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Dietary isoflavones differentially induce gene expression changes in lymphocytes from postmenopausal women who form equol as compared with those who do not

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Abstract

Human and animal studies suggest that dietary soy isoflavones reduce cancer risk, ameliorate postmenopausal syndrome and decrease bone resorption in postmenopausal women. The capacity to form the metabolite equol from daidzein is suggested as an important modulator of response to isoflavones; this capacity depends on gut colonization with appropriate bacteria. We administered a dietary supplement containing high-dose purified soy isoflavones (genistein, 558 mg/day; daidzein, 296 mg/day; and glycitein, 44 mg/day) to 30 postmenopausal women for 84 days and collected peripheral lymphocytes at timed intervals. Using microarray analysis, we determined whether changes in gene expression associated with this treatment support existing hypotheses as to isoflavones' mechanisms of action. Expression of a large number of genes was altered by isoflavone treatment, including induction of genes associated with cyclic adenosine 3′,5′-monophosphate (cAMP) signaling and cell differentiation and decreased expression of genes associated with cyclin-dependent kinase activity and cell division. We report that isoflavone treatment in subjects who have the capacity to produce equol differentially affects gene expression as compared with nonproducers, supporting the plausibility of the importance of equol production. In general, isoflavones had a stronger effect on some putative estrogen-responsive genes in equol producers than in nonproducers. Our study suggests that, in humans, isoflavone changes are related to increased cell differentiation, increased cAMP signaling and G-protein-coupled protein metabolism and increased steroid hormone receptor activity and have some estrogen agonist effects; equol-production status is likely to be an important modulator of responses to isoflavones.

Keywords: Soy; Isoflavones; Gene expression; Postmenopausal women; Estrogen-responsive genes

1. Introduction

Case–control studies conducted in China [1] and in Asian Americans [2,3] and a prospective study from Japan [4] reported an inverse relationship between soy intake and breast cancer risk. The incidence of breast cancer is lower in Asian countries (where the intake of soy is higher than in Western countries) [5]. In addition, postmenopausal symptoms are less frequent in Asian women than in women living in Western countries [5]. The proposed mechanisms of action of soy isoflavones are varied, and it is unclear which of these mechanisms are important in humans.

Some investigators suggest that equol, a bacterial metabolite of the isoflavone daidzein, is an important bioactive agent responsible for some isoflavone effects [6–10]. Equol binds to both alpha and beta estrogen receptors and remains in circulation longer than genistein or daidzein [7]. The capacity to form equol depends on colonization of the intestine with certain bacteria, and it is present in approximately 30–40% of humans [11].

The soy isoflavone genistein has estrogen agonist and antagonist actions; there is considerable controversy as to whether isoflavones are net estrogen agonists [12–14] or whether they act via antiestrogenic effects [15]. Other properties that may result in isoflavones' effects include antioxidant activity [16], inhibition of growth factor receptor signaling via tyrosine kinases [17–19], induction of apoptosis [20,21] and/or induction of cell differentiation [22].

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Many of the above-proposed mechanisms for isoflavones' actions eventually result in changes in gene expression; these changes have not been extensively investigated in humans in vivo. There are multiple reports of gene expression changes when human cells are treated with isoflavones in vitro [23–36]. The aim of the present study was to assess changes in gene expression in postmenopausal women treated with isoflavone dietary supplements (genistein, 558 mg/day; daidzein, 296 mg/day; and glycitein, 44 mg/day). This gene expression study is part of a Phase I multiple-dose clinical study of soy isoflavones in healthy postmenopausal women, sponsored by the National Cancer Institute (NCI, Division of Cancer Prevention). Moreover, to investigate whether equol producers respond differently compared with equol nonproducers, we performed the microarray analysis by stratifying the subjects based on their equol-producer status.

2. Methods and materials

2.1. Subjects

Study subjects were healthy, nonobese, postmenopausal women recruited from the local population of the Raleigh-Durham, NC metropolitan area. Postmenopausal status was defined as the last spontaneous menstrual bleeding >12 months previous to recruitment and follicular stimulating hormone (FSH) >27 mIU/ml. Nonobese was defined as a body mass index <35 kg/m^2. Women taking hormone/estrogen therapy or selective estrogen receptor modulators within 3 months of enrollment or those at high risk of breast cancer (5-year risk of 1.9% or higher) as assessed by NCI's Breast Cancer Risk Assessment Tool (http://bcra.nci.nih.gov) were excluded. Serious intercurrent medical illness, significant cardiac disease, abnormalities on the physical examination, use of oral antibiotics within the 3 months prior to enrollment, supplements containing isoflavones within 1 month prior to enrollment, current tobacco use and routine alcohol ingestion >2 drinks/day were also reasons for exclusion. Before acceptance into this study, the volunteer's health was verified by medical history, physical examination by a licensed medical doctor, screening laboratory tests, chest X-ray and electrocardiogram. In addition, the subject was required to furnish copies of negative mammogram and Pap smear reports dated within the last year.

Seventy-two women met eligibility criteria based on an initial telephone screen and were scheduled for a two-part screening appointment. Thirty-eight subjects met initial eligibility after screening, and 36 women were enrolled. One subject was terminated from the study due to FSH levels that were too low. Another two subjects completed the study, but their information was dropped because it was later determined that their mammogram results were not normal. Three other subjects decided not to complete the study. One subject moved to another state; one stated that the study supplement made her irritable, and another claimed personal reasons for not completing the study. The remaining 30 subjects successfully completed the study.

The subjects provided written informed consent, and the study was conducted in accordance with the guidelines of the Institutional Review Board of the School of Medicine at the University of North Carolina (UNC) at Chapel Hill. We operated under an Investigational New Drug application (No. 54137) for the isoflavone preparation, which was obtained from the Food and Drug Administration by the NCI. Westat (Rockville, MD), the monitoring agency designated by NCI, conducted a complete evaluation of study procedures and study event documentation.

2.2. Equol status

We determined equol-producer status using an equol challenge test in which each subject was given 473.2 ml of soy milk containing 50 mg of daidzein [8]. After 18 h, blood was collected and serum equol was determined by Kenneth Setchell at Cincinnati Children's Hospital Medical Center using high-pressure liquid chromatography mass spectrophotometry [7]. Equol producers were defined as individuals with plasma levels >20 μg/L; nonequol producers were defined as those with plasma levels <10 μg/L. Individuals with intermediate equol values (>10 to <20 μg/L) were excluded from the study.

2.3. Isoflavone formulation and dosage

Protein Technologies International (PTI; St. Louis, MO), via the NCI, provided PTI G-2535 (Unconjugated Isoflavones −70) hard gelatin capsules that contained ≥70% active substance as total unconjugated isoflavones. Isoflavones were produced under Good Manufacturing Practices guidelines. University Pharmaceuticals of Maryland, Inc. (Baltimore, MD) formulated the capsules to contain 150 mg genistein activity. The analytical data for PTI G-2535 capsules (Lot # UPM 9809-021) used in this clinical study are as follows: genistein, 139.5 mg/capsule; daidzein, 74 mg/capsule; and glycitein, 11 mg/capsule. Two laboratories (Ralston Analytical Laboratories, St. Louis, MO; Sigma Chemical Laboratories, St. Louis, MO) independently analyzed isoflavone composition and concentrations. The preparation was stable at 40°C and 70°C for at least 6 months and at 25°C for 3 years (assays were performed at University Pharmaceuticals of Maryland, Inc.). The placebo capsules were identical in size and color, contained excipients from the active formulation and were provided by the Solae Company.

2.4. Study design

The study was conducted at the UNC General Clinical Research Center. Subjects were randomized into two groups using a random numbers table. Group 1 (n=20) received four capsules of a dietary supplement (PTI G-2535, which contains genistein, 558 mg/day; daidzein, 296 mg/day; and glycitein, 44 mg/day) divided into two equal daily doses.

Group 2 ($n=10$) received the same number of placebo capsules. Subjects self-administered the capsules with food both in the morning and in the evening for 84 days. Compliance was determined using capsule counts and blood genistein concentrations. Subjects were instructed not to consume diets containing >20 mg genistein/day while enrolled in the study. Most subjects chose to totally abstain from soy during their study participation. All subjects were followed up for 28 days after the cessation of treatment until 112 days after the initiation of the study.

Blood was collected via venipuncture using a CPT Vacutainer tube with sodium citrate as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ) on Day 1 (prestudy drug), Day 84 (end of dosing period) and Day 112 (28 days poststudy drug). Blood collection was performed in the morning under fasting conditions. Samples were subjected to centrifugation for 30 min at 1500 rcf at room temperature (18°C). Plasma was removed, and the lymphocytes were washed twice with phosphate-buffered saline, transferred to another tube and was again subjected to centrifugation (1500 rcf, 5 min at room temperature). The supernatant was removed, and the lymphocyte pellet was lysed in 1 ml Trizol (Invitrogen, Carlsbad, CA) and stored at −80°C for future RNA extraction.

2.5. RNA extraction and quality control

RNA was isolated following the manufacturer's instructions (Invitrogen). RNA quality was verified at the UNC Genomics and Bioinformatics Core (http://cancer.med.unc.edu/genomicscore/), using a 2100 Bioanalyzer (RNA chip) with Degradometer software (Agilent Technologies, Palo Alto, CA). Only samples that were found to be of good quality were used for hybridization. The RNA amount provided for subsequent microarray hybridization was between 440 and 4100 ng/sample.

2.6. Microarray hybridization and data retrieval

All subsequent procedures were performed at the UNC Genomics and Bioinformatics Core. Two hundred fifty nanograms from each sample was first amplified using an Eberwine protocol [37]; then, labeled cDNA was synthesized from the amplified RNA [38] using a Low RNA Input Linear Amplification Kit (Agilent Technologies). The labeled cDNA from each subject at baseline was combined with the labeled cDNA from the same subject at the end of the study so that paired samples from the same subjects were hybridized on human 16K oligo arrays produced at the UNC Genomics and Bioinformatics Core using 60-mer

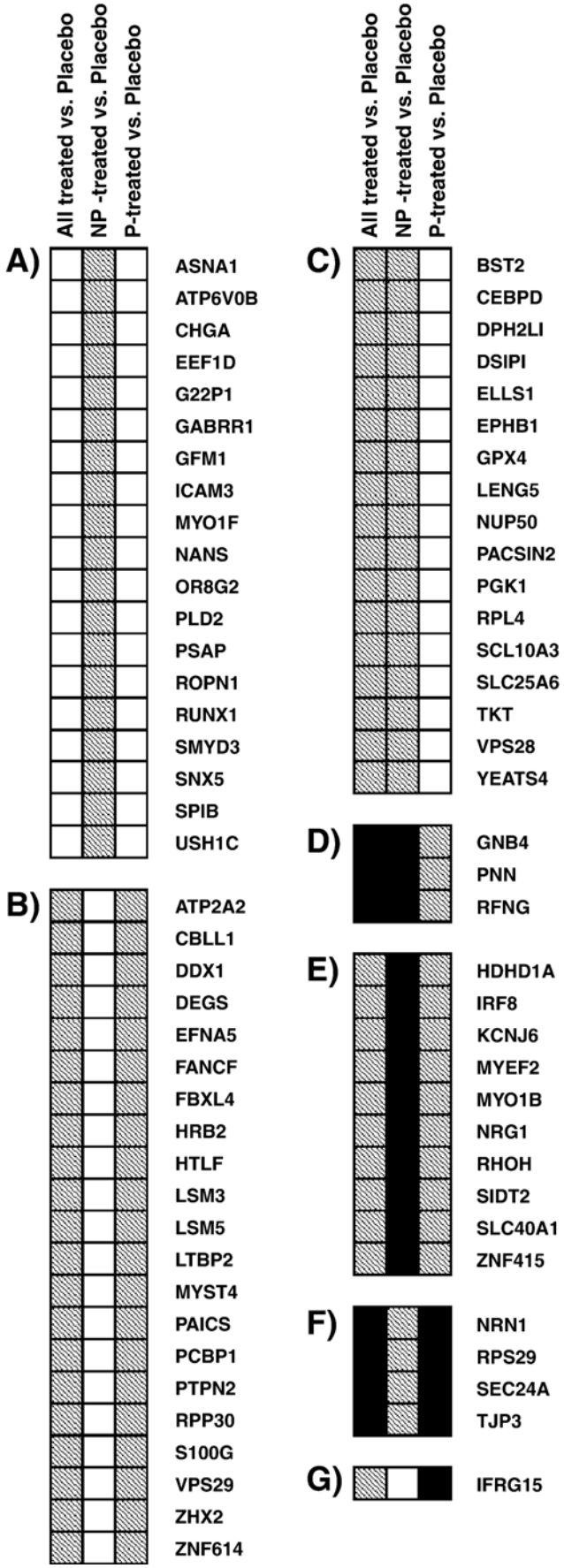

Fig. 1. Treatment with isoflavones induced selective changes in gene expression. Selected clusters represent seven different patterns of change in gene expression between equol producers (P-treated) and equol non-producers (NP-treated), versus placebo, respectively. When responses of these two groups were lumped together for analyses (All treated), some of these changes are not reflected. Black box, overexpression versus placebo group; white box, underexpression versus placebo group; hatched box, no change versus placebo group.

Table 1
Dietary soy isoflavones induced changes in gene expression in human lymphocytes after 84 days of daily treatment

Overexpressed genes			Underexpressed genes		
Gene symbol	Gene name	*d* score	Gene symbol	Gene name	*d* score
KCNG2	Potassium voltage-gated channel, subfamily G, member 2	4.5	*TNFAIP6*	Tumor necrosis factor, alpha-induced protein 6	−6.1
IFRD2	Interferon-related developmental regulator 2	4.1	*SGPP1*	Sphingosine-1-phosphate phosphatase 1	−6.0
BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	4.0	*ESPN*	Espin	−5.6
OLFML3	Olfactomedin-like 3	3.8	*ABCA1*	ATP-binding cassette, subfamily A (ABC1), member 1	−5.5
PNN	Pinin, desmosome associated protein	3.7	*POU4F2*	POU domain, class 4, transcription factor 1	−5.2
TACR3	Tachykinin receptor 3	3.6	*STC1*	Stanniocalcin 1	−5.2
PDLIM2	PDZ and LIM domain 2 (mystique)	3.6	*UCHL1*	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	−5.1
FMN2	Formin 2	3.5	*DOCK3*	Dedicator of cytokinesis 3	−4.5
EPHA1	EPH receptor A1	3.4	*ATP6V0A2*	ATPase, H+ transporting, lysosomal V0 subunit a isoform 2	−4.1
IRF2BP1	Interferon regulatory factor 2 binding protein 1	3.4	*ADD2*	Adducin 2 (beta)	−4.1

The 10 genes with the highest positive *d* score (overexpressed) and the 10 genes with the lowest negative *d* score (most underexpressed), according to SAM analysis of gene arrays (All treated group vs. Placebo group), are shown. The *d* score reflects the averaged ratio of change in gene expression to its variance [39], where values are rounded off to the nearest decimal.

oligonucleotides (Compugen USA, Jamesburg, NJ). After excluding the RNA samples and the hybridized arrays of poor quality, a total of 26 arrays were available for further analysis: 10 for the placebo group (All placebo; of these, 2 subjects were identified as equol producers and included in the array analysis within the group), 11 for equol non-producers who received isoflavones (NP-treated) and 5 for equol producers who received isoflavones (P-treated). All arrays originated from the same printing batch. The arrays were scanned using a GenePix 4000B Scanner (Axon Instruments, Union City, CA). Detailed protocols are available at http://cancer.med.unc.edu/genomicscore/. Images obtained were analyzed by superimposing a grid for each array using GenePix Pro 5.0 software (Axon Instruments). All spots of poor quality (as determined by visual inspection) were flagged as bad and removed from further analysis.

2.7. Microarray data analysis

Raw expression data files collected were further processed by uploading them into the UNC Microarray Database (http://genome.unc.edu/), and these data were filtered and retrieved according to the following criteria: all 26 arrays were uploaded; data retrieved by SUID to collapse replicate spots by gene name; spots were selected if they had both Channel 1 and Channel 2 Lowess normalized mean at least 30% above background; genes were selected only if they had >70% good data (each gene had good spots for 19 or more arrays out of 26). No cutoff values were selected. The final data were expressed as log(base 2) of end of treatment/baseline Lowess normalized ratio (mean), with 5269 unique genes that qualified for further analysis.

2.7.1. Statistical analysis of gene expression

Significance analysis of microarrays (SAM) [39] was applied to the final data using TIGR MeV software version 3 [40]. We used the one-class response type and 100 or

Table 2
Changes in gene expression were influenced by the capacity to form equol

Gene symbol	All treated vs. Placebo	NP-treated vs. Placebo	P-treated vs. Placebo
ABCA1	−5.5	−3.4	−2.2
ADD2	−4.1	−3.0	=
ATP6V0A2	−4.1	−2.4	−2.5
BAZ1A	4.0	=	=
DOCK3	−4.5	−3.1	−2.3
EPHA1	3.4	=	=
ESPN	−5.6	−3.5	−2.8
FMN2	3.5	=	=
IFRD2	4.1	=	=
IRF2BP1	3.4	=	=
KCNG2	4.5	=	=
OLFML3	3.8	=	=
PDLIM2	3.6	=	=
PNN	3.7	3.4	=
POU4F2	−5.2	−3.2	−4.1
SGPP1	−6.0	−4.4	−2.8
STC1	−5.2	−3.0	−3.8
TACR3	3.6	=	=
TNFAIP6	−6.1	−4.3	−2.7
UCHL1	−5.1	−3.4	−3.5

The 20 genes with the highest and lowest relative expression *d* scores (All treated vs. Placebo), sorted in alphabetical order, are shown. The *d* score reflects the averaged ratio of change in gene expression to its variance [39], where values are rounded off to the nearest decimal. An equal sign signifies no change (see Section 2 for the assessment of change within treatment groups vs. placebo). Negative *d* scores represent underexpression, while positive *d* scores represent overexpression. NP-treated, equol nonproducers; P-treated, equol producers.

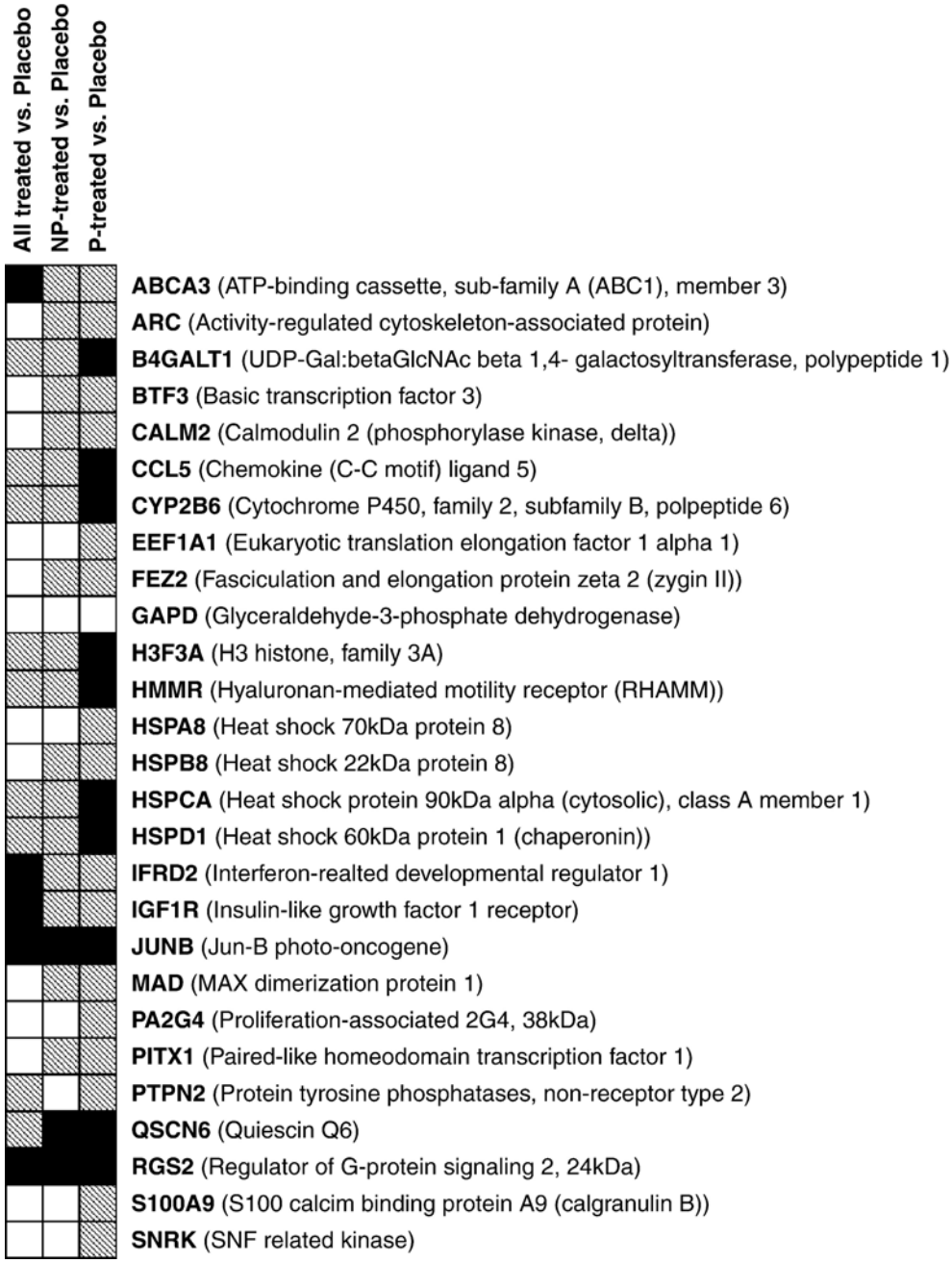

Fig. 2. Treatment with isoflavones induced changes in expression of estrogen-responsive genes. Isoflavones induce a stronger estrogenic response in equol producers (P-treated) than in the nonproducers (NP-treated). Results are also shown for responses of these two groups when lumped together for analyses (All treated). All genes were previously reported to be overexpressed by estrogen, except for *QSCN6* [71–75], based on the search using ERGDB (available at http://research.i2r.a-star.edu.sg/promoter/Ergdb-v11/index.htm). Black box, overexpression versus placebo group; white box, underexpression versus placebo group; hatched box, no change versus placebo group.

maximum allowed permutations (if less than 100) to select the genes that were significantly changed across all groups (all treated with placebo, Placebo; all isoflavones treated, All treated; equol nonproducers treated, NP-treated; equol producers treated, P-treated). An arbitrary false discovery rate (FDR) with a maximum of 5% was chosen, and the closest threshold value Δ was selected for each group. The software generated a list of significantly over- and underexpressed genes (d-score assessment), and q values were computed for each gene (the lowest FDR at which the gene is called significant). The data generated by SAM were converted to logical operators for subsequent use in gene ontology (GO) classification (−1 for underexpression, 0 for no change, 1 for overexpression) and for the analysis of the differentially expressed genes between groups (All treated vs. All placebo; NP-treated vs. All placebo; P-treated vs. All placebo).

2.7.2. GO classification

GoMiner 1.22 (http://www.miblab.gatech.edu/gominer/) was used to construct a GO list of the significantly changed genes [41], using gene symbols as identifiers. Data from the SAM output file were converted to text files, and GoMiner generated a list of genes classified by their inclusion criteria in various GO classes, based on the default database (com.mysql.jdbc.Driver at jdbc:mysql://discover.nci.nih.gov/GEEVS). Fisher's Exact Tests were performed to assess the extent of change within the total number of genes in each GO class.

2.7.3. Estrogen-responsive genes search

The Dragon Estrogen Responsive Genes Database (ERGDB; available at http://research.i2r.a-star.edu.sg/promoter/Ergdb-v11/index.htm) was used to identify genes that were previously reported to be responsive to estrogen and to contain estrogen-responsive elements within their promoters [42]. A total number of 373 genes were reported to be regulated in various human models by estrogen receptor activation (last checked on February 13, 2006).

3. Results

3.1. Differentially expressed genes

Out of 5629 unigenes for which data were retrieved, the isoflavone treatment induced changes in gene expression for a number of genes across all comparison groups: 562 significantly changed genes for All treated versus Placebo comparison group, 322 genes for NP-treated versus Placebo and 319 genes for P-treated versus Placebo. A complete list of changes is available at http://www.unc.edu/zeisel_lab/. The different patterns in gene expression changes between the equol producers (P-treated) and equol nonproducers (NP-treated) versus the placebo group (Placebo) are presented in Fig. 1. A selected list of genes with the highest *d* scores (higher overexpression) and with the lowest *d* scores (the most underexpressed) for changes with isoflavone treatment is given in Table 1. However, when the analysis was further stratified based on the equol-producing status (equol producers, P-treated; equol nonproducers, NP-treated), we found significant differences from placebo that were not apparent in the initial analysis when these subjects were lumped together (All treated vs. Placebo). Table 2 indicates the same 20 genes, where relative expression scores (*d* scores) are given for all three comparison groups, versus placebo. Cluster affinity search technique analysis using TIGR MeV revealed 14 different patterns of change between P-treated and NP-treated subgroups when compared with the All treated group. Fig. 1 depicts seven selected clusters where changes are different between the two subgroups (P-treated and NP-treated).

Of special interest are potential expression changes among the estrogen-responsive genes. Twenty-seven of these genes were responsive to the soy isoflavone treatment (Fig. 2). Interestingly, 10 genes were overexpressed within the equol-producer group (P-treated), while only 3 genes were overexpressed within the equol-nonproducer group (NP-treated). Conversely, one gene was underexpressed within the P-treated group, while seven genes were underexpressed within the NP-treated group. Ten other genes were significantly changed within the All treated versus

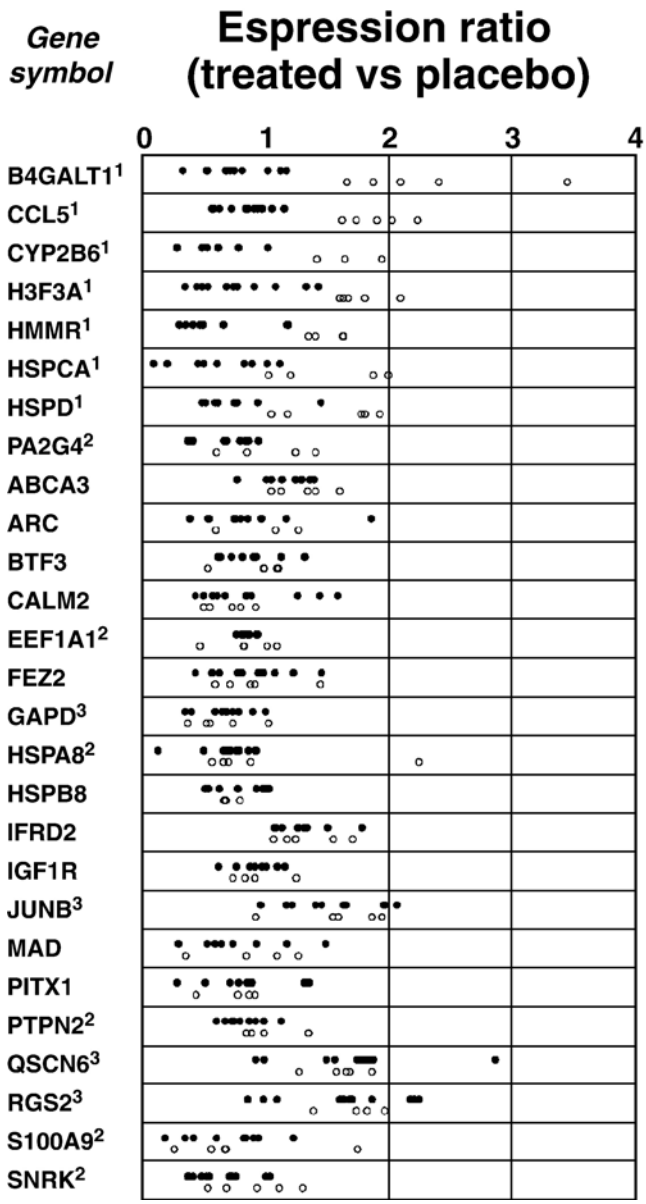

Fig. 3. For certain estrogen-responsive genes, the capacity to form equol changes the magnitude of change in gene expression after isoflavone treatment. Raw gene expression ratios (treatment vs. placebo, before SAM) are plotted for all 27 genes that were considered significantly changed by SAM. Each spot represents the ratio from each individual array. Black circles, NP-treated; white circles, P-treated. Superscript numbers reflect statistical significance of the change in gene expression versus placebo (via SAM analysis): 1 for P-treated group only, 2 for NP-treated group only and 3 for both P-treated and NP-treated group separately. The absence of a superscript number reflects lack of statistical significance for each group but significance of change for All treated (pooled NP-treated and P-treated).

Table 3
Expression of genes involved in cAMP metabolism, cell differentiation, G-protein-coupled protein metabolism and signaling, tyrosine kinase activity and steroid transport was significantly changed after isoflavone treatment

GO ID	Term	All treated vs. Placebo	NP-treated vs. Placebo	P-treated vs. Placebo
30552	3′,5′-cAMP binding	↑	↑	
16208	AMP binding	↑	↑	
19933	cAMP-mediated signaling	↑		
30154	Cell differentiation	↑		
51301	Cell division	↓		
4693	Cyclin-dependent protein kinase activity	↓		
4930	G-protein-coupled receptor activity			↑
16526	G-protein-coupled receptor activity, unknown ligand			↑
7186	G-protein-coupled receptor protein signaling pathway		↑	↑
7188	G-protein-signaling, coupled to cAMP nucleotide second messenger	↑		
30296	Protein tyrosine kinase activator activity		↑	
30971	Receptor tyrosine kinase binding		↑	
8277	Regulation of G-protein-coupled receptor protein signaling pathway		↑	
3707	Steroid hormone receptor activity			↑
15248	Sterol transporter activity	↓		

Changes in gene expression were grouped by GO classes as described in Section 2. Isoflavones induced different responses in gene expression when equol-producer status (NP-treated, equol nonproducers; P-treated, equol producers) was used as a stratification criterion. The up arrow indicates that the number of overexpressed genes was significantly changed for the respective GO class; the down arrow indicates that the number of underexpressed genes was significantly changed for the respective GO class (Fisher's Exact Test, $P<.05$).

Placebo analysis, but the equol-producer stratification did not reveal any significant changes (likely due to smaller cell size and loss of power). The raw data (before SAM analysis) for these estrogen-responsive genes are given in Fig. 3, where raw ratios (treatment vs. placebo) are plotted.

GO analysis revealed that for 397 GO classes, the Fisher's Exact Test was significant ($P<.05$). A selected list of significantly changed GO classes is shown in Table 3, where selection criteria were related to several potential mechanisms of action for genistein: tyrosine kinase inactivation, changes in cyclic adenosine 3′,5′-monophosphate (cAMP) metabolism, estrogen-related activation, G-protein-coupled metabolic processes and cell differentiation and proliferation.

4. Discussion

Two important questions as to the actions of isoflavones were addressed in these studies: (a) Are isoflavones net estrogen receptor agonists in postmenopausal women? and (b) Does the capacity to form equol influence the response to isoflavones in humans? For the first time in humans, we show that treatment with isoflavones changes the expression of genes known to be responsive to estrogen and that equol status is an important modifier of gene expression responses to treatment with isoflavones. Our findings are based on studies in lymphocytes from treated women, and we caution that these do not necessarily reflect changes that might occur in organs such as breast and endometrium.

Traditionally, studies on the effects of isoflavones have not always differentiated between humans who are equol producers and those who are not. When we lump all subjects in one group, we observed that genes with the highest relative expression changes (Table 1), including *IFRD2* (interferon-related developmental regulator 2) and *IRF2BP1* (IRF-2 binding protein 1), previously reported to be underexpressed in blood cells [43], increased their expression with isoflavones. IRF2BP1 binds to the C-terminal repression domain and has the properties of IRF-2-dependent transcriptional corepressors that can inhibit both enhancer-activated and basal transcription [44]. *KCNG2* encodes a gamma subunit of the potassium channel, voltage-gated, subfamily G and was characterized in rat and human myocardium [45]. The bromodomain adjacent to zinc finger domain, 1A gene (*BAZ1A*) is involved in chromatin-dependent regulation of transcription. Bromodomain proteins are integral components of chromatin remodeling complexes and frequently possess histone acetyltransferase activity [46]. The olfactomedin-like 3 gene (*OLFML3*) was reported to be underexpressed in various leukemia cells [47], although its function in blood cells is unknown. The pinin, desmosome associated protein gene (*PNN*) was hypothesized to act as a tumor suppressor in certain types of cancers [48]. Tachykinin receptor 3 gene (*TACR3*), although initially identified predominantly in the central nervous system [49], has been recently reported to be expressed in the platelet's membrane and to possibly be involved in platelet aggregation [50]. The PDZ and LIM domain 2 (mystique) gene (*PDLIM2*) is an IGF-IR-regulated adapter protein located at the actin component of the cytoskeleton and is necessary for the migratory capacity of cells of epithelial origin [51]. The fromin 2 gene (*FMN2*) was extensively studied, especially with regard to its role in oocyte meiosis [52]. The EPH receptor A1 gene (*EPHA1*) belongs to the ephrin receptor subfamily of the protein tyrosine kinase family and was previously reported to be differently expressed in various leukemia cells [47].

Among the genes with the highest relative decreases in expression changes after isoflavone treatment (Table 1), *ABCA1* [adenosine triphosphate (ATP)-binding cassette

transporter A1] is implicated in cAMP-dependent and sulfonylurea-sensitive anion transport. ABCA1 deficiency is associated with the Scott syndrome, a bleeding disorder characterized by a failure to expose phosphatidylserine on the outer leaflet of the platelet plasma membrane [53]. Another underexpressed gene, *STC1* (stanniocalcin 1) was directly correlated to acute leukemia at diagnosis and relapse [54]. The tumor necrosis factor, alpha-induced protein 6 gene (*TNFAIP6*) was underexpressed in isoflavone-treated subjects. Normally expressed in peripheral blood mononuclear cells, it is possibly involved in cell–cell and cell–matrix interactions during inflammation and tumorigenesis [55]. Sphingosine-1-phosphate phosphatase 1 (*SGPP1*) regulates the activity of sphingosine-1-phosphate by controlling the phosphorylation status of sphingosine-1-phosphate. It has been suggested that regulation of this gene could have important implications in cell proliferation, angiogenesis and apoptosis [56]. The ATPase, H+ transporting, lysosomal V0 subunit a isoform 2 (*ATP6V0A2*) is an ATP-dependent proton pump responsible for acidification of intracellular compartments and appears to be involved in the modulation of the immune response within the macrophages [57,58]. Another underexpressed gene, adducin 2 (beta) (*ADD2*) appears to be involved in complex reciprocal relationship regulation with calmodulin binding and phosphorylation by protein kinase A (PKA) and PKC [59]. All other underexpressed genes (*ESPN*, *POU4F2*, *UCHL1* and *DOCK3*) appear to be highly specific for the nervous tissue, and little, if any, information is available on their roles in other tissues, according to our PubMed and SOURCE searches (http://smd.stanford.edu/cgi-bin/source/sourceResult).

Our gene expression analysis indicates that the response to soy isoflavones is not homogeneous and can be related to the equol-producing status (Table 2 and Fig. 1). When genistein-treated subjects are stratified according to their equol-producing status, the two subgroups show a different response to isoflavones (Fig. 1).

To better understand and integrate changes in gene expression to isoflavone treatment, we used GO analysis to classify genes with significant changes according to their ontology (Table 3). Several mechanisms are involved when discussing soy isoflavones' effects: (a) effects on cell proliferation and apoptosis including tyrosine kinase inhibition, (b) effects on cell differentiation, (c) estrogenic effects and (d) effects related to DNA damage and repair. Expression of *PCNA*, a surrogate index for cellular proliferation, was inhibited by isoflavones in endometrial cell cultures [60]. Genistein inhibits tyrosine-specific protein kinase activity [25,61]. However, genistein also exerts a tyrosine-kinase-independent inhibition of cAMP phosphodiesterase [62] with subsequent accumulation of cAMP [63]. cAMP activates PKA by binding to the regulatory subunit of PKA and releasing active catalytic subunits [64]. The roles of PKA in lymphocytes and immune response have been reviewed recently [65].

A human study in which subjects were given single doses of genistein (2, 4 or 8 mg/kg; each dose was separated by 1 week) reported that after one dose, tyrosine phosphorylation was significantly changed, suggesting that soy can modulate cell-signaling pathways in vivo [66]. In our study, we found that genes involved in cAMP binding were significantly overexpressed in the equol nonproducers. Interestingly, the treated group significantly underexpressed two genes involved in cyclin-dependent kinase activity: *CDK7* (cyclin-dependent kinase 7), which is expressed in lymphocytes and involved in nucleotide excision DNA repair [67], and *CKS2* (CDC28 protein kinase regulatory subunit 2), underexpressed in the NP-treated group, which significantly correlates with risk for acute lymphoblastic leukemia [68]. In equol nonproducers (NP-treated), one gene, *NRG1* (Neuregulin 1), classified into "Protein kinase activator activity" and "Receptor tyrosine kinase binding," was overexpressed. This gene is expressed in blood cells and acts as a direct ligand for erbb3 and erbb4 tyrosine kinase receptors, resulting in ligand-stimulated tyrosine phosphorylation and activation of the erbb receptors [69]. In equol producers, the "steroid hormone receptor activity" GO member genes were significantly overexpressed: *ESRRB* (estrogen-related receptor beta) and four other orphan nuclear receptors (*NR0B2*, *NR2C1*, *NR2F2* and *NRII3*). Genistein modulated the expression of a wide variety of genes in cultured prostate cancer cells, including cell growth, apoptosis, angiogenesis and metastasis [30,35]. Some similar effects on the expression of genes involved in cell growth and apoptosis were reported in whole animal studies using genistein treatment of rats and studying gene expression in uterine tissue [70].

In our studies, isoflavones induced various responses in the expression of estrogen-responsive genes (Fig. 2). Of the 27 responsive genes identified as significantly changed, 10 were overexpressed in equol producers (Fig. 2), while only 3 were overexpressed in the equol nonproducers (*JUNB*, *QSCN6* and *RGS2*). Conversely, more genes (seven genes) were underexpressed in the nonproducers, while only one gene (*GAPD*) was underexpressed in the equol producers. Among overexpressed genes, *B4GALT1* encodes Type II membrane-bound glycoproteins that appear to have exclusive specificity for the donor substrate UDP-galactose; it appears to be involved in B-cell maturation [76]. Another estrogen-responsive, overexpressed gene was *CYP2B6* (of the cytochrome P450 superfamily of enzymes, moderately expressed in lymphocytes [43]), involved in the metabolism of xenobiotics. JUNB is a member of JUN family members that can dimerize with one another or with members of Fos and ATF families to form AP-1 transcription factor. However, the transactivation activity of JUNB is much weaker when compared with JUN. A number of studies demonstrated that JUNB antagonizes the functions of JUN in cell cycle regulation, proliferation and transformation by competing with JUN to form less-efficient transactivating dimers. Thus, JUNB may be considered to be a conditional tumor suppressor [77].

Recent reports suggest that soy isoflavones, particularly genistein, can induce gene damage. Genistein induced mammalian topoisomerase-II-dependent DNA cleavage in purified broken cell preparations [78] and induced increased DNA strand breaks as detected by COMET assay and micronucleus formation in mouse lymphoma cells in culture [79]. It is proposed that genotoxicity arises from alterations of the DNA topoisomerase II activity, resulting in a stabilization of DNA double-strand breaks at topoisomerase II–DNA binding sites [80]. However, in our previous human study, no significant genotoxicity was observed at high doses (300 or 600 mg) of a purified soy isoflavone mixture administered for 84 days to 15 men with prostate cancer [81]. In our current study, although the number of changed genes within the "DNA repair" GO class as a whole was not significant (data not shown), some of the genes involved in DNA repair were significantly changed: *ABL1*, *PTTG1*, *CDK7*, *GTF2H3*, *G22P1* and *RAD9A* were underexpressed, while *XRCC2* was overexpressed (All treated vs. Placebo), and no significant changes were found within the two subgroups (P-treated and NP-treated). It is possible that our small study does not have sufficient power to detect changes in these subgroups.

In conclusion, dietary soy isoflavones induced changes in gene expression in postmenopausal women. These genes are involved in a variety of pathways. Importantly, some of the changes were related to increased cell differentiation, increased cAMP signaling and G-protein-coupled protein metabolism and increased steroid hormone receptor activity. In addition, the response to isoflavones was different between equol producers and equol nonproducers, with enhanced expression of some of the estrogen-responsive genes mainly occurring within the equol producers, suggesting that the capacity to form equol may be an important determinant in responsiveness to isoflavone treatment. Further studies are required to specifically assess the functional significance of these changes relative to the metabolism of postmenopausal women.

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