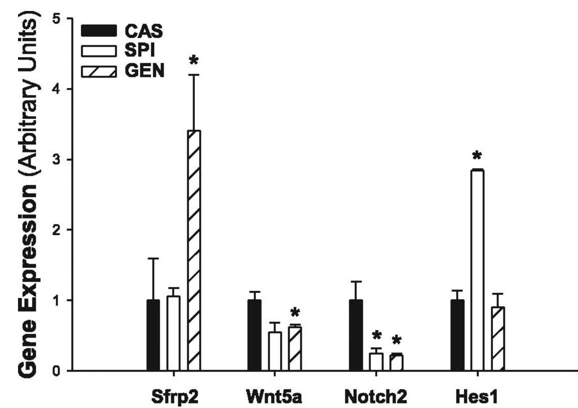

Fig. 4. Wnt and Notch signaling components are regulated by dietary SPI and Gen in rat mammary epithelial cells. Transcript levels were quantified by QPCR. $*P < 0.05$ relative to Cas group.

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*-methyl-*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 (*Pkib*); *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

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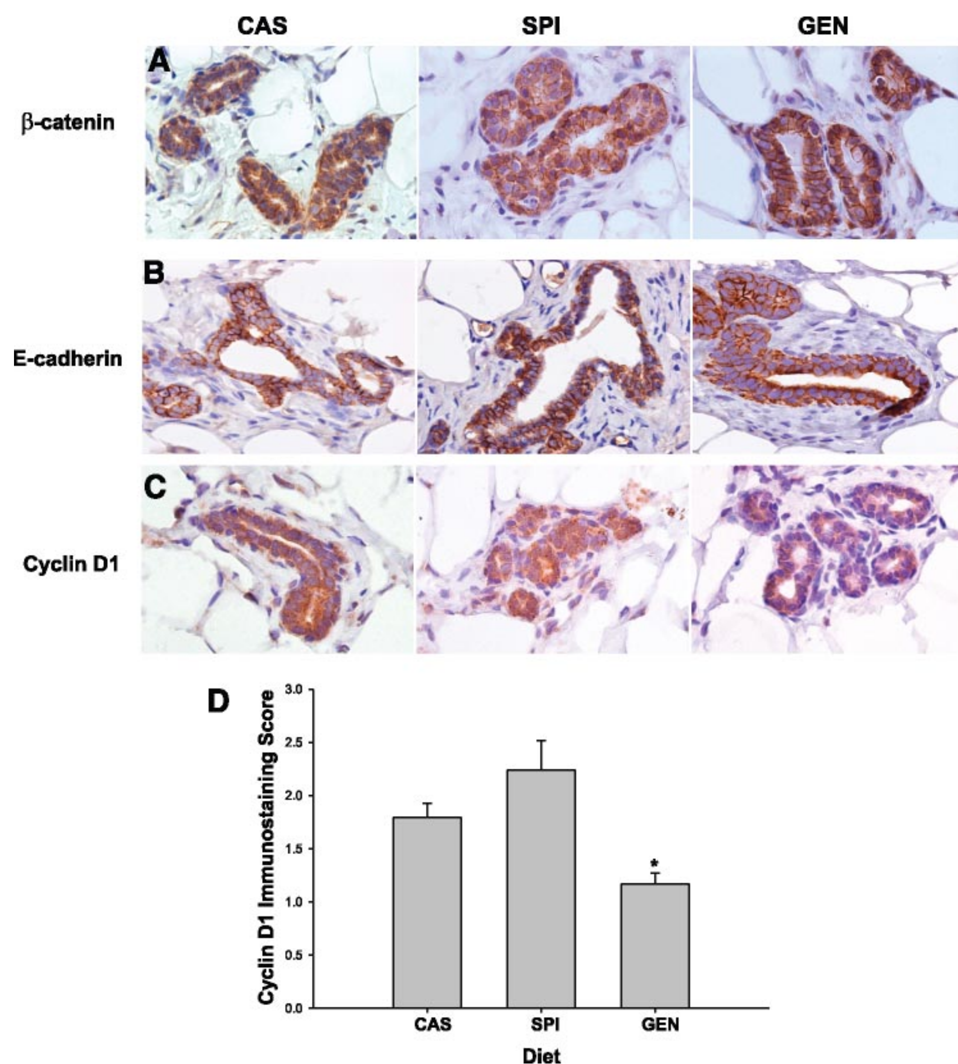

Fig. 5. Expression of β-catenin-associated proteins in rat mammary glands of diet groups. Mammary sections representing 3 or 4 animals of each diet group were analyzed. Representative micrographs of ductal epithelium immunostained with β-catenin (*A*), E-cadherin (*B*), and cyclin D1 (*C*) are presented. Graphical representation of immunostaining intensities (means ± SE) for cytoplasmic cyclin D1, using a scoring range of 1 (weak), 2 (moderate), and 3 (strong). $*P < 0.05$ relative to Cas group.

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*

Gene Name	Gene Symbol	Diet[1,3]	NMU[2,3]
Carbonic anhydrase 2	Ca2	SPI (+), Gen (+)	(+)
Interleukin 17B	Il17b	SPI (−)	(+)
RT1class Ib, locus Aw2	RT1-Aw2	SPI (−)	(+)
Fatty acid binding protein 3	Fabp3	SPI (+), Gen (+)	(+)
Wingless-type MMTV integration site 5A	Wnt5a	Gen (−)	(+)
Protein kinase(cAMP dependent, catalytic) inhibitor beta	Pkib	SPI (−)	(+)
Cadherin 22	cdh22	SPI (−), Gen (−)	(−)
Collagen, type V, alpha3	Col5a3	SPI (−)	(−)
Myelin basic protein	Mbp	Gen (+)	(−)
Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Tnfrsf11b	SPI (−)	(−)
Thyroid hormone responsive protein	Thrsp	Gen (+)	(−)

NMU, *N*-methyl-*N*-nitrosourea. [1]Relative to Cas. [2]Adapted from Ref. 8; relative to normal mammary gland. [3]Induced (+); Repressed (−).

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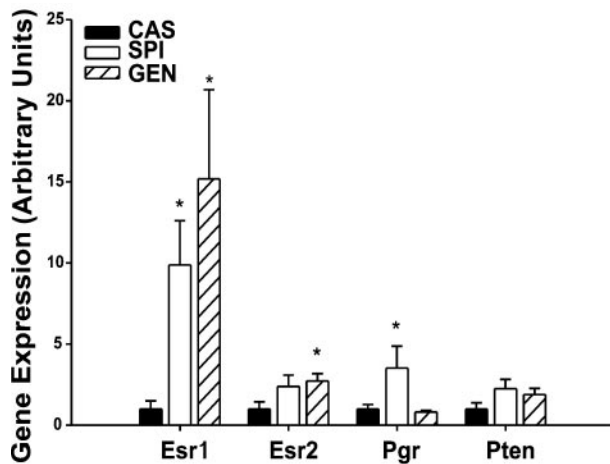

Fig. 6. Dietary changes in expression of growth regulatory genes in rat mammary epithelial cells. Transcript levels were quantified by QPCR. *$P < 0.05$ relative to Cas group.

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 make-up 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 mammary 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 metaplastic/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 W*nt5a* (decreased), S*frp2* (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), *Qscn6* (quiescent-induced gene; 14), *Ros1* (receptor tyrosine kinase; 10), *Tfrc* (requisite for maintenance of tumor cells in a high metabolic state; 64), and W*ap* (associated with mammary gland differentiation state; 48). Furthermore, using the candidate gene approach, we found that the expression levels 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

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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, Pk1b*, *RT1 class 1b, 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 multimember 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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REFERENCES

1. **Adlercreutz H, Mazur W.** Phyto-oestrogens and Western diseases. *Ann Med* 29: 95–120, 1997.
2. **Anderson E, Clarke RB.** Steroid receptors and cell cycle in normal mammary epithelium. *J Mammary Gland Biol Neoplasia* 9: 3–13, 2004.
3. **Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, Mandinova A, Raffoul W, Fiche M, Dotto GP, Brisken C.** Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc Natl Acad Sci USA* 103: 3799–3804, 2006.
4. **Badger TM, Ronis MJ, Simmen RC, Simmen FA.** Soy protein isolate and protection against cancer. *J Am Coll Nutr* 24: 146S–149S, 2005.
5. **Barker DJ.** Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* 3: 807–813, 1997.
6. **Bianchi S, Dotti MT, Federico A.** Physiology and pathology of notch signalling system. *J Cell Physiol* 207: 300–308, 2006.
7. **Cassidy A, Albertazzi P, Lise N, I, Hall W, Williamson G, Tetens I, Atkins S, Cross H, Manios Y, Wolk A, Steiner C, Branca F.** Critical review of health effects of soyabean phyto-oestrogens in post-menopausal women. *Proc Nutr Soc* 65: 76–92, 2006.
8. **Chan MM, Lu X, Merchant FM, Iglehart JD, Miron PL.** Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. *Carcinogenesis* 26: 1343–1353, 2005.
9. **Chang EC, Frasor J, Komm B, Katzenellenbogen BS.** Impact of estrogen receptor β on gene networks regulated by estrogen receptor α in breast cancer cells. *Endocrinology* 147: 4831–4842, 2006.
10. **Charest A, Wilker EW, McLaughlin ME, Lane K, Gowda R, Coven S, McMahon K, Kovach S, Feng Y, Yaffe MB, Jacks T, Housman D.** ROS fusion tyrosine kinase activates a SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice. *Cancer Res* 66: 7473–7481, 2006.
11. **Chung MJ, Jung SH, Lee BJ, Kang MJ, Lee DG.** Inactivation of the PTEN gene protein product is associated with the invasiveness and metastasis, but not angiogenesis, of breast cancer. *Pathol Int* 54: 10–15, 2004.
12. **Civenni G, Holbro T, Hynes NE.** Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. *EMBO Rep* 4: 166–171, 2003.
13. **Clarke RB, Anderson E, Howell A.** Steroid receptors in human breast cancer. *Trends Endocrinol Metab* 15: 316–323, 2004.
14. **Coppock D, Kopman C, Gudas J, Cina-Poppe DA.** Regulation of the quiescence-induced genes: quiescin Q6, decorin, and ribosomal protein S29. *Biochem Biophys Res Commun* 269: 604–610, 2000.
15. **Dai Q, Shu XO, Jin F, Potter JD, Kushi LH, Teas J, Gao YT, Zheng W.** Population-based case-control study of soyfood intake and breast cancer risk in Shanghai. *Br J Cancer* 85: 372–378, 2001.
16. **Daniel CW, Silberstein GB, Strickland P.** Direct action of 17 β-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. *Cancer Res* 47: 6052–6057, 1987.
17. **Dave B, Eason RR, Till SR, Geng Y, Velarde MC, Badger TM, Simmen RC.** The soy isoflavone genistein promotes apoptosis in mammary epithelial cells by inducing the tumor suppressor PTEN. *Carcinogenesis* 26: 1793–1803, 2005.
18. **Dressel R, Walter L, Gunther E.** Genomic and functional aspects of the rat MHC, the RT1 complex. *Immunol Rev* 184: 82–95, 2001.
19. **Duncan AW, Rattis FM, DiMascio LN, Congdon KL, Pazianos G, Zhao C, Yoon K, Cook JM, Willert K, Gaiano N, Reya T.** Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immun* 6: 314–322, 2005.
20. **Hakkak R, Korourian S, Shelnutt SR, Lensing S, Ronis MJ, Badger TM.** Diets containing whey proteins or soy protein isolate protect against

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7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in female rats. *Cancer Epidemiol Biomarkers Prev* 9: 113–117, 2000.
21. **Han B, Dong Z, Liu Y, Chen Q, Hashimoto K, Zhang JT.** Regulation of constitutive expression of mouse PTEN by the 5'-untranslated region. *Oncogene* 22: 5325–5337, 2003.
22. **Hennighausen L, Robinson GW.** Signaling pathways in mammary gland development. *Dev Cell* 1: 467–475, 2001.
23. **Hilakivi-Clarke L, de Assis S.** Fetal origins of breast cancer. *Trends Endocrinol Metab* 17: 340–348, 2006.
24. **Hwang SY, Kim HY.** Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. *Mol Cell* 19: 180–184, 2005.
25. **Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ.** Cancer statistics. *CA Cancer J Clin* 56: 106–130, 2006.
26. **Jimenez BD, Maldonado L, Dahl RH, Quattrochi LC, Guzelian PS.** Ectopic expression of MHC class II genes (RT1. B(I) beta/alpha) in rat hepatocytes in vivo and in culture can be elicited by treatment with the pregnane X receptor agonists pregnenolone 16α-carbonitrile and dexamethasone. *Life Sci* 71: 311–323, 2002.
27. **Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW, Smith EP.** Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res* 51: 159–186, 1996.
28. **Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT.** The Wnt/Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet* 16: 279–283, 2000.
29. **Li G, Robinson GW, Lesche R, Martinez-Diaz H, Jiang Z, Rozengurt N, Wagner KU, Wu DC, Lane TF, Liu X, Hennighausen L, Wu H.** Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development* 129: 4159–4170, 2002.
30. **Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R.** PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275: 1943–1947, 1997.
31. **Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung MC.** Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc Natl Acad Sci USA* 97: 4262–4266, 2000.
32. **Liu B, Edgerton S, Yang X, Kim A, Ordonez-Ercan D, Mason T, Alvarez K, McKimmey C, Liu N, Thor A.** Low-dose dietary phytoestrogen abrogates tamoxifen-associated mammary tumor prevention. *Cancer Res* 65: 879–886, 2005.
33. **Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW.** Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9: 2266–2278, 1995.
34. **Messina M, Barnes S.** The role of soy products in reducing risk of cancer. *J Natl Cancer Inst* 83: 541–546, 1991.
35. **Michaelson JS, Leder P.** β-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland. *Oncogene* 20: 5093–5099, 2001.
36. **Morin PJ.** β-catenin signaling and cancer. *Bioessays* 21: 1021–1030, 1999.
37. **Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM.** Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 289: 1751–1754, 2000.
38. **Nelson WJ, Nusse R.** Convergence of Wnt, β-catenin, and cadherin pathways. *Science* 303: 1483–1487, 2004.
39. **Nusse R, Varmus HE.** Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31: 99–109, 1982.
40. **Okahara F, Ikawa H, Kanaho Y, Maehama T.** Regulation of PTEN phosphorylation and stability by a tumor suppressor candidate protein. *J Biol Chem* 279: 45300–45303, 2004.
41. **Peeters PH, Keinan-Boker L, van der Schouw YT, Grobbee DE.** Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. *Breast Cancer Res Treat* 77: 171–183, 2003.
42. **Polakis P.** Wnt signaling and cancer. *Genes Dev* 14: 1837–1851, 2000.
43. **Pullan SE, Streuli CH.** The mammary gland epithelial cell. In: *Epithelial Cell Culture,* edited by Harris A. Cambridge University, 1996, p. 97–121.
44. **Reeves PG, Nielsen FH, Fahey GC Jr.** AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939–1951, 1993.
45. **Reya T, Clevers H.** Wnt signalling in stem cells and cancer. *Nature* 434: 843–850, 2005.
46. **Reya T, Morrison SJ, Clarke MF, Weissman IL.** Stem cells, cancer, and cancer stem cells. *Nature* 414: 105–111, 2001.
47. **Riggs BL, Hartmann LC.** Selective estrogen-receptor modulators–mechanisms of action and application to clinical practice. *N Engl J Med* 348: 618–629, 2003.
48. **Robinson GW, McKnight RA, Smith GH, Hennighausen L.** Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development* 121: 2079–2090, 1995.
49. **Russo J, Russo IH.** Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia* 5: 187–200, 2000.
50. **Russo J, Russo IH.** The role of estrogen in the initiation of breast cancer. *J Steroid Biochem Mol Biol* 102: 89–96, 2006.
51. **Russo J, Tahin Q, Lareef MH, Hu YF, Russo IH.** Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. *Environ Mol Mutagen* 39: 254–263, 2002.
52. **Schwartz S, Beaulieu JF, Ruemmele FM.** Interleukin-17 is a potent immuno-modulator and regulator of normal human intestinal epithelial cell growth. *Biochem Biophys Res Commun* 337: 505–509, 2005.
53. **Shen Q, Brown PH.** Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways. *J Mammary Gland Biol Neoplasia* 8: 45–73, 2003.
54. **Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben Ze'ev A.** The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* 96: 5522–5527, 1999.
55. **Shu XO, Jin F, Dai Q, Wen W, Potter JD, Kushi LH, Ruan Z, Gao YT, Zheng W.** Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. *Cancer Epidemiol Biomarkers Prev* 10: 483–488, 2001.
56. **Simmen RC, Eason RR, Till SR, Chatman L Jr, Velarde MC, Geng Y, Korourian S, Badger TM.** Inhibition of NMU-induced mammary tumorigenesis by dietary soy. *Cancer Lett* 224: 45–52, 2005.
57. **Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE.** The consensus coding sequences of human breast and colorectal cancers. *Science* 314: 268–274, 2006.
58. **Strom A, Arai N, Leers J, Gustafsson JA.** The Hairy and Enhancer of Split homologue-1 (HES-1) mediates the proliferative effect of 17β-estradiol on breast cancer cell lines. *Oncogene* 19: 5951–5953, 2000.
59. **Su Y, Eason RR, Geng Y, Till SR, Badger TM, Simmen RC.** In utero exposure to maternal diets containing soy protein isolate, but not genistein alone, protects young adult rat offspring from NMU-induced mammary tumorigenesis. *Carcinogenesis* 28: 1046–1051, 2007.
60. **Thanos J, Cotterchio M, Boucher BA, Kreiger N, Thompson LU.** Adolescent dietary phytoestrogen intake and breast cancer risk (Canada). *Cancer Causes Control* 17: 1253–1261, 2006.
61. **Watson CJ, Burdon TG.** Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. *Rev Reprod* 1: 1–5, 1996.
62. **Wu AH, Wan P, Hankin J, Tseng CC, Yu MC, Pike MC.** Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. *Carcinogenesis* 23: 1491–1496, 2002.
63. **Xiao R, Badger TM, Simmen FA.** Dietary exposure to soy or whey proteins alters colonic global gene expression profiles during rat colon tumorigenesis. *Mol Cancer* 4: 1, 2005.
64. **Yang DC, Jiang XP, Elliott RL, Head JF.** Inhibition of growth of human breast carcinoma cells by an antisense oligonucleotide targeted to the transferrin receptor gene. *Anticancer Res* 21: 1777–1787, 2001.
65. **Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A, Veerkamp JH.** Members of the fatty acid binding protein family are differentiation factors for the mammary gland. *J Cell Biol* 127: 1097–1109, 1994.
66. **Yin YJ, Katz V, Salah Z, Maoz M, Cohen I, Uziely B, Turm H, Grisaru-Granovsky S, Suzuki H, Bar-Shavit R.** Mammary gland tissue targeted overexpression of human protease-activated receptor 1 reveals a novel link to β-catenin stabilization. *Cancer Res* 66: 5224–5233, 2006.

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