**Uterine Responses to Feeding Soy Protein Isolate and Treatment with 17 β -Estradiol Differ
in Ovariectomized Female Rats**

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Short Title: Selective estrogen receptor modulator effects of soy in rat uterus

Abstract

There are concerns regarding reproductive toxicity from consumption of soy foods, including an increased risk of endometriosis and endometrial cancer, as a result of phytoestrogen consumption. In this study, female rats were fed AIN93G diets made with casein (CAS) or soy protein isolate (SPI) from postnatal day (PND) 30, ovariectomized on PND 50 and infused with 5 µg/kg/d 17β-estradiol (E2) or vehicle. E2 increased uterine wet weight ($P<0.05$). RNAseq analysis revealed that E2 significantly altered expression of 1991 uterine genes ($P<0.05$). SPI feeding had no effect on uterine weight and altered expression of far fewer genes than E2 at 152 genes ($P<0.05$). Overlap between E2 and SPI genes was limited to 67 genes. Functional annotation analysis indicated significant differences in uterine biological processes affected by E2 and SPI and little evidence for recruitment of estrogen receptor (ER)α to the promoters of ER-responsive genes after SPI feeding. The major E2 up-regulated uterine pathways were carcinogenesis and extracellular matrix organization, whereas SPI feeding up-regulated uterine peroxisome proliferator activated receptor (PPAR) signaling and fatty acid metabolism. The combination of E2 and SPI resulted in significant regulation of 504 fewer genes relative to E2 alone. The ability of E2 to induce uterine proliferation in response to the carcinogen dimethylbenz(a)anthracene (DMBA) as measured by expression of PCNA and Ki67 mRNA was suppressed by feeding SPI ($P<0.05$). These data suggest SPI is a selective estrogen receptor modulator (SERM) interacting with a small sub-set of E2-regulated genes and is anti-estrogenic in the presence of endogenous estrogens.

Key Words: Uterus; Soy; Estradiol; Endometrial Cancer; Selective Estrogen Receptor Modulator

INTRODUCTION

Hormone replacement therapy (HRT) with estrogens used to be standard therapy for treatment of menopausal symptoms such as hot flushes and depression. However, findings from the Women's Health Initiative and subsequent studies revealed increased risks of heart disease, breast cancer and uterine conditions such as hyperplasia, endometriosis and endometrial cancer (Rossouw et al., 2002). As a consequence, many American women have turned to use of plant based dietary supplements containing phytoestrogens. A recent survey indicated that about 42% of women were using soy products including soy protein and isoflavones such as genistein (Mahady et al., 2003). The majority of epidemiological studies and clinical trials in post-menopausal women consuming soy foods have suggested either no effects or decreased risk of endometrial cancer and a lack of effects on uterine morphology or endometrial proliferation (Mahady, 2005; Tsuchiya et al. 2007; Myung et al., 2009; Ollberding et al., 2012; Quaas et al., 2013; Alekel et al. 2015; Budhathoki et al., 2015). However, Unfer et al. (2004) reported significantly higher endometrial hyperplasia after consumption of soy isoflavones in a 5-year randomized double-blind, placebo controlled clinical trial of post-menopausal women, and there are several case reports of endometriosis in patients with a high intake of soy products and concentrated soy isoflavones (Noel et al. 2006; Chandrareddy et al. 2008). Although published data suggest anti-estrogenic actions of soy and soy isoflavones in animal models and premenopausal women (Tansey et al. 1998; Mahady, 2005; Qin et al., 2009; Ronis et al., 2012; Douglass et al., 2013; Maskarinec et al. 2013; Gonzales et al., 2014), concerns persist on the potential risks of soy products and isoflavone consumption on female reproductive health, including uterine function, during early development and post-menopause when endogenous estrogens are present at low levels (Mahady 2005, Badger et al., 2009). These concerns center mainly around the potential

65 estrogenicity of isoflavone supplements containing genistein and daidzein and of soy protein
66 isolate (SPI) which is used extensively in processed foods and is the sole protein source in soy
67 infant formula (Jeffersen and Newbold, 2000; Allred et al. 2001; Doege and Sheehan 2002;
68 Jeffersen et al. 2007; Andrade et al. 2015). In this regard, the National Toxicology Program
69 recently increased its level of concern about the potential reproductive toxicity of soy infant
70 formula from negligible to minimal (McCarver et al., 2011). Numerous mechanistic animal
71 studies purporting to relate to the estrogenic effects of soy on uterine function such as
72 appearance of hyperplasia; increased cell proliferation; and increased endometrial
73 carcinogenesis, have utilized phytochemical extracts or purified isoflavones as opposed to soy
74 foods such as SPI, and have differed significantly in developmental stage, route and duration of
75 exposure and dose (Cotroneo et al., 2005; Ford et al. 2006; Diel et al. 2006; Chang et al. 2007;
76 Rimoldi et al. 2007; Aidoo et al., 2009; Moller et al., 2009, 2010, 2012; Jefferson et al., 2009;
77 Bliedtner et al. 2010; Moller et al. 2010; Gaete et al. 2012; Kakehasgi et al., 2012; Salleh et al.
78 2013; Norrby et al., 2013; Zin et al, 2013; Chinigarzadeh et al., 2014). One study using soy
79 protein isolate feeding of adult female Sprague Dawley rats for 14 weeks reported histological
80 evidence of squamous metaplasia in one out of 10 animals (Nakai et al. 2005). However, the
81 same group did not observe similar effects on uterine histology in ovariectomized Sprague
82 Dawley rats or in the more estrogen sensitive Fischer 344 rat strain (Nakai et al. 2005; Bahr et al.
83 2005). In addition , the only study which has compared developmental effects of dietary SPI on
84 the rat uterus with pure genistein at nutritionally relevant doses, observed minimal effects of
85 either treatment on uterine morphology or proliferation but that genistein induction of apoptosis
86 was not mimicked by SPI (Eason et al., 2005). In the current study, we directly compared the
87 effects of feeding SPI and 17 β -estradiol (E2) treatment, alone or in combination, on uterine gene

expression profiles and estrogen receptor alpha (ER α) binding to ERE elements on the promoters of estrogen responsive genes in ovariectomized (OVX) adult female rats to address the issue: Does SPI act as a weak estrogen in the uterus in the absence of endogenous estrogens? In addition we examined the effects of SPI and E2 on expression of cytochrome P450 (CYP1) enzymes and cell proliferation in the uterus of OVX rats treated with the model mutagen and carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) to determine if there is any evidence for adverse effects of SPI feeding on chemical carcinogenesis in the uterus.

MATERIALS AND METHODS

Animal Care and experimental design. Animal studies received prior approval from the Institutional Animal Care and Use Committee at UAMS. Female Sprague-Dawley rats, postnatal day (PND) 30, were obtained from Charles River Laboratories (Wilmington, MA) and were housed in an AAALAC approved animal facility under a 12 h light-dark cycle. The rats were given *ad libitum* access to semi-purified diets made according to the AIN-93G formula (Reeves et al. 1993) except that corn oil replaced soybean oil and the protein source varied. Half of the animals were fed diets made using casein (CAS) as the protein source, the other half were fed the same diet made with soy protein isolate (SPI) (Singhal et al. 2009a). The soy diet contained 430 mg total isoflavones/kg (276 mg/kg genistein and 132 mg/kg daidzein). Daily consumption of genistein averaged 19.3 mg/kg/d and that of daidzein averaged 9.2 mg/kg/d. Total isoflavones in 24-h urine pools averaged ~40 μ mol/l (Ronis et al. 2001).

Rats were fed CAS or SPI diets from PND30 until PND65. On PND50, rats were ovariectomized (OVX) to remove endogenous estrogens. Half of each diet group were

subcutaneously infused with 5µg/kg/d 17β-estradiol (E2) in polyethyleneglycol vehicle and half were infused with vehicle alone via Alzet 2002 mini-osmotic pumps (Alza Corp., Mountain View, CA, USA). On PND64 half of each group of rats were gavaged with either 50 mg/kg of the carcinogen DMBA in sesame oil vehicle or with vehicle alone. 24 h later, all rats were anesthetized with Nembutal (100 mg/kg) followed by decapitation (Singhal et al. 2009b). Blood and uterus were collected and frozen at -80° C until analysis. Treatment groups (N = 6/group), were designed as follows: 1) Control, CAS diet (CAS); 2) CAS-E2, E₂ treatment on CAS background; 3) SPI, SPI diet; 4) SPI-E2, E₂ treatment on SPI background; 5) CAS-DMBA, DMBA treatment on a CAS background; 6) CAS-E2-DMBA, DMBA and E₂ treatment on a CAS background; 7) SPI-DMBA, DMBA treatment on a SPI background; 6) SPI-E2-DMBA, DMBA and E₂ treatment on a SPI background.

Serum Analysis of Isoflavones and E2. Serum concentrations of soy associated isoflavones and their metabolites (genistein, daidzein, dihydrodaidzein, glycitein, o-desmethylangolansin and equol) were measured by LC-MS and have been reported previously (Singhal et al., 2009a). Serum E2 was measured according to manufacturer's instructions by radioimmunoassays (DLS-4800, Beckman Coulter, Indianapolis, IN, USA).

Poly-A RNA isolation and Preparation of RNA-seq libraries. Total RNA was isolated from each uterus using RNeasy-mini columns (Qiagen, Valencia, CA), including on-column DNase digestion. RNA quality and integrity was confirmed by A260/A280 ratio (> 1.9) and visualization using Experion RNA Std-Sens chips (BioRad, Hercules, CA). Three biologically separate pools containing equal amounts of RNA from 2 individual uteri were utilized. Thus N= 6 uteri samples were represented over three biological replicate pools. RNA-seq libraries were constructed as follows. Poly-A RNA was isolated from 5 µg of total RNA using Dynabeads®

133 mRNA-Direct kit (Invitrogen, Carlsbad, CA) and procedures described previously (1). Briefly,
134 poly-A RNA was captured by addition of 100 μ l of Oligo-(dt)25 Dynabeads in 150 μ l of lysis
135 buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8, 1% LiDS, 5 mM
136 dithiothreitol). The mixture was incubated on a rotary shaker for 20 min at room temperature.
137 mRNA-bead complexes were washed twice with 100 μ l of wash buffer A (10 mM Tris-HCl, pH
138 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), followed by two washes (100 μ l each) with wash
139 buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). RNA was eluted from the beads
140 in 11 μ l of nuclease free water by heating to 65° C for 5 min. Following purification, mRNA was
141 sheared to ~ 150 bp fragments using a Covaris S2 instrument (120 μ l volume, 10% DC, 5
142 intensity, 200 cpb, 7.5 min). Fragmented poly-A RNA was precipitated using sodium
143 acetate/ethanol and reconstituted in 14 μ l of nuclease free water. RNA-seq library construction
144 was carried out using NEB-Next reagents (New England Biolabs, Ipswich, MA). First and
145 second strand cDNA synthesis, end-filling using Klenow fragment, and dA-tailing were carried
146 out using manufacturer's recommendations. Ligation with Illumina's paired-end adapters for
147 multiplexed sequencing was performed with 1 μ l of T4 DNA ligase, 0.3 μ M of annealed
148 adapters, in a 50 μ l reaction volume for 30 min at room temperature. Ligated products were
149 separated using a high-resolution 2% agarose gel (Bio-Rad, Cat # 161-3107), and products
150 around 200 bp ($\pm$ 50 bp) were excised and purified using Qiagen gel extraction kit (Qiagen,
151 Valencia, CA). Size-selected cDNA libraries were amplified using indexed primers containing a
152 6-bp barcode. PCR was carried out for 12-14 cycles using 29 μ l of template, 1 μ l of forward and
153 reverse primers (25 μ M), and 1 U Phusion high-fidelity DNA polymerase (New England
154 Biolabs, Ipswich, MA). PCR products were purified using Qiaquick PCR purification columns
155 (Qiagen, Valencia, CA) and eluted in 30 μ l final volume. A small aliquot (~1 μ l) was evaluated

using DNA 1000 chip (Experion automated electrophoresis, Bio-Rad, Hercules, CA) to confirm the absence of primer-dimers and other spurious products. Quantification of the RNA-seq libraries was done via quantitative real-time PCR using SYBR green chemistry (Kapa Biosystems, Woburn, MA). Diluted libraries (1:10,000) were quantitated using standards ranging from 0.0002 – 20 pM.

Sequencing, Alignment and Data Analysis. Libraries from the same group were indexed with unique 6-bp barcodes, combined and clustered on a single-read flow cell. Clustered libraries consisting of 36-bp single read plus an index read were sequenced with a Genome Analyzer II_x using TruSeq v5 reagents (Illumina Inc., San Diego, CA). Image analysis including base calls, was performed by Real-time Analysis software (RTA v2.6, Illumina). Individual .qseq files containing reads and base-quality information per tile of the flow-cell were demultiplexed based on their respective barcodes using scripts in the CASAVA v1.7 suite (Illumina, San Diego, CA). After demultiplexing each lane into biological replicates, three (fastq) files were obtained for each experimental condition. Reads' quality control was performed with the program FastQc v 0.10. Phred quality scores above 25 were considered acceptable for a given nucleotide. To standardize and remove possible uninformative reads, a quality filter was applied using the tool fastq_quality_filter (FASTX toolkit v 0.0.13.2) with parameter values p=0.95 and q=25 producing an average of approximately 10 million reads per fastq file. Reads were aligned to the rn4 rat genome using TopHat v. 2.0.9 and Bowtie 2.1.0 and have an average alignment rate of 79.53 percent. To assemble individual transcripts from RNA-seq reads the program Cufflinks (v 2.1.1) (Trapnell et al. 2014) was used. The statistical model for differential expression included in the program Cuffdiff (part of Cufflinks) was used to select genes with significant changes in the summed FPKM values of their transcripts with an FDR-adjusted P-value less than 0.05.

179 Individual differentially expressed gene lists were generated for each of the diet groups keeping
180 casein as control. The data was further annotated and integrated with routines from the library
181 cummeRbund in R (v 3.1.1). Gene lists were further cleaned up imposing a two-fold change
182 cutoff of the estimated gene-expression FPKM values (Fragments Per Kilobase of exon per
183 Million fragments mapped) on a specific experimental group with respect to the estimated
184 FPKM value of the casein (control) group. Complete lists of the differentially expressed genes
185 with these conditions implemented were used for subsequent analysis and are presented in a
186 supplementary file (Supplemental Data file 1). Data files were also deposited in the Gene
187 Expression Omnibus (GEO) repository at the National Center for Biotechnology Information
188 (<http://www.ncbi.nlm.nih.gov/geo/>) under the GEO accession number GSE69819.

189 Functional analysis from our gene lists was performed using the functional annotation
190 tool DAVID (v. 6.7) (Huang, et al. 2009,b). The gene id was transformed internally in DAVID,
191 and the rat KEGG pathways for each of the comparisons are presented as a supplement (Data in
192 Brief). We further organized gene lists into a network of interactions within specific pathways
193 using the Reactome Functional Interaction Network plugin (2013) in Cytoscape (v.3.1.1). Highly
194 significant and relevant pathways were selected for graphical representation in Circos (v. 0.64).
195 Heatmaps were generated from the FPKM values in cummeRbund. Network interactions were
196 extracted from the so-called edge files within the Reactome FI tool in Cytoscape, and were
197 divided into three categories: activation (catalyze), repression (inhibition) or predicted
198 (complex). Scripts in Python (v 2.7.5) were developed to integrate the heatmaps and network
199 interactions into Circos (Krzewinski et al. 2009). The list of the selected pathways is presented in
200 Data in Brief.

201 ***Real-time quantitative PCR.*** Total RNA (1 µg) was reverse-transcribed using iSCRIPT cDNA
202 synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. cDNA
203 samples were amplified using previously described conditions (Singhal et al. 2008a,b).
204 Quantitative real-time PCR was performed on genes that were of particular interest to this study
205 (see Table 1 for primer list). Expression levels of genes were normalized to the gene levels of
206 control genes *Rps13* and *Spr14*, averaged with geNorm (Vandesompele et al. 2002).

207 ***Western Blotting.*** Tissue lysates were prepared from 100 mg of individual frozen uterine tissues
208 homogenized in RIPA buffer. 20 µg of each sample was applied to 10% SDS-PAGE gels.
209 Western blots were performed samples from n = 5/group. Proteins were transferred onto a PVDF
210 membrane. Primary antibody against estrogen receptor (ER)α (sc-542) was from Santa Cruz
211 Biotechnology, (Dallas, TX). Polyclonal antibodies to Indian hedgehog (Ihh) (MABF23) and
212 matrix metalloproteinase (Mmp)7 (SAB451894) were obtained from Millipore and Sigma
213 Aldrich respectively. Secondary antibodies were obtained from Santa Cruz and signals were
214 detected by chemoluminescence (SuperSignal West Femto Chemiluminescent Substrate, Thermo
215 Scientific, Rockford, IL, USA) for immunoquantitation.

216 ***Chromatin Immunoprecipitation.*** Chromatin immunoprecipitation was performed as previously
217 described (Ronis et al. 2012). Briefly, chromatin was extracted with the ChIP-It Enzymatic
218 Express kit (ActiveMotif, Carlsbad, CA, USA) from pools of 300 mg of uterine tissues following
219 the manufacturer's protocol. The tissue was fixed in 20 ml fixing solution (20 mL PBS, 0.54 mL
220 37% formaldehyde) for 10 min and stopped with addition of Glycine Stop solution. Tissues were
221 pelleted and resuspended in 2 mL ChIP Lysis Buffer (Santa Cruz Biotechnology), and
222 homogenized using 20 strokes of a Dounce homogenizer. Homogenate was passed through a 100
223 µm cell strainer and nuclei were pelleted then resuspended in 1 mL Digestion Buffer. Shearing

224 was achieved by sonicating with 2 pulses of 10 sec each at setting of 6 on a Fisher 50 Sonic
 225 Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) with 15 sec of icing between pulses,
 226 followed by 5 min of icing then adding 50 μ L Shearing Enzyme Cocktail (0.5 μ L enzyme,
 227 49.5 μ L 50% glycerol) and incubation at 37°C for 10 min. The reaction was stopped with addition
 228 of 20 μ L of ice cold 0.5 M EDTA and icing for 10 min. followed by centrifugation.
 229 Immunoprecipitation was performed using 2 μ g of chromatin and 2 μ g of antibody in a 200 μ L
 230 reaction at 4°C overnight. An antibody against ER α (Santa Cruz Biotechnology, Dallas, TX,
 231 USA) was used with normal rabbit IgG as a negative control. Beads were washed twice with
 232 CHIP Buffer 1 and twice with CHIP Buffer 2. DNA was eluted in 50 μ L AM2 and reverse cross-
 233 linked by addition of 50 μ L Reverse Cross-Linking Buffer and heating at 95°C followed by
 234 treatment with Proteinase K then Proteinase K Stop Solution. As previously published (45), ER
 235 binding to estrogen response elements (EREs) was analyzed to known sites in the *ESR1* (+90 to
 236 +156, intron 3), *Clca3* (-336 to -347), *Igfbp2* (-388 to -456) and *Hsd11b1* (-18 to -120) genes.
 237 The 5' UTR of *Aldh3a2* was utilized as a negative control. Semi-quantitative PCR was
 238 performed on immunoprecipitated sequences using the following primer pairs: forward
 239 TGGGCAAC-TTCTGTTACCAAG, reverse TCTCACCAGCTGAGCTAAGTAT, *ESR1*,
 240 amplicon 133 bp; forward TGGAGTGGGTGGGTTCAGTGT, reverse
 241 ACGCTGATCCAGGAAAAGCA, *Ccl3*, amplicon 91 bp; forward
 242 GCTACGAGGTGCCCTTTGG, reverse TCACACCA-GCCCTCGTGTT, *Igfbp2*, amplicon 100
 243 bp; forward CTCCCTGACAAGCCATTCTTG, reverse GCTGGGTTCCTGAGCATCTC,
 244 *Hsd11b1*, amplicon 102 bp were visualized on 1.5% agarose gels.

245 **Statistical Analysis.** Data analysis of RNAseq data are described above. Experimental outcomes
 246 were summarized as mean $\pm$ standard errors (SE). A three factor, diet (Cas/Soy), E2 treatment

(yes/no) and DMBA treatment (yes/no), analysis of variance (ANOVA) was used in analysis of body weight, organ weight, serum E2, real time RT-PCR mRNA expression and Western immunoblot data, to test main effects and interactions. In the presence of a significant interaction, the effects of one factor were interpreted at each of the levels of the other factor. Pair-wise comparisons using Tukey-Kramer post-hoc tests were performed for findings significant at $\alpha=0.05$ to identify groups whose means differed significantly while retaining the family-wise error rate at 5% for all the models. Statistical analyses were performed using the Stata statistical package version 12.0 (Stata Corporation, College Station, TX, USA).

RESULTS

Body weight, uterine wet weight, serum Isoflavones and E2. Serum values of E2 were measured in the first OVX mouse study. Values in ovariectomized Cas and SPI fed rats were similarly low in the range of 1-5 pg/ml. E2 treatment via osmotic minipump significantly increased serum E2 to values similar to those found in intact females, in both the E2 and SPI + E2 groups, compared to Cas ($P<0.05$). However, serum E2 values were higher in the SPI + E2 group compared to the E2 group ($P<0.05$) (Table 2). Serum concentration of the different isoflavones and isoflavone metabolites in these animals have been reported previously (Singhal et al. 2009a). Genistein, daidzein, dihydrodaidzein, glycitein, o-desmethylangolansin and equol were detected in the serum of SPI-fed animals almost exclusively in their conjugated form, but were undetectable in casein-fed animals. The isoflavone found at the highest level was equol (2207 nM), followed by genistein (922 nM) and daidzein (522 nM), indicating a high level of conversion of daidzein into the more potent xenoestrogen equol. Neither DMBA treatment nor

269 feeding SPI had significant effects on body weight. CAS + E2 and SPI + E2 groups had
270 decreased body weights compared to the CAS group in both the presence and absence of
271 additional DMBA treatment ($P < 0.05$) (Table 2). Both absolute uterine wet weight and uterine
272 weight expressed as % body weight were unaffected by SPI feeding or DMBA treatment. In
273 contrast, both measures were elevated by E2 treatment irrespective of diet or DMBA treatment
274 ($p < 0.05$) (Table 2).

275 ***Uterine gene expression profiles in SPI-fed and E2-treated OVX female mice.*** A dendrogram of
276 the Jensen-Shannon distances between gene expression profiles of the different diet and
277 treatment groups demonstrated that samples from the SPI-fed group grouped with samples from
278 the CAS group while E2 treatment differentiated E2 and SPI + E2 groups from the other two
279 groups (Data in Brief). Using a 2-fold cut-off and FDR adjusted p-value < 0.05 , E2 treatment
280 resulted in significant effects on 1991 genes (1098 up-regulated and 893 down-regulated)
281 relative to the CAS control group. In contrast, SPI feeding only significantly affected expression
282 of 152 genes (133 up-regulated and 19 down-regulated) relative to CAS. The combination of E2
283 treatment and SPI feeding resulted in significant changes in expression of 1487 genes (908 up-
284 regulated and 579 down-regulated) relative to CAS. Complete gene lists are shown in
285 Supplemental Data File 1. Overlap between the E2 and SPI groups was only 67 genes (3% of E2,
286 44% of SPI). In addition, SPI feeding reduced the number of E2-regulated genes from 1991 to
287 467 (Figure 1). Pathways analysis revealed that the major biological effects of E2 treatment in
288 the uterus were associated with pathways in cancer; extracellular matrix and cytoskeleton
289 organization; Toll-like receptor signaling; HIF-1 signaling; immune responses, EGF receptor,
290 cytokine and chemokine signaling-signaling and aldosterone-regulated sodium reabsorption
291 (Data in Brief, Tables 2 and 3 and Figure 2). In contrast, the major biological pathways in the

uterus affected by feeding SPI were complement and coagulation cascades, PPAR signaling; fatty acid metabolism; and adipocytokines (Supplemental Data File 2, Tables 4 and 5). Closer examination of the two major E2-regulated pathways revealed that many E2-up-regulated genes were either unaffected or down-regulated by SPI feeding while a larger proportion of E2-down-regulated genes were also down-regulated to some degree in the SPI-fed group. Among cancer pathway and extracellular matrix organization genes only 2 of 23 and 1 of 29 E2-up-regulated genes were regulated similarly by SPI feeding, while 22 of 25 and 13 of 21 E2-down-regulated genes were regulated similarly by SPI.

The top 30 uterine mRNAs up-regulated by E2 are shown in Table 3. A cluster of 12 highly expressed genes were undetectable in the CAS group, this included a highly expressed micro RNA *Mir21*. 8 of these genes were also detected at very low levels (1-4% of E2) after SPI-feeding. This group included a variety of structural proteins including casein kappa (*Csn3*), glycoprotein 2 (*Gp2*) and amelotin (*Amtn*); the lectins *Reg3b* and *Reg3g*; the ion channel *Tmc5*; the G coupled galanin receptor *Galr3* and two genes associated with the extracellular matrix: the transmembrane serine protease *Tmprss11g* and matrix metalloproteinase *Mmp20*. The only member of this cluster with significant expression in the SPI group (20%) relative to the E2 group was the lectin *Clec2d*. The remainder of the top E2 up-regulated genes were induced 50-360-fold and included the micro RNA *Mir147*, genes encoding the enzymes lipase *Lipf* and lactoperoxidase (*Lpo*), protein arginine deamidase (*Padi1*); the chloride ion channel *Clca1*; the calcium binding protein calbindin (*S100g*); the secreted globulin *Scgb1a1*; the hormone oxytocin (*Oxt*); a variety of genes associated with cell/cell communication, regulation of apoptosis and cell proliferation and tumorigenesis including *Mmp7*, *Olfm4*, *Cuzd1*, *Lcn2*, *Fam3b*, *Gjb2*, placenta expressed transcript 1 (*Plet1*) and the transcriptional co-repressor *Kap*. Of these genes

only two, *Kap* and *Plet1* were also significantly up-regulated by >2-fold in the SPI-fed compared to the CAS group.

The top 30 uterine mRNAs up-regulated by SPI feeding are shown in Table 4. In general, the SPI-regulated genes were affected much less than the E2 regulated genes with induction only in the range of 3- to 9-fold. Many of the SPI-regulated genes are associated with lipid metabolism. These include the transcription factor peroxisome proliferator activated receptor (*PPAR*) γ , phospholipase A2 (*Pla2g2a*), fatty acid binding protein 4 (*Fabp4*), steroyl CoA desaturase (*Scd1*); malic enzyme (*Me1*), acyl CoA synthase (*Acs1l*), the fat droplet coating protein perilipin (*Plin*) and the adipokines adiponection (*Adipoq*) and resistin (*Retn*) and the proteolipid ion channel regulator neuronatin (*Nnat*). In addition, enzymes involved in amino acid metabolism (*Nat8l*, *Mosc1*, *Cdo1*); binding proteins (*S100b*, *Rbp4*); transporters (*Aqp7*) and genes involved in regulation of cell apoptosis/proliferation: (fibroblast growth factor 1 (*Fgf1*) and the cell death factor *Cidec* and genes involved in muscle contraction/vasoconstriction: the oxytocin receptor *Oxtr* and angiotensinogen (*Agt*) were also induced significantly by SPI feeding. Only 6 of these genes (*Fcn1*, *Adipoq*, *Oxtr*, *Ces1d*, *Agt* and *lfi204*) were also up-regulated significantly by E2. In contrast, *Scd1*, *Retn* and *Rbp4* were actually down-regulated by E2 treatment.

We have confirmed many of the significant gene expression changes shown by RNAseq at the individual mRNA level using real time RT-PCR (Figures 3, 4 and 5). Many highly E2 up-regulated mRNAs such as those encoding lactoperoxidase, olfactomedin 4 and S100g were unaffected by feeding SPI and the combination of E2 treatment and SPI feeding had no effect relative to E2 alone (Figure 3). Another smaller group of mRNAs including those coding for

337 mmp7, oxytocin and Clca1/3 expression which were induced significantly by E2, were further
 338 increased by the combination of E2 and SPI feeding ($P < 0.05$) (Figure 4, Figure 5). In contrast,
 339 another cluster of mRNAs including those encoding aquaporin 2 and the tumorigenesis-related
 340 genes, S100 calcium binding proteins A8 and A9 (S100A8, S100A9) (Carlsson et al. 2005) were
 341 increased by E2 ($P < 0.05$) and unaffected by SPI feeding but the E2 effect was reversed by the
 342 combination of E2 and SPI ($P < 0.05$) (Figure 4). Another cluster of mRNAs including those
 343 encoding genes regulating epithelial-mesenchymal transition (EMT) - indian hedgehog (*Inh*) and
 344 the zinc finger protein *Gli1* (Katoh and Katoh, 2008); the low density lipoprotein receptor related
 345 protein 2 (*Ldlrrp2*), Wnt inhibitory factor 1 (*Wif1*) and estrogen receptor alpha (*Esr1*) were
 346 down-regulated by E2 treatment ($P < 0.05$) and also to a lesser but significant degree by SPI
 347 feeding but were not repressed in an additive fashion by E2 + SPI (Figure 4, Figure 5). A group
 348 of mRNAs including those encoding neuropeptide Y (*Npy*), fatty acid binding protein (*Fabp4*)
 349 and carbonic anhydrase 3 (*Car3*) were elevated by SPI feeding relative to CAS or E2 groups but
 350 were significantly suppressed by the combination of E2 and SPI relative to SPI alone (FDR
 351 adjusted $P < 0.05$) (Figure 3).

352 ***ChIP Analysis of ER α Binding to EREs in Uterine Genes from SPI-fed and E2-treated OVX***
 353 ***female mice.*** We examined the effects of feeding SPI and of E2 treatment on recruitment of
 354 ER α to EREs in selected estrogen-responsive uterine genes identified in the RNAseq analysis.
 355 The results for *Esr1*, *Clca3*, *Igfbp2* and *Hsd11b1* are shown in Figure 5 in comparison to mRNA
 356 expression measured by real time RT-PCR. E2 treatment significantly repressed mRNA
 357 expression of *Esr1*, the gene encoding ER α itself, equally well in the presence of absence of SPI
 358 feeding ($P < 0.05$). SPI-feeding also appeared to suppress expression of this gene ($P < 0.05$). ChIP
 359 analysis indicated increased binding of ER α to an ERE in this gene after E2 treatment. However,

360 binding of ER α appeared reduced in the SPI relative to CAS group and in the E2 + SPI vs. the
361 E2 group. E2 treatment upregulated *Igfbp2* mRNA (P<0.05) accompanied by increased promoter
362 binding of ER α . SPI feeding had no effect by itself but appeared to increase binding of ER α to an
363 ERE in this gene when combined with E2 treatment. However, rather than having an additive
364 effect on *Igfbp2* mRNA, the combined treatment appeared to reduce E2-mediated expression.
365 E2 treatment upregulated *Clca3* mRNA (P<0.05) accompanied by increased promoter binding of
366 ER α . SPI feeding had no effect by itself but appeared to both increase binding of ER α to an ERE
367 in this gene when combined with E2 treatment and have an additive effect on *Clca3* mRNA
368 expression. Similarly, SPI had little effect on binding of ER α to an ERE in the *Hsd11b1* gene, but
369 appeared to increase binding of ER α after E2 treatment. However, in this case changes in mRNA
370 expression after either E2 or SPI treatment were not significant.

371 ***Effects of E2 treatment and SPI feeding on expression of estrogen responsive uterine proteins***
372 ***in OVX female rats.*** We also examined the effects of E2 and SPI feeding on expression of three
373 uterine proteins by Western immunoblot: ER α , indian hedgehog (Ihh) and MMP7, shown to be
374 highly E2-responsive at the mRNA level by RNAseq and confirmed by real time RT-PCR
375 (Figures 4 and 5). In agreement with mRNA expression, E2 treatment downregulated ER α
376 protein (P<0.05) and upregulated MMP7 (P<0.05). E2 treatment also suppressed mean
377 expression of Ihh protein but this did not reach statistical significance. However, despite modest
378 but significant decreases in mRNA expression, SPI feeding had no effect on ER α or Ihh at the
379 protein level and whereas MMP7 mRNA was increased in the SPI+E2 group relative to the E2
380 group (P<0.05), at the protein level, the combination of E2 + SPI actually had slightly lower
381 MMP7 protein expression (P<0.05) (Figure 6).

Effects of E2 and SPI on Uterine Responses to DMBA in OVX Female Rats. In additional groups of mice we examined the effects of E2 treatment and SPI feeding on uterine responses to the carcinogen DMBA. DMBA by itself had no effect on body weight or uterine wet weight in OVX female rats fed either casein or SPI (Table 1). In addition, identical decreases in body weight and increases in uterine wet weight were observed after E2 treatment of OVX female rats irrespective of DMBA treatment (Table 1). DMBA induced cytochrome P450 CYP1 family, CYP1A1 and CYP1B1 mRNA expression in the uterus ($P<0.0001$). There was no significant interaction between DMBA and diet on uterine CYP1 induction (Table 5). In contrast, E2 treatment significantly reduced DMBA induction of both CYP1A1 and CYP1B1 mRNA in the uterus in the presence of either casein or SPI-feeding ($P<0.05$). mRNA expression of Ki67 and of PCNA were measured by real time RT-PCR as measures of cellular proliferation. DMBA by itself had no effect on either mRNA. Ki67 mRNA was increased after E2 treatment and there was an additive effect when E2 treated mice were given DMBA ($P<0.05$). This effect was significantly reduced in SPI relative to CAS-fed groups ($P<0.05$). Similar effects were observed on expression of PCNA mRNA (Table 5).

DISCUSSION

Soy foods are part of the traditional Asian diet and have been suggested to possess many health beneficial properties including reduced risk of cardiovascular disease, osteoporosis and various cancers including endometrial cancer (Anthony et al., 1998; Wei et al. 2012; Clair and Anthony 2005; Badger et al., 2005; Myung et al. 2009; Ollberg et al. 2012). Isoflavones, in particular genistein and daidzein, which are naturally found in soy have been shown to have estrogen-like

properties and have been suggested to be the major bioactive components responsible for health beneficial effects (Messina, 2014; Saha et al. 2014). Given the health risks associated with HRT post-menopause, many women have taken to consumption of soy foods, isoflavone extracts or genistein as an alternative to estradiol treatment (Mahandy, 2003). However, recent animal studies using pure isoflavones and isoflavone extracts have demonstrated reproductive toxicity and estrogenic effects in reproductive tissues such as the mammary gland and uterus; including increased proliferation, dysplasia and promotion of chemically-induced mammary and endometrial carcinogenesis (Jeffersen et al., 2007; 2009; Aidoo et al. 2010; Kakehashi et al. 2012). Despite epidemiological data suggesting health benefits and lack of toxicity of soy foods, concerns persist regarding safety of soy products such as SPI, which is widely used as an additive in processed foods and is the sole protein in soy infant formula, when these products are consumed in infants and post-menopausal women where endogenous estrogens are low. However, far less is known regarding the potential estrogenicity and toxicity of soy foods which are complex mixtures containing potentially bioactive proteins, peptides and hundreds of phytochemicals in addition to isoflavones (Fang et al. 2004; Badger et al. 2009; Kang et al. 2010).

The goal of this study was to compare the estrogenic effects of feeding a diet containing SPI as the sole protein source with that of exogenous E2 supplementation in the OVX female rat where endogenous estrogen concentrations are minimized and similar to those seen prior to puberty or post-menopause. The control diet against which SPI was compared was AIN-93G with casein as the sole protein source. This is a standard diet used in nutritional research (Reeves et al., 1993). There has been debate with regards to what constitutes a control in diet studies. It has been suggested that casein may have biological effects of its own (Shah, 2000; Hakkak et al.

2000). It has also been suggested that a more appropriate control diet to compare the estrogenicity of phytochemical constituents in SPI against would be SPI-, where SPI has been stripped of all phytochemicals by successive ethanolic washes (Bahr et al., 2005; Ronis et al. 2009). However, such processing has been shown to alter the protein/peptide structure of SPI which might affect its biological properties. Moreover, the current study addresses questions regarding the potential estrogenicity and safety of a foodstuff, soy infant formula, as opposed to the estrogenicity of isoflavones or soy phytochemicals. In this regard casein is the most appropriate control since it is the major protein source in other infant formulas made with cow's milk. Total isoflavone concentrations after SPI feeding were in the range reported in soy formula fed infants (Setchell, et al. 1997). However, the major form was the most estrogenic of the isoflavones, the daidzein metabolite equol, which is formed by gut bacteria (Franke et al., 2014) with lower levels of genistein and daidzein. Equol is not found in infants prior to weaning and is only found in ~20% of adult soy consumers (Setchell, et al. 1997; Franke et al. 2014). We have previously published data from these animals showing that E2 treatment to restore physiological concentrations decreased body weight and produced uterine hyperplasia; whereas, SPI feeding had no significant effects on these estrogen sensitive physiological parameters (Singhal et al. 2009a). Similar results were obtained in rats treated additionally with DMBA. In order to determine if SPI acts like a weak estrogen in the uterus, we next measured gene expression profiles using RNAseq. As expected, E2 treatment resulted in a robust change in expression of nearly 2000 genes many of which regulate cellular proliferation and other pathways linked to cancer; pathways linked to organization of the extracellular matrix and to inflammation. We verified many of the changes in gene expression at the level of individual mRNAs using real time RT-PCR and several of the more highly regulated mRNAs – ER α (Esr1), MMP7 and Ihh at

the protein level using Western immunoblotting. We also confirmed that E2 treatment recruited ER α to EREs on flanking sequences of several of the E2 regulated genes using ChIP. In contrast, only 3% of E2-regulated mRNAs were up- or down-regulated >2-fold after SPI feeding. There was also no concordance between major pathways affected or the top 30 mRNAs up- or down-regulated by the two treatments. These data are in agreement with our previously reported findings on gene expression in the liver and mammary gland of these animals using Affymetrix arrays; with our microarray data from the mammary glands of prepubertal male and female rats fed SPI (Singhal et al. 2009a; Ronis et al. 2012; Miousse et al., 2013) and from the livers of neonatal piglets fed soy infant formula (Ronis et al. 2011). These data are also consistent with a lack of estrogenic effects of SPI in uterine histology in previous SPI feeding studies in adult ovariectomized Sprague-Dawley and intact adult female Fisher 344 rats (Bahr et al. 2005; Nakai et al 2005) and with lack of effects on the size of reproductive organs including the uterus of soy formula-fed infants measured by ultrasound compared to dairy formula-fed and breast-fed infants reported in the “Beginnings” longitudinal clinical study of breast and formula feeding (Gilchrist et al., 2010; Andres et al. 2015). Similar to findings in the mammary gland, many SPI-regulated genes were specific to SPI and unaffected by E2 treatment.

Approximately half of the SPI-regulated mRNAs were E2-regulated. These were predominantly downregulated genes. This pattern suggests that SPI has unique effects independent of E2 which may be related to non-isoflavone components of SPI including phytosterols, saponins and bioactive peptides (Kang et al. 2010; Burris et al., 2014). In addition, SPI also appears to act as a weak selective estrogen receptor modulator affecting a small proportion of E2-regulated genes rather than being a weak estrogen agonist. The lack of overlapping signaling between E2 and SPI is perhaps not surprising given that isoflavones such

as genistein have a much higher affinity for ER β than ER α whereas E2 has comparable affinity for both receptors. However, it should be noted that ER β is only expressed at low levels in the adult rat uterus (Nakajima et al., 2015). The combination of E2 treatment and SPI-feeding resulted in significant regulation of 679 fewer mRNAs than E2 alone and 20 fewer mRNAs than SPI alone. This suggests a degree of negative cross-talk between the two treatments. Interestingly, and in contrast to our previous ChIP results in the mammary gland (Ronis et al., 2012), there was no evidence of increased recruitment of ER α to the EREs examined in the SPI compared to the casein group, even to the ERE in the *Esr1* gene, the mRNA of which was significantly downregulated by both E2 and SPI-feeding in both organs. Our ChIP data suggested increased recruitment of ER α to EREs in 3 of the 4 genes examined by the combination of SPI feeding and E2 treatment. However, the effects of combined treatment on mRNA expression of these genes was inconsistent. A similar disparity between ER recruitment and mRNA expression was previously observed in mammary gland genes in response to the combination of E2 treatment and SPI feeding (Miousse et al. 2013). This may reflect differential recruitment of co-activators such as Sp1 or co-repressors such as Fos and Jun (Petz et al, 2000; 2004).

SPI-specific, ER-independent responses in the uterus appeared to be primarily associated with the complement system and with fatty acid metabolism. We have previously reported that SPI feeding had effects on fatty acid homeostasis in the mammary gland and liver (Singhal et al, 2009a; Ronis et al, 2009; 2011; 2012; Miousse et al, 2013). In particular, evidence of activation of PPAR-regulated pathways and elevation of PPAR mRNA expression was previously observed in weanling rats of both sexes fed SPI compared to casein for 14 days (Ronis et al, 2009).

Previous studies with pure isoflavones and isoflavone extracts from soy have suggested that although E2 and these phytoestrogens appear not to be genotoxic themselves, they can act as promoters of endometrial carcinogenesis as a result of increasing cellular proliferation and dysplasia in the uterus after co-exposure with the model pro-carcinogen DMBA (Aidoo et al, 2010; Kakehashi et al. 2012). We have previously reported that E2 can increase the carcinogenicity of DMBA in the liver as a result of increased induction of CYP1 enzymes mediated via recruitment of AhR and ER α to xenobiotic response elements (Singhal et al, 2008a). This effect could be blocked by feeding SPI as a result of AhR degradation and anti-estrogenic actions on ER recruitment (Singhal et al, 2007; 2008b; 2009b). In the current study, we examined the effects of E2 and feeding SPI alone or in combination together with DMBA on uterine CYP1 induction and cellular proliferation markers. Although we found CYP1A1 and CYP1B1 to be highly inducible by DMBA in the uterus, unlike liver, we observed that E2 treatment actually significantly reduced CYP1 induction in the uterus and that feeding SPI had no modulatory effect on CYP1 induction in this tissue. DMBA, E2 and feeding SPI by themselves had no effect on markers of cellular proliferation in the uterus. However, despite the reduced oxidative activation of DMBA by CYP1s, the combination of DMBA and E2 resulted in significant increases in proliferation markers. In contrast to studies with isoflavones, SPI feeding in this model suppressed cellular proliferation after combined E2/DMBA treatment. Although only a small number of animals was used in the current study, the suppressive effects of SPI on uterine proliferation markers in E2 and DMBA treated mice was robust and statistically significant. These data are consistent anti-estrogenic and anti-carcinogenic effects of SPI-feeding previously reported in the rat uterus by Tansey et al. (2005); in rat mammary glands (Ronis et al, 2012; Miousse et al, 2013), in rat models of DMBA-induced mammary carcinogenesis (Hakkak

et al, 2000) and with epidemiological studies in Asian consumers of traditional soy-rich diets (Badger et al. 2005; Myung et al, 2009; Messina 2014; Nagata et al. 2014; Wu et al. 2015).

In conclusion, feeding SPI as the sole protein source resulted in total serum isoflavone concentrations similar to those found in infants fed soy formula but with higher potential estrogenicity as result of high equol production. However, this did not result in either uterine hyperplasia or an estrogenic gene expression signature in the uterus of adult OVX female rats even in the absence of most endogenous estrogens. Very limited overlap was observed with E2-responsive genes and only a small sub-set appeared to be suppressed by both E2 and SPI treatment. This suggests SERM-like effects resulting in recruitment of co-repressors rather than co-activators to EREs on some uterine genes after SPI feeding. Reduced E2-associated gene expression and blockage of E2/DMBA-induced uterine cell proliferation, which is known to be ER α -dependent, suggests some anti-estrogenic effects of SPI feeding. These effects may reflect the weak estrogenic potency of the serum isoflavones found after feeding SPI and competition for ER α with endogenous estrogens. Most of the isoflavones are conjugated in the intestinal mucosa to glucuronides and sulfates which appear in serum rather than the bioactive aglycones (Gu et al, 2005; 2006; Ronis et al, 2006). However, further studies are required in other estrogen-responsive organs especially in males during early development to assess the estrogenicity and safety of SPI feeding to neonates. Our data do not support the suggestion that isoflavones consumed in the context of soy foods act as promoters of endometrial carcinogenesis. They suggest that the endocrine effects of purified isoflavones/isoflavone supplements and soy foods are quite different. These data also illustrate the difficulties associated with toxicological assessment of complex mixtures such as foods and suggest that a reductionist approach focused on single components results in misleading conclusions.

541

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803 **Figure Legends**

804 **Figure 1.** Overlap in uterine mRNA profiles between E2, SPI and E2 + SPI female OVX rat
805 groups. Number of significantly upregulated or downregulated genes that are specific or
806 common to each treatment.

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808 **Figure 2.** RNAseq heat maps of uterine genes changed by > 2-fold in OVX female rats by
809 treatment with E2, feeding SPI or E2+SPI treatment relative to rats fed casein. Genes were
810 separated in circular bands. Outer band: E2+ SPI, Middle band: SPI, Inner band: E2. Detailed
811 gene expression lists can be found online in Supplemental Data files 1 and 2.

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813 **Figure 3.** mRNA quantitation of gene expression clusters in E2, SPI and E2 + SPI female OVX
814 rat groups revealed by RNSseq using quantitative (q)RT-PCR. 1) E2 induced/SPI –independent:
815 lactoperoxidase; Olfactomedian 4; S100g. 2) SPI increased/E2 repressed: neuropeptide Y; fatty
816 acid binding protein; carbonic anhydrase 3 (n = 6/group). Relative gene expression normalized

to Srp14 and Rps13 averaged with geNorm. Different letters indicate a significant difference, $P < 0.05$, by 2-way ANOVA followed by pair-wise Tukey-Kramer post-hoc analysis, $a > b$.

Figure 4. mRNA quantitation of gene expression clusters revealed by RNSseq in E2, SPI and E2 + SPI female OVX rat groups using quantitative (q)RT-PCR. 1) E2 induced/induction enhanced by SPI: Matrix metalloproteinase 7; oxytocin. 2) E2 repressed/SPI repressed: Indian hedgehog; Gli 1; low density lipoprotein receptor related protein 2; Wnt inhibitory factor 1. 3) E2 induced/reversed by SPI: Aquaporin 3; S100A9, S100A8 ($n = 6$ /group). Relative gene expression normalized to Srp14 and Rps13 averaged with geNorm. Different letters indicate a significant difference, $P < 0.05$, by 2-way ANOVA followed by pair-wise Tukey-Kramer post-hoc analysis, $a > b$.

Figure 5. mRNA quantitation of E2 and SPI regulated uterine genes revealed by RNAseq ($ER\alpha$ /ESR-1); insulin-like growth factor binding protein 2 (Igfbp2); Clca1/3 and hydroxysteroid dehydrogenase 11b1 (Hsd11b1) using quantitative (q)RT-PCR ($n = 6$ /group). Relative gene expression normalized to Srp14 and Rps13 averaged with geNorm. Different letters indicate a significant difference, $P < 0.05$, by 2-way ANOVA followed by pair-wise Tukey-Kramer post-hoc analysis, $a > b$. Comparison with ChIP analysis of $ER\alpha$ binding to ERE elements in regulatory regions of these genes. Details of ChIP analysis found in the text.

Figure 6. Western immunoblot analysis of protein expression for three rat uterine genes regulated by E2 and SPI as measured by RNAseq and qRT-PCR. ER α ; indian hedgehog (Ihh) and matrix metalloprotease 7. Different letters indicate a significant difference, $P < 0.05$, by 2-way ANOVA followed by pair-wise Tukey-Kramer post-hoc analysis, $a > b$.

Table 1. Primer sequences used in the current study

Gene	Forward	Reverse
Aqp3	GGTGGCTTCCTCACCATCAA	GTTCAAGTGGGCTCCAGACACT
Car3	GCTGAGCTTCACCTGGTTCAC	AATGCCAACCACAGCAATCC
Clca1/3	CAAGCAAACCACTCCCATGAC	TCAAGAACGAGGCACACGATT
Esr1	TCTGACAATCGACGCCAGAA	ACAGCACAGTAGCGAGTCTCCTT
Fabp4	CCAAGCCCAACTTGATCATCAG	TGGTCGACTTTCCATCCCACT
Gli1	CCAGCCTCGACCTGCAAA	GTAGGAACCGCCTGGAGATGT
Hsd11b1	GGTGTCTCGCTGCCTTGAAC	TCCTGCCTCAACAACAAATCG
Igfbp2	CCTGCATATCCCCAACTGTGA	CTGGATTGGCTTCCCAGTATTG
Ihh	TCAGACCGCGACCGAAAT	GTGGGCCTTGGACTCGTAATAC
Lpo	GCCAGAAATGATCCACCTGCCAAA	ATCCAGGAAGGCCTTGTTGACTGA
Lrp2	ATTCTCGGACGAAGCTAACTG	AGACGTAGGCGCGAAGGATA
Mmp7	GGCGGAGATGCTCACTTTGA	GTGGCAACAAACAGGAAGTTCA
Npy	CCATGTGGTGATGGGAAATG	ATTGGTGGGACAGGCAGACT
Olfm4	CTCCCTCAGGCTGCCTACAA	CCACAGCAAGGTCAATGTCTTG
Oxt	AGTCTTGCTTGCTGCCTGCTT	AGGGAAGACACTTGCGCATATCCA
Rpn1	GTCATCATCGTCCGTCTAGACTT	GTGATACAGGCCACTTTCATCCT
S100a8	GCCTTGAGCAACGTCATTGA	CATTTTCCTGAAGTCATCCCTGTA
S100a9	GGACACAAACCAGGACAATCAA	TGGGTTGTTCTCATGCAGCTT
S100g	CACTGACAGCAAGCAGCACAGAAA	CAGCTGGTTTGGATCGCCTTCTTT
Wif1	TCCTTCGGACCCCTCAAAAT	ACCCGCCTTTCGTTACAAAA
ClCa3-ChIP	TGGAGTGGGTGGGTCAAGTGT	ACGCTGATCCAGGAAAAGCA
Igfbp2-ChIP	GCTACGAGGTGCCCTTTGG	TCACACACAGCCCTCGTGTT
Hsd11b1-ChIP	CTCCCTGACAAGCCATTCTTG	GCTGGGTTCTGAGCATCTC
Esr1-ChIP	TGGGCAACTTCTGTTACCAAG	TCTCACCAGCTGAGCTAAGTAT

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Table 2. Physiological Values

	Bwt	Utw	%Utw	Serum E2
Cas	291 ± 20^{a,A}	0.21 ± 0.07^{b,B}	0.07 ± 0.022^{b,B}	3.82 ± 0.94^c
Cas E2	235 ± 23^b	0.62 ± 0.06^a	0.27 ± 0.040^a	18.27 ± 2.39^b
SPI	287 ± 17^a	0.22 ± 0.03^b	0.08 ± 0.010^b	2.89 ± 2.39^c
SPI E2	231 ± 11^b	0.60 ± 0.30^a	0.26 ± 0.135^a	41.82 ± 7.20^a
Cas DMBA	274 ± 15^A	0.19 ± 0.06^B	0.07 ± 0.022^B	
Cas E2 DMBA	221 ± 22^B	0.58 ± 0.15^A	0.27 ± 0.077^A	
SPI DMBA	283 ± 21^A	0.23 ± 0.02^B	0.08 ± 0.014^B	
SPI E2 DMBA	231 ± 24^B	0.52 ± 0.11^A	0.23 ± 0.065^A	

Data represent mean ± SEM. Means with differing letters are significantly different P<0.05, a > b > c; Means with differing capital letters are significantly different P<0.05, A > B.

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Table 3. Top 30 E2 Up Regulated Genes

Gene	Cas (FPKM)	E2 (FPKM)	Fold Change E2	SPI (FPKM)	Fold Change SPI	SPI+E2 (FPKM)	Fold Change SPI+E2
Tmc5	0	7	∞	0	0	6	∞
Gp2	0	14	∞	1	∞	16	∞
Mir21	0	379	∞	0	∞	0	0
Tmprss11g	0	23	∞	1	∞	26	∞
Amtn	0	12	∞	1	∞	14	∞
Csn3	0	16	∞	1	∞	15	∞
Reg3b	0	50	∞	1	∞	27	∞
Clec2d	0	10	∞	2	∞	11	∞
Reg3g	0	131	∞	1	∞	109	∞
Galr3	0	9	∞	1	∞	12	∞
Mmp20	0	7	∞	0	0	4	∞
Rbp2	0	34	∞	0	0	69	∞
Lipf	2	575	362	4	2*	727	458
Clca1	1	94	339	1	-1	94	340
Lpo	4	1152	297	7	2	1248	322
Mmp7	44	11095	251	68	2	12056	273
Olfm4	2	422	242	3	2	400	230
Scgb1a1	8	1950	238	8.26	1	2946	360
Kap	31	6470	209	100	3	4842	156
Cuzd1	1	2151	207	13.06	1	2184	210
Noxo1	1	55	150	1	2*	45	124
Lcn2	14	1690	123	21	2	799	58
S100g	31	3807	123	49	2	1657	54
MGC105649,Mir147	2	171	95	3	2	311	172
Padi1	1	24	87	1	1	34	122
Ccl11	5	452	86	11	2*	246	47
Oxt	7	551	76	10	1	673	93
Fam3b	4	295	67	6	1	289	65.16
Gjb2	3	147	56	4	2	195	74
Plet1	50	2740	55	106	2	2488	50

Bold-Fold change ≥ 2 in SPI

*Did not reach significance in SPI vs CAS

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Table 4. Top 30 SPI UP Regulated Genes

Gene	CAS (FPKM)	SPI (FPKM)	Fold Change		Fold Change E2	SPI+E2 (FPKM)	Fold Change SPI+E2
			SPI	E2 (FPKM)			
Pla2g2a	9	77	9	10	1	10	1
C6	4	26	6	5	1	4	1
Fabp4	151	815	5	261	2	217	1
Grifin	28	142	5	16	-2	18	-2
Fcn1	7	34	5	14	2	8	1
Car3	174	793	5	241	1	139	-1
Fgf1	2	11	5	4	2	2	1
Adipoq	85	376	4	690	8	1211	14
Ifit1	3	15	4	7	2*	10	3
Scd1	262	1115	4	65	-4	109	-2
Aqp7	5	21	4	7	1	4	-1
Oxtr	11	46	4	84	8	64	6
Ces1d,LOC291863	10	43	4	27	3	9	-1
Plin	14	53	4	17	1	9	-2
S100b	28	107	4	34	1	29	1
Cdo1	30	115	4	20	-2	17	-2
Cidec	20	76	4	20	1	17	-1
Pparg	3	11	4	5	2	4	1
Me1	27	100	4	20	-1	19	1
Mpo	5	17	4	2	-3*	2	-2
Retn	24	88	4	7	-3	15	-2
Angptl4	4	13	4	6	2	5	1
Mosc1	4	15	4	2	-2*	2	-2
Tmem179	3	10	4	3	1	3	1
Nat8l	2	7	3	1	-2	1	-2
Rbp4	120	402	3	51	-2	53	-2
Nnat	28	94	3	51	2	41	1
Agt	7	23	3	15	2	10	1
Acsl1	48	155	3	53	1	45	1
Ifi204	3	9	3	8	3	5	2

Bold-Fold change ≥ 2 in E2 VS CAS

*Did not reach significance in E2 vs CAS

Highlight-Opposite regulation between SPI and E2

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Table 5. Effects of diet, E2 and DMBA on Expression of CYP1, Ki67 and PCNA mRNAs in the Uterus of OVX Female Rats

	Means (SE)							
	CAS	CAS+E2	CAS+E2+DMBA	CAS+ DMBA	SPI	SPI +E2	SPI +E2+DMBA	SPI + DMBA
CYP1A1	0.01 (0.00)	0.01 (0.000)	0.75 (0.17)	0.94 (0.16)	0.1 (0.01)	0.02 (0.01)	0.73 (0.13)	1.4 (0.15)
CYP1B1	0.29 (0.05)	0.22 (0.08)	3.17 (0.66)	4.32 (0.70)	0.33 (0.05)	0.13 (0.06)	3.00 (0.60)	8.15 (2.08)
KI67	0.99 (0.31)	1.65 (0.07)	4.17 (0.54)	0.97 (0.27)	0.58 (0.15)	0.98 (0.18)	1.60 (0.47)	0.61 (0.07)
PCNA	1.60 (0.26)	1.57 (0.16)	2.37 (0.09)	1.64 (0.19)	1.50 (0.12)	1.53 (0.10)	1.65 (0.38)	1.40 (0.09)

	ANOVA effect <i>p</i> -values						
	Main effects			Interactions			
	SPI	E2	DMBA	SPI*E2	SPI*DMBA	E2* DMBA	SPI*E2* DMBA
CYP1A1	0.18	0.02	<0.0001	0.17	0.22	0.02	0.15
CYP1B1	0.15	0.01	<0.0001	0.10	0.14	0.02	0.12
KI67	0.0001	<0.0001	0.001	0.01	0.042	0.001	0.03
PCNA	0.04	0.06	0.09	0.39	0.11	0.06	0.29

FIGURE 2

Figure 3

Figure 4

Figure 5

Figure 6

Uterine Total Lysate