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Feeding soy protein isolate and treatment with estradiol have different effects on mammary gland morphology and gene expression in weanling male and female rats

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Miousse IR, Sharma N, Blackburn M, Vantrease J, Gomez-Acevedo H, Hennings L, Shankar K, Cleves MA, Badger TM, Ronis MJJ. Feeding soy protein isolate and treatment with estradiol have different effects on mammary gland morphology and gene expression in weanling male and female rats. *Physiol Genomics* 45: 1072–1083, 2013. First published September 17, 2013; doi:10.1152/physiolgenomics.00096.2013.—Isoflavones are phytochemical components of soy diets that bind weakly to estrogen receptors (ERs). To study potential estrogen-like actions of soy in the mammary gland during early development, we fed weanling male and female Sprague-Dawley rats a semipurified diet with casein as the sole protein source from postnatal day 21 to 33, the same diet substituting soy protein isolate (SPI) for casein, or the casein diet supplemented with estradiol (E2) at 10 μg/kg/day. In contrast to E2, the SPI diet induced no significant change in mammary morphology. In males, there were 34 genes for which expression was changed ≥2-fold in the SPI group vs. 509 changed significantly by E2, and 8 vs. 174 genes in females. Nearly half of SPI-responsive genes in males were also E2 responsive, including adipogenic genes. Serum insulin was found to be decreased by the SPI diet in males. SPI and E2 both downregulated the expression of ERα (*Esr1*) in males and females, and ERβ (*Esr2*) only in males. Chromatin immunoprecipitation revealed an increased binding of ERα to the promoter of the progesterone receptor (*Pgr*) and *Esr1* in both SPI- and E2-treated males compared with the casein group but differential recruitment of ERβ. ER promoter binding did not correlate with differences in *Pgr* mRNA expression. This suggests that SPI fails to recruit appropriate co-activators at E2-inducible genes. Our results indicate that SPI behaves like a selective estrogen receptor modulator rather than a weak estrogen in the developing mammary gland.

soy; xenoestrogen; mammary gland; 17β-estradiol; microarray; chromatin immunoprecipitation

SOY IS PART OF THE TRADITIONAL diet in Asia and has been introduced in the West relatively recently, where it is gaining in popularity. The Soyfoods Association of North America reports an increase from $1 billion to $4.9 billion in sales of soy foods from 1996 to 2010 (54). One of the factors contributing to this trend is the many health benefits attributed to soy. Soy protein consumption has been demonstrated by several meta-analyses to lower serum total cholesterol and LDL cholesterol (1, 58, 64). In addition, epidemiological studies have repeatedly associated soy consumption in adult women with a reduction in the risk and recurrence of breast cancer in Asian populations (11, 23, 63). Early soy consumption has been proposed to be more protective with regard to breast cancer risk than exposure in adulthood. Moreover, soy consumption appears to persistently reduce mammary cancer risk, even if soy diets were restricted to childhood and adolescence (23, 27).

Despite these apparent health benefits, the National Toxicology Program recently raised its level of concern about the potential reproductive toxicity of soy infant formula from negligible to minimal (31). This re-evaluation of soy infant formula, which has been in use in the US for over 50 yr and which is consumed by nearly 1 million infants/year, was motivated by a series of in vitro and in vivo results evaluating the estrogenic properties of the soy isoflavone genistein (31). Although epidemiological studies in human infants consuming soy formula revealed normal growth and development (13, 55), there is a concern that infants consuming soy formula are exposed to dangerously high levels of genistein and are at increased risk for reproductive tract malformations and for estrogen-sensitive cancers such as breast and uterine cancer, similarly to children of women exposed to diethylstilbestrol in utero (49). Genistein and other soy-associated isoflavone phytochemicals, such as daidzein, and the daidzein metabolite equol have a structural similarity with estrogens and are able to bind weakly to the estrogen receptors alpha and beta (ERα and ERβ), with a preference for ERβ (24, 30). Genistein has been shown to have estrogen-like properties: stimulating growth in the MCF7 breast cancer cell line, inducing an increase in uterus weight, and inducing reproductive toxicity in rodents (18, 20, 31). However, genistein is only one of the hundreds of phytochemicals associated with soy. In industrially processed soy products such as soy protein isolate (SPI, the sole protein source in soy infant formula), isoflavones constitute only 7% of phytochemicals, which also include oxygenated fatty acids, soyasaponins and lysophospholipids (12).

It has been proposed that the potential effects of isoflavones on estrogen receptor signaling pathways the mammary are dependent on developmental window of exposure. Several animal studies have shown that genistein given subcutaneously before puberty reduced the number and delayed the appearance of mammary tumors induced by DMBA (6, 17, 25, 40). Moreover, in contrast to adult exposure, prepubertal exposure to purified genistein and neonatal exposure to equol has also been associated with a reduction in the number of terminal end

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buds (TEBs, a marker of increased mammary gland differentiation) (5, 6, 16, 32, 37). Therefore, consumption of isoflavones during early development when endogenous estrogens are low may actually increase differentiation in mammary tissues, which in turn may be responsible for the decrease in breast cancer risk associated with early soy consumption (47).

Some animal studies have tested SPI rather than genistein. These studies provide a closer parallel with consumption of soy infant formula. Dietary exposure to SPI in ovariectomized adult female rats was associated with no morphological change other than a decrease in epithelial/fat pad area and an overlap of only 1% in gene expression change between estradiol (E2) and SPI, suggesting that SPI has minimal estrogenic actions in the adult female mammary gland even in the absence of endogenous estrogens (45). Exposure to SPI continuously through gestation and early development, indirectly via the dam and directly following weaning, has been shown to decrease the number of TEBs, increase the number of lobules, and reduce mammary adipose cell size in female rat mammary. It has been suggested that these effects are the result of activation of mammary estrogen signaling pathways by SPI isoflavones during early development (46, 56). However, many other hormones and molecular pathways other than estrogens are also involved in mammary development (10, 22, 25), and the effects of SPI and estrogen treatment on early mammary development have never been directly compared.

Unfortunately, direct nutritional intervention in rodents during the neonatal period to mimic soy formula feeding in infants is technically challenging. The current study compares mammary morphology and the mammary gene expression profiles of weanling male and female rats after feeding SPI or after E2 treatment for 14 days. This period of exposure corresponds to prepubertal soy consumption in Asian children who are generally breast fed. Since isoflavones do not appear at appreciable levels in breast milk, phytoestrogen exposure in this population would begin with consumption of soy foods postweaning. We have previously identified highly sexually dimorphic effects of estradiol on mammary morphology and gene expression during this developmental window and demonstrated that the weanling male mammary gland was especially responsive to E2, with increasing TEB number and proliferative gene expression even at low levels, making it an ideal tissue to study the potential estrogenic actions of SPI during early development (35).

METHODS

Animal care and experiment design. Time-impregnated Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were fed AIN-93G diets at a level of 20% protein, with casein as the sole protein source. Corn oil replaced soybean oil (43, 44, 45). After birth, dams continued to be fed AIN-93G throughout lactation. Pups were weaned on postnatal day (PND)21, at which time the animals were separated by sex, implanted subcutaneously with Alzet 2002 mini-osmotic pumps (Alza, Mountain View, CA) and were assigned to one of three treatment groups ($n = 10$ for each group in males, $n = 7$ for females): One group was assigned to the same AIN-93G casein diet as the dams and infused sc with polyethylene glycol (PEG) vehicle (CAS), a second group was fed a diet identical to AIN-93G except that casein was replaced by SPI as the sole protein source and infused sc with PEG vehicle (SPI). SPI contained 3.21 g/kg total isoflavones, including 1.87 g/kg genistein-containing compounds and 1.22 g/kg daidzein-containing compounds, corresponding to 1.08 and 0.69 g/kg aglycone equivalents of genistein and daidzein, respectively, per kg (56). The third group of rats was fed the AIN-93G casein diet but were infused sc with an E2 dose of 10 μg/kg/day dissolved in PEG vehicle (Sigma-Aldrich, St. Louis, MO). Vaginal opening was monitored from PND26 until death. On PND34, animals were killed by anesthesia with Nembutal (100 mg pentobarbital/kg ip) followed by decapitation. This corresponds to the average time of vaginal opening in control Sprague-Dawley females. Blood was collected and frozen at −20°C until analysis. One abdominal mammary gland from each animal was frozen at −80°C, another was used for preparation of whole mounts (46) and determination of mammary gland morphology by light microscopy by a trained veterinary pathologist (L. Hennings) as described previously (35, 45). In brief, the mammary tissues were fixed flat and defatted in acetone for a minimum of 48 h. Tissues were then rehydrated through a series of alcohols, stained overnight with carmine alum, then dehydrated through a series of alcohols, and cleared in xylene. Image J was utilized to identify and measure TEBs and epithelial and fat pad areas under a dissecting microscope with identical settings and a ruler bar for every image. All animal protocols were approved by the Institutional Care and Use Committee at University of Arkansas for Medical Sciences.

Hormone serum concentrations. Hormone serum concentrations were measured according to manufacturer's instructions: 17β-E2, testosterone (T), and progesterone were measured with radioimmunoassays (DLS-4800, DSL-4100, and DSL-3400; Beckman Coulter, Indianapolis, IN). Insulin was measured with the Rat/Mouse Insulin ELISA kit (EZRMI-13K; Millipore, Billerica, MA).

Mammary microarray set. Microarray analysis of gene expression was performed as previously described (35). In brief, 8 μg purified mRNA was extracted from frozen mammary tissue using TRI reagent (Molecular Research Center, Cincinnati, OH) and cleaned up with the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was processed according to manufacturer's instruction (one-cycle cDNA synthesis kit and GeneChip IVT labeling kit; Affymetrix, Santa Clara, CA). Resulting cRNAs from rat mammary glands ($n = 3$ different pools from each treatment group, each containing mRNA extracted from 2 or 3 individual rats) were hybridized to an Affymetrix GeneChip Rat Genome 230 2.0 following the manufacturer's protocol (Affymetrix). The probe array was washed and stained using the Affymetrix kit in a GeneChip fluidics station 450 and scanned using GeneChip Scanner 3000. Data on probe-level intensities were extracted using GeneChip operating software obtained from Affymetrix, and .CEL data files were generated. Files were deposited in the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (http:/www.ncbi.nlm.nih.gov/geo/) under GEO accession no GSE40713. The files containing the probe-level intensities were processed using the robust multiarray analysis algorithm (GeneSpring 11.5.1; Agilent Technologies, Wilmington, MA) for background correction, normalization, and log transformation (19). A list of differentially expressed genes was generated by performing an unpaired *t*-test. This list was used to make comparisons between various treatments and the cutoff was set at variation greater than twofold and $P < 0.05$. Results were based on unadjusted P values. Males and females were analyzed separately.

Microarray validation by real-time quantitative PCR. Total RNA (1 μg) was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. cDNA samples were amplified using previously described conditions (52). Quantitative real-time PCR (qRT-PCR) was performed on genes that were of particular interest to this study (see Table 1 for primer list). Expression levels of genes were normalized to the gene levels of control genes *Rps13* and *Spr14*, averaged with geNorm (60).

Western blotting. Tissue lysates were prepared from 100 mg of individual frozen mammary tissues homogenized in RIPA buffer. We applied 20 μg of each sample to 10% SDS-PAGE gels. Western blots were also performed with pooled samples from $n = 3$–5/group for illustrative purposes. Proteins were transferred onto a PVDF mem-

Table 1. *Primers used in this study*

Genes	Forward	Reverse
	Quantitative PCR	
Rps13	5′-CCCCGAGGATCTCTACCATTT-3′	5′-TCAGAATCAGGCGGAATTTAGC-3′
Srp14	5′-GAACAAGTTTCAGATGGCCTATTCA-3′	5′-GTGCTGGTTTGCTCTTCTTACTCTT-3′
Areg	5′-AGCCTAGCTGATGGCAATTCAGGA-3′	5′-ACTTCTGGAGCCTTCGCTGAAAGA-3′
Pgr	5′-TGGTTCCGCCACTCATCA-3′	5′-TGGTCAGCAAAGAGCTGGAAG-3′
Csn2	5′-TATGCCCAGACCATCTCTTGCAGT-3′	5′-AGGAAGGGCATCTGTTTGTGCTTG-3′
Esr1	5′-GACAATCGACGCCAGAA-3′	5′-ACAGCACAGTAGCGAGTCTCCTT-3′
Esr2	5′-AGAGTCCCTGGTGTGAAGCAAG-3′	5′-GACAGCGCAGAAGTGAGCATC-3′
Pcna	5′-AGC AAC TTG GAA TCC CAG AAC AGG-3′	5′-TAA GGT CCC GGC ATA TAC GTG CAA-3′
Id2	5′-ATGGAAATCCTGCAGCACGTCATC-3′	5′-ACGTTTGGTTCTGTCCAGGTCTCT-3′
FolR1	5′-TACAAGCTCAGCAACTACAGCCGA-3′	5′-ATAGAACCTCGCCACTTCCTCGTT-3′
Dusp1	5′-CTGGTAGTGACCCTCAAAGTGGTT-3′	5′-CTTACCATGCTTCCCGGAAAG-3′
Dguok	5′-GGGCAGAGAAGAAGAGAAAGG-3′	5′-TGGAGCTTGGTAGTCTTGTTAAT-3′
Mup5	5′-AACCAAGACTGATCGCTGTCTCCA-3′	5′-ATCGCAGAATTTGGCTCAGGATGC-3′
	Chromatin immunoprecipitation	
Pgr	5′-ATGACTGAGCTGCAGGCAAAGGAT-3′	5′-TATTGGCGAGACTACAGACGATGC-3′
Esr1	5′-TGGGCAACTTCTGTTACCAAG-3′	5′-TCTCACCAGCTGAGCTAAGTAT-3′
Aldh3a2	5′-GGGTACGCACTTTGTCGTTAGC-3′	5′-GGCAGGTGAAGCAGCACAATAC-3′

brane. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and detected by chemiluminescence (SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific, Rockford, IL) for immunoquantitation.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as previously described (45). Briefly, chromatin was extracted with the ChIP-It Enzymatic Express kit (Active-Motif, Carlsbad, CA) from pools of 300 mg of minced male mammary tissues following the manufacturer's protocol. The tissue was fixed in 20 ml fixing solution (20 ml PBS, 0.54 ml 37% formaldehyde) for 10 min and stopped with addition of Glycine Stop solution. Tissues were pelleted and resuspended in 2 ml ChIP lysis buffer (Santa Cruz Biotechnology) and homogenized using 20 strokes of a Dounce homogenizer. Homogenate was passed through a 100 μm cell strainer, and nuclei were pelleted and then resuspended in 1 ml digestion buffer. Shearing was achieved by sonicating with 2 pulses of 10 s each at setting of 6 on a Fisher 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) with 15 s of icing between pulses, followed by 5 min of icing and then the addition of 50 μl Shearing enzyme cocktail (0.5 μl enzyme, 49.5 μl 50% glycerol) and incubation at 37°C for 10 min. The reaction was stopped with addition of 20 μl of ice-cold 0.5 M EDTA and icing for 10 min followed by centrifugation. Immunoprecipitation was performed using 2 μg of chromatin and 2 μg of antibody in a 200 μl reaction at 4°C overnight. Antibodies against ERα and ERβ (Santa Cruz Biotechnology, Dallas, TX) were used with normal rabbit IgG as a negative control. Beads were washed twice with ChIP *buffer 1* and twice with ChIP *buffer 2*. DNA was eluted in 50 μl AM2 and reverse cross-linked by addition of 50 μl reverse cross-linking buffer and heating at 95°C followed by treatment with proteinase K then proteinase K stop solution. As previously published (45), ER binding to estrogen response elements (ERE) was analyzed in estrogen-responsive elements in the first exon of the progesterone receptor gene *Pgr* and the fourth intron of the ERα gene *Esr1*. The 5′-untranslated region of *Aldh3a2* was utilized as a negative control. Semiquantitative PCR results were visualized on 1.5% agarose gels.

Table 2. *Physiological values*

	CAS	CAS+ E2	SPI	*P* Value
		Males		
Serum estradiol	5.52 (0.63)[a]	12.75 (2.19)[b]	6.22 (0.22)[a]	0.0025
Progesterone	1.42 (0.29)	2.08 (0.32)	1.28 (0.25)	0.1929
Testosterone	0.32 (0.13)[a]	0.03 (0.01)[b]	0.23 (0.09)[a]	0.0117
Insulin	1.49 (0.32)[a]	1.32 (0.24)[a]	0.33 (0.13)[b]	0.0069
Body weight	152.00 (5.35)	139.60 (4.61)	135.00 (4.06)	0.0955
Terminal end buds, *n*	24.40 (8.47)[a]	53.00 (3.16)[b]	11.70 (2.20)[a]	0.0035
Epithelial area	12.76 (1.59)[a]	19.78 (1.13)[b]	15.70 (1.50)[a,b]	0.0098
Fat pad area	21.61 (1.77)	25.34 (1.08)	22.88 (1.54)	0.221
Epithelial-to-fat area ratio	0.59 (0.06)[a]	0.78 (0.03)[b]	0.68 (0.03)[a,b]	0.0369
		Females		
Serum estradiol	6.89 (0.90)[a]	19.18 (1.56)[b]	12.69 (3.21)[a,b]	0.0115
Progesterone	48.33 (3.58)[a]	2.05 (0.36)[b]	3.91 (0.64)[b]	0.0029
Insulin	1.46 (0.28)	1.61 (0.34)	0.96 (0.11)	0.1704
Body weight	136.86 (7.09)[a]	117.43 (3.99)[b]	115.14 (4.14)[b]	0.0256
Terminal end buds, *n*	54.33 (9.88)	39.67 (3.92)	48.17 (9.68)	0.2891
Epithelial area	17.49 (1.64)	18.67 (1.49)	14.74 (1.63)	0.2286
Fat Pad area	24.93 (2.28)	23.53 (1.80)	21.56 (1.94)	0.5127
Epithelial-to-fat area ratio	0.70 (0.04)[a,b]	0.79 (0.02)[b]	0.68 (0.02)[a]	0.0174

Data are presented by a nonparametric (Kruskal-Wallis) analysis. Different superscript letters indicate that the groups are significantly different ($P \leq 0.05$). Diet groups: CAS, casein; CAS + E2 casein + estradiol; SPI, soy protein isolate.

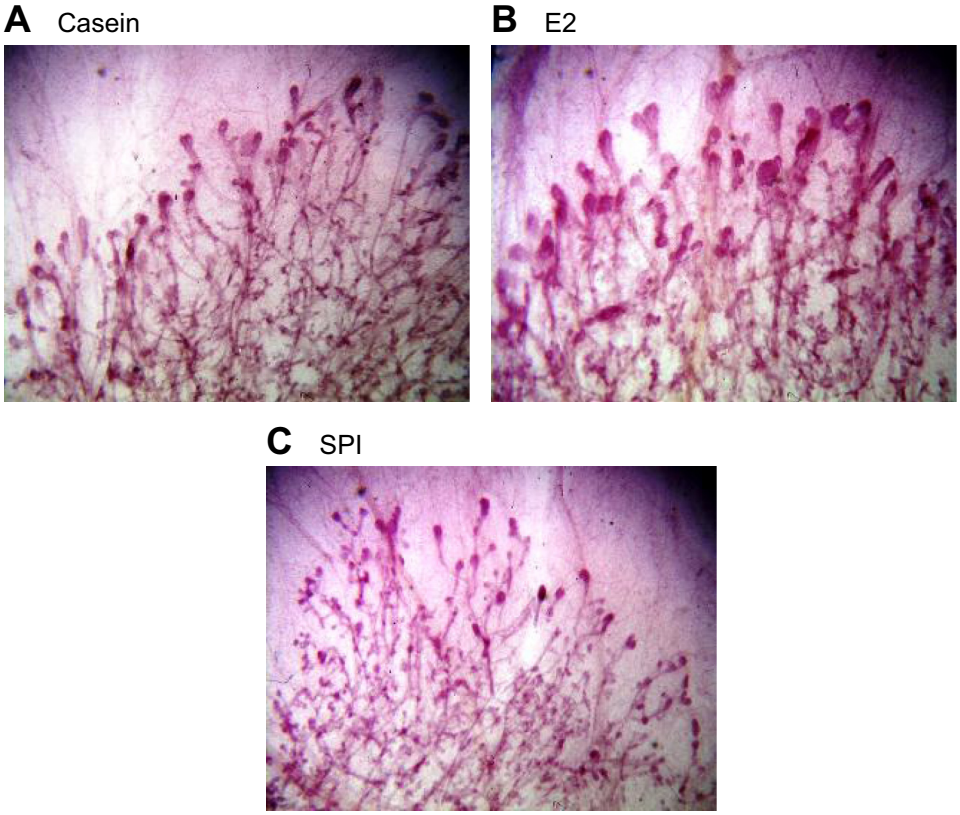

Fig. 1. Mammary whole mounts (×3 magnification) from male weanling rats fed casein (*A*), casein + estradiol treatment (E2) (*B*), or soy protein isolate (SPI) (*C*). Representative mammary gland images from male rats on postnatal day (PND)33.

Statistical analysis. Experimental outcomes are summarized as means ± SE. Data analysis was stratified by sex. Parametric one-way analysis of variance (ANOVA) was used to compare means across experimental groups; alternatively nonparametric, Kruskal-Wallis tests were used when the data were not normally distributed. Post hoc analysis of significant ANOVAs ($P \leq 0.05$) were performed with a post hoc Tukey's or Dunn's range test. Time to vaginal opening was compared across groups by the log-rank test. Accelerated failure time log-normal and Weibull models were used to compute average time-shift in vaginal opening between groups. Statistical analyses were performed using the Stata statistical package version 12.0 (Stata, College Station, TX).

RESULTS

Serum isoflavones, endocrinology, and mammary morphology. In females, both E2 and SPI groups had decreased body weight compared with the CAS group ($P < 0.05$). In males, body weight was lower in the SPI group compared with the CAS group ($P < 0.05$) (Table 2). Serum concentration of the different isoflavones and isoflavone metabolites from this experiment have been reported previously (65). Genistein, daidzein, dihydrodaidzein, glycetein, 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 (2,207 nM), followed by genistein (922 nM) and daidzein (522 nM), indicating a high level of conversion of daidzein into the more potent xenoestrogen equol.

E2 treatment resulted in vaginal opening in all female rats from the second day this was assessed (PND27), whereas complete vaginal opening occurred in the CAS control group at PND33 ($P < 0.05$ vs. CAS controls). Although vaginal opening occur on average 0.8 days earlier in the SPI group than in the CAS group, this time acceleration was not statically significant (log-rank $P = 0.2673$).

All endocrinological and morphological measurements are reported in Table 2, and representative images of male mammary whole mounts are shown in Fig. 1. Serum E2 was increased by E2 treatment in males and in females ($P < 0.05$),

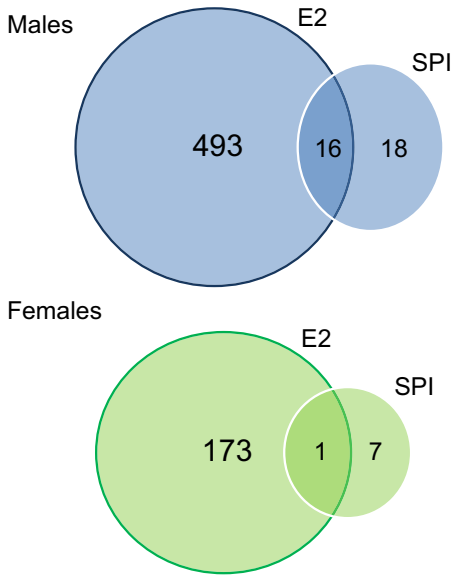

Fig. 2. Overlap between E2 and SPI. Number of significantly upregulated or downregulated genes that are specific or common to the E2 treatment and the SPI diet in males and females.

while SPI feeding did not produce significant differences relative to the CAS groups. In males, serum T values were not normally distributed. T was decreased by E2 treatment relative to the other two groups ($P < 0.05$). There was no difference in T in males from the SPI group compared with the CAS group. There was a marked decrease in serum progesterone in both E2 and SPI groups compared with the CAS group but only in females ($P < 0.001$). Serum insulin was significantly decreased in the SPI group relative to the other two groups but only in males ($P < 0.05$). There was no significant difference in the mammary epithelial area, fat pad area, or epithelial to fat pad area between SPI and CAS groups in either males or females. As reported previously, E2 caused a significant increase in TEB number in males ($P < 0.005$) (35). However, SPI feeding had no significant effect on male mammary TEB number.

Mammary gland gene expression. With a threshold of twofold and an unadjusted $P < 0.05$, the expression of a total of 34 genes was changed in males and 8 in females in the SPI groups compared with their respective CAS controls (Fig. 2, Table 3). In both sexes, no gene was changed by more than fourfold in SPI groups compared with the CAS groups. Since these effects were only statistically significant with an unadjusted analysis, the overall effects of SPI on mammary transcription profile were relatively weak. Three-quarters of SPI-regulated genes were downregulated. We confirmed the significant downregulation of dual-specificity protein phosphatase 1 (*Dusp1*), a MAP kinase phosphatase (57), by SPI with qRT-PCR (Fig. 3). We have previously identified that the upregulated genes amphiregulin (*Areg*) and the progesterone receptor (*Pgr*) were very sensitive indicators of exogenous estrogenic action in the developing male mammary gland (35). Both microarray and qRT-PCR revealed that these genes were unchanged by SPI feeding (Fig. 3). Similarly, we have previously demonstrated the milk protein gene *Csn2* was dose-dependently upregulated by E2 in the developing mammary gland (35). In the current study, *Csn2* was upregulated in the E2 group of both sexes by qRT-PCR analysis but was not upregulated in the SPI groups

Table 3. *Gene expression changes in the SPI diet*

Gene Symbol	Name	Fold Change
	Males	
Nek1	NIMA-related kinase 1	2.30
Sfrs3	splicing factor, arginine/serine-rich 3	2.30
Zbtb41	zinc finger and BTB domain containing 41	2.26
Mtmr1	myotubularin-related protein 1	2.18
Bhlhe40	basic helix-loop-helix family, member e40	2.17
Kpna1	karyopherin alpha 1	2.10
Dhx36	DEAH (Asp-Glu-Ala-His) box polypeptide 36	2.05
Gdf1/Lass1	growth differentiation factor 1/LAG1 homolog, ceramide synthase 1	2.03
Chst12	carbohydrate (chondroitin 4) sulfotransferase 12	−2.01
Cadm3	cell adhesion molecule 3	−2.02
Nov	nephroblastoma overexpressed	−2.02
Fgfr1	fibroblast growth factor receptor 1	−2.03
Gaa	glucosidase, alpha; acid	−2.04
Slc31a1	solute carrier family 31 (copper transporters), member 1	−2.05
Ms4a2	membrane-spanning 4-domains, subfamily A, member 2	−2.05
Thy1	thymus cell antigen 1, theta	−2.07
Tpm3	tropomyosin 3	−2.07
App	amyloid beta (A4) precursor protein	−2.10
Sdccag3	serologically defined colon cancer antigen 3	−2.11
Slc31a2	solute carrier family 31 (copper transporters), member 2	−2.12
Aebp1	adipocyte enhancer binding protein 1	−2.15
Mesdc2	mesoderm development candidate 2	−2.15
Ckap4	cytoskeleton-associated protein 4	−2.18
Akirin2	akirin 2	−2.20
Hspb8	heat shock protein B8	−2.20
Shox2	short stature homeobox 2	−2.24
Mbnl2	muscleblind-like 2	−2.30
Abcc9	ATP-binding cassette, subfamily C (CFTR/MRP), member 9	−2.34
Fstl1	follistatin-like 1	−2.35
Armcx3	armadillo repeat containing, X-linked 3	−2.45
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	−2.64
Mbp	myelin basic protein	−3.31
Dusp1	dual-specificity phosphatase 1	−3.39
Mpz	myelin protein zero	−3.51
	Females	
Dguok	deoxyguanosine kinase	3.81
Il1rl1	interleukin 1 receptor-like 1	2.26
C6	complement component 6	2.26
Mup5	major urinary protein 5	2.14
Tmem35	transmembrane protein 35	2.10
Nipbl	nipped-B homolog	−2.09
Rcn1	reticulocalbin 1, EF-hand calcium binding domain	−2.38
Vps52	vacuolar protein sorting 52 homolog	−2.84

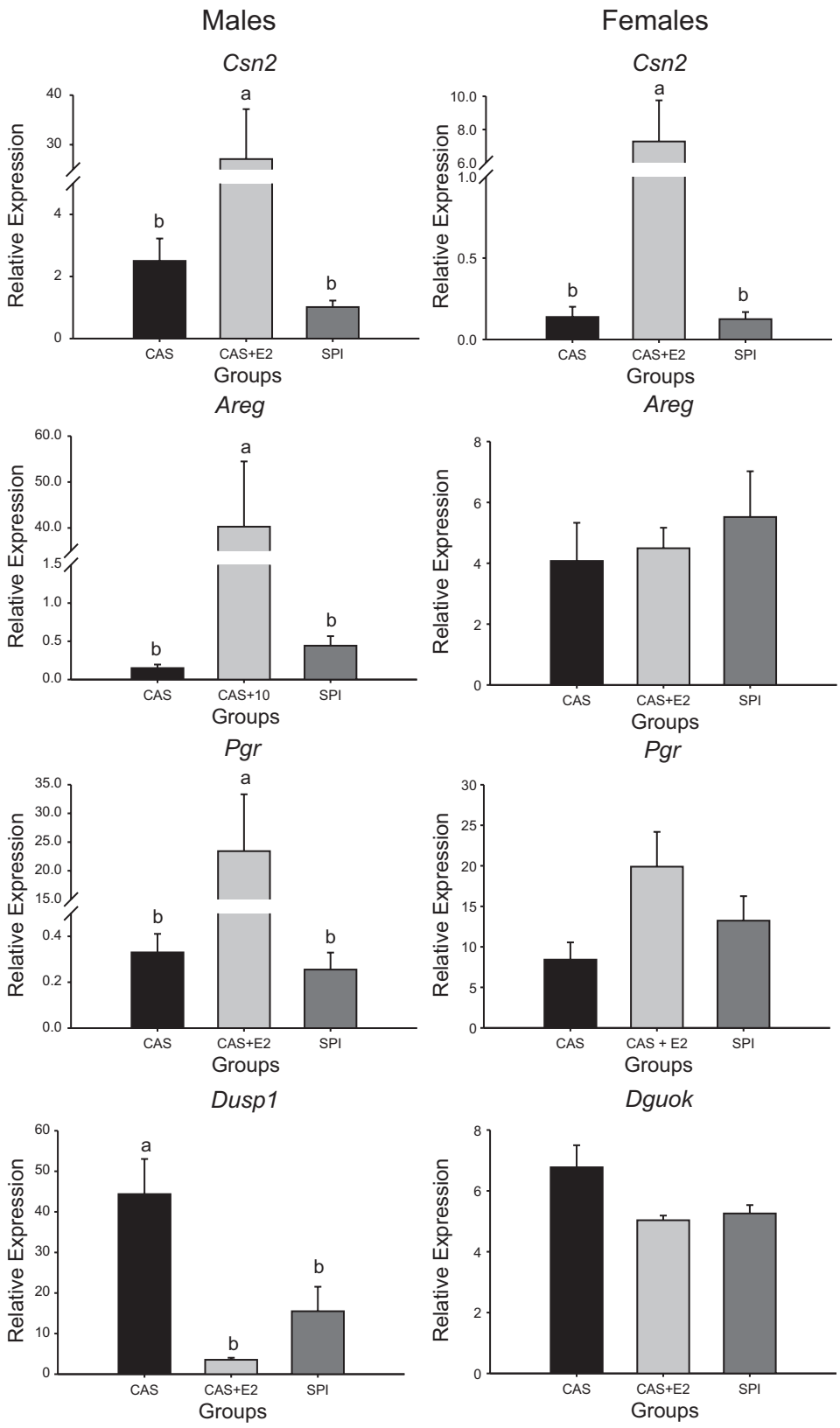

Fig. 3. Confirmation of gene expression changes. Relative gene expression normalized to Srp14 and Rps13 by quantitative (q)RT-PCR (n = 10 for males and n = 7 for females). Different letters indicate a significant difference by 1-way ANOVA followed by Dunn's post hoc analysis.

compared with expression in the CAS groups (Fig. 3). The deoxyguanosine kinase (*Dguok*) gene was downregulated as predicted by the array analysis but did not reach significance per qRT-PCR (Table 3, Fig. 3).

Comparison with E2. We previously reported dose-response data for mammary gene expression changes in the developing mammary gland of weanling rats treated with E2 (35). We observed significant, dose-dependent up- and downregulation

of key genes particularly in males. Overall, the overlap in genes significantly regulated by both E2 and SPI was only 3% of E2 dose-dependently regulated genes in the developing male mammary gland and only 0.5% of E2-regulated genes in the developing female mammary gland (Fig. 2); 16 of 34 (47%) of SPI-regulated male mammary genes were also regulated in a similar fashion by E2 (Table 4). However, this represented only 16 of 506 genes significantly regulated by E2 in the male mammary. Only the gene *Rcn1* was dose-dependently downregulated by both SPI and the E2 treatment in the developing female mammary gland (Table 4).

Proliferation and differentiation. To assess the impact of the SPI and E2 treatments on cell proliferation, we measured mRNA and protein abundance of the proliferating cell nuclear antigen (*Pcna,* PCNA) (Fig. 4). As we have previously reported, gene expression levels were higher in the male E2 group compared with the male CAS group and male SPI group ($P < 0.05$) but was unchanged by E2 in females. Immunoquantitation of PCNA protein expression in individual male mammary homogenates adjusted for loading using Amido black staining of total protein gave similar results: 1.02 ± 0.17 (CAS) vs. 1.19 ± 0.15 (E2) vs. 0.56 ± 0.17 (SPI) ($P < 0.05$, E2 vs. SPI). We did not observe significant differences in PCNA mRNA or protein expression between SPI and CAS groups in the mammary glands of females. We also examined the gene expression of the proliferation factor *FolR1* and the inhibitor of differentiation *Id2*. *FolR1* and *Id2* are part of the cMyc pathway previously shown to be upregulated by E2 in the adult female mammary gland (45). Both mRNAs were increased in the E2 group compared with the CAS group in the developing male mammary gland ($P < 0.05$) but were unchanged in the male SPI group relative to the male CAS group. In the developing female mammary gland, results for *FolR1* gene expression were the same as observed in males, while *Id2* mRNA expression displayed no significant difference between the three treatment groups (Fig. 4).

Estrogen receptor expression and promoter occupancy. Because of the affinity of soy isoflavones for the estrogen receptors, we measured the expression of mRNAs encoding ERα and ERβ (*Esr1* and *Esr2*) in our model (Fig. 5). As previously reported, E2 treatment decreased the mRNA expression of both *Esr1* and *Esr2* in E2 males relative to the CAS group ($P < 0.05$) and decreased mRNA and protein expression of *Esr1* in E2 females relative to the CAS group ($P < 0.05$) (35). ERα protein was undetectable in males. SPI groups of both sexes displayed reduced mammary expression of *Esr1* mRNA, and the male SPI group also expressed a lower level of *Esr2* mRNA than the male CAS group ($P < 0.05$). We also compared the genes whose expression was significantly changed by SPI with a list of genes previously shown to be regulated by either ERα or ERβ in the human MCF7 mammary epithelial cell line (15). Three genes were downregulated in the male SPI group compared with the male CAS group, which were previously described as ERβ-regulated in this cell line: heat shock protein B8 (*Hspb8*), muscleblind-like 2 (*Mbnl2*), and CCAAT/enhancer binding protein alpha (*Cebpa*). To identify whether ERE occupation was correlated with the expression of E2-regulated genes in the developing male mammary gland after E2 treatment or SPI feeding, we performed ChIP with antibodies against ERα and ERβ in male tissues. Binding of ERα to EREs present in *Esr1* and *Pgr* were increased by both E2 and SPI (Fig. 6). In contrast, although binding of ERβ to the ERE on the *Pgr* promoter was also increased in both E2 and SPI groups relative to the CAS group, binding of ERβ to the ERE on the *Esr1* promoter was detectable only in the SPI group. Despite similarities in ER occupancy of ERE sites on the promoter of the *Pgr* gene in E2 and SPI groups compared with the CAS group, this did not correlate with the dramatic difference in induction of *Pgr* mRNA in these groups (Fig. 3). Similarly, no correlation was observed between differential ERβ ERE occupancy and comparable downregulation of *Esr1* mRNA expression in both the E2 and SPI groups (Fig. 5). ChIP results were replicated with material from a separate chromatin extraction from mammary tissue (data not shown).

Table 4. *Genes regulated by both E2 and SPI*

Gene Symbol	E2	SPI
	Males	
Mpz	−2.81	−3.51
Dusp1	−2.01	−3.39
Mbp	−3.16	−3.31
Cebpa	−3.07	−2.64
Abcc9	−2.51	−2.34
Mbnl2	−2.49	−2.30
Shox2	−2.86	−2.24
Aebp1	−2.25	−2.15
Slc31a2	−2.16	−2.12
Ms4a2	−2.36	−2.05
Nov	−2.98	−2.02
Cadm3	−3.04	−2.02
Chst12	−2.07	−2.01
Zbtb41	2.58	2.26
Sfrs3	2.33	2.30
Nek1	2.22	2.30
	Females	
Rcn1	−2.44	−2.38

DISCUSSION

Because isoflavones found in soy foods are recognized as having estrogen-like properties, concerns have been raised about the safety of soy-based infant formula and of soy consumption in young children. The goal of this analysis was to compare potential estrogenic effects of an isoflavone-containing SPI diet on mammary morphology and gene expression to the effects of exogenous E2 supplementation of a control casein diet in the male and female weanling rat, a developmental window analogous to the period from weaning to puberty in children. Previous studies have demonstrated very limited overlap between responses to E2 and responses to feeding SPI, the sole protein source in soy infant formula, in adult female rats (45). However, questions remain with regard to the potential interactions of SPI with estrogen receptor signaling pathways in the female mammary gland prior to puberty and in the developing male mammary gland, situations where normal exposure to endogenous estrogens is low. As an earlier report from our group identified important sexually dimorphic differences in the estrogenic response in the mammary gland between male and female weanling rats (35), we analyzed sexes separately.

Both E2 and SPI feeding showed a trend toward decreased body weight in weanling rats compared with CAS controls. A

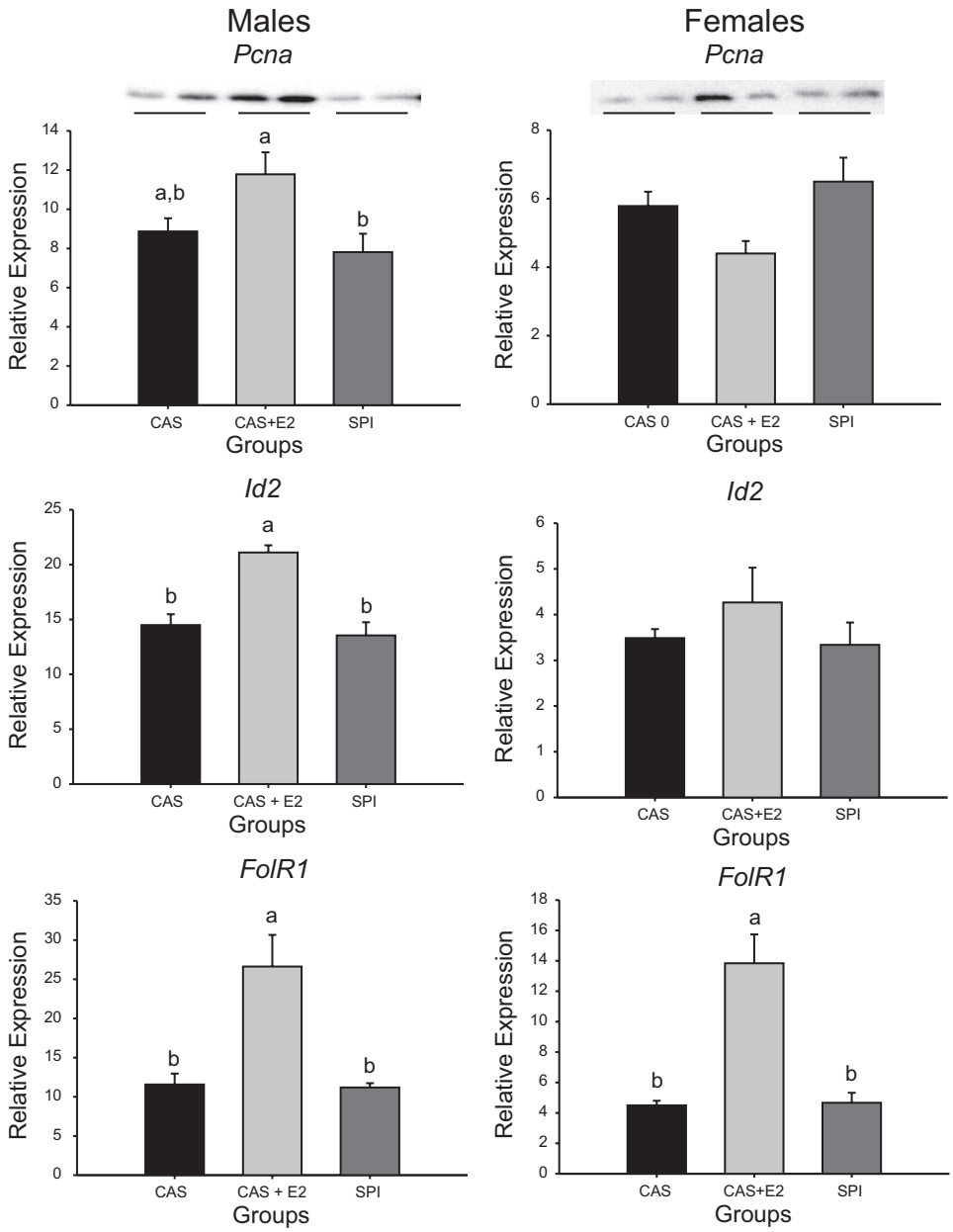

Fig. 4. Proliferation and differentiation. Relative gene expression normalized to *Srp14* and *Rps13* by quantitative RT-PCR [$n = 10$ for proliferating cell nuclear antigen (*Pcna*), $n = 5$ pools of 2 for *Id2* and *FolRI* in males and $n = 7$ for females]. Different letters indicate a significant difference by 1-way ANOVA followed by Tukey test post hoc analysis in males and Dunn's in females. Representative Western blot results are displayed for PCNA in males and females, each lane represents data from a pool of $n = 3$–5 mammary homogenates, above the corresponding mRNA groups in the bar graph.

decrease in body weight was previously observed in the offspring of dams fed SPI (56), but not in adults fed SPI (45). However, since the endocrine effects and the morphological and molecular effects of E2 and SPI observed in the mammary gland differed substantially, these differences cannot be secondary to reduced weight gain. E2 increased the number of TEB, but only in males. In contrast, SPI feeding in either males or females had no statistically significant effect on any aspect of mammary gland morphology. If anything, there was a trend toward decreased TEB number in males ($P < 0.1$). This is opposite to the increase in TEB previously reported for weanling male rat mammary glands following treatment with E2 (35), but in agreement with observations in female rats following perinatal SPI feeding via maternal diet and with data on the effects of neonatal dietary exposure to isoflavones (5, 7, 56). Serum T was decreased only by E2, but progesterone was markedly decreased in female rats by both SPI feeding and by E2 treatment as previously reported in rats and humans (29, 50). As described by others, SPI feeding was also associated with a decrease in serum insulin (59).

We previously reported that the weanling male mammary gland is more responsive to exogenous E2 treatment than the female mammary gland (35). Similarly, the changes in mammary gene expression in response to feeding SPI were small in males and negligible in females. However, in males, only 3% of genes significantly altered by E2 were also regulated more than twofold by SPI. In females, only one gene modulated by E2 was also present in the SPI group. This minimal overlap between gene expression patterns after E2 and SPI treatment, even under analysis with relaxed stringency where the chance of false positives is higher, in line with earlier results from our group in SPI-fed adult female rat mammary gland and liver (45, 53). We previously identified three genes as very sensitive dose-dependent indicators of estrogenic action in the develop-

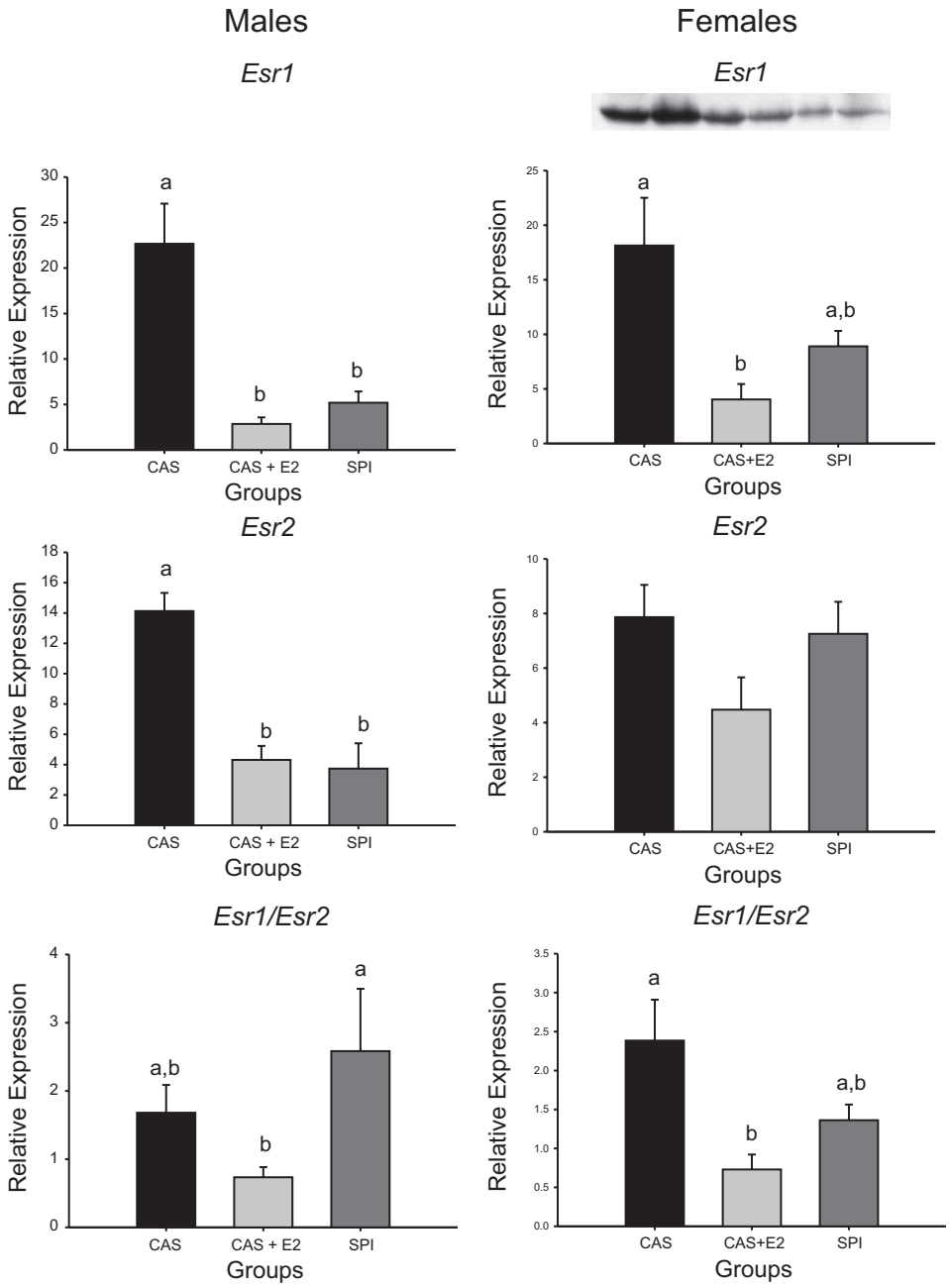

Fig. 5. Estrogen receptor expression. Relative gene expression normalized to *Srp14* and *Rps13* by qRT-PCR ($n = 5$ pools of 2 in males and $n = 7$ for females). Different letters indicate a significant difference by 1-way ANOVA followed by a Tukey test post hoc analysis (Dunn's post hoc analysis for ERα in female). Representative Western blot results are displayed for *Esr1* in females, above the corresponding mRNA groups in the bar graph.

ing rat mammary gland: *Areg* and *Pgr* in males and *Csn2* in females (35). *Areg*, particularly, has been identified as a mediator of increases in the number of TEBs, a risk factor for neoplastic growth in the mammary gland. There was no change in either *Areg* or *Pgr* in the male mammary gland or in *Csn2* expression in either male or female mammary gland measured by either microarray or qRT-PCR in the SPI diet groups compared with CAS controls, and there was no association with morphological changes in the mammary gland in either sex. Consistent with these data, we also observed no effects of SPI on the E2-responsive c-myc pathway and indications of reduced mammary proliferation in the form of reduced expression of PCNA in SPI-fed compared with E2-treated males.

Interestingly, although only a small number of E2-responsive genes were also significantly affected by SPI-feeding, almost half of the SPI-modulated genes in males were also found to be E2 regulated. Almost all of these were downregulated by both treatments with the same degree of potency. If SPI were acting as a weak E2 agonist, we would expect a much larger proportion of E2-responsive genes to have been affected by SPI feeding in both directions but less robustly than by E2 itself. Instead, a very small subset of E2-regulated genes appears to have been selectively downregulated in response to SPI in a similar fashion the response observed with E2. This pattern suggests that SPI may act as a selective estrogen receptor modulator. While we observed more downregulation than upregulation with E2 treatment (315 downregulated vs. 194 upregulated), this is not enough to explain the overwhelming trend toward downregulation in the overlapping gene set with SPI (35). Of the male mammary genes downregulated by

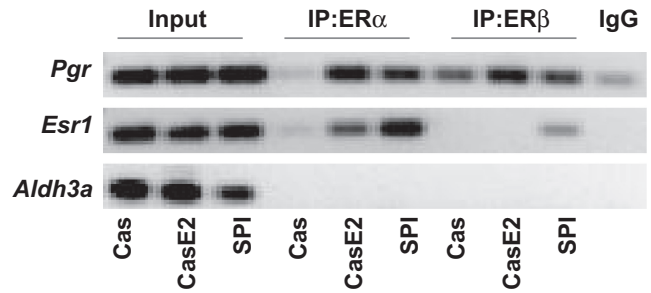

Fig. 6. Chromatin immunoprecipitation in male mammary tissue. PCR products from total input chromatin are displayed on the *left* and from ERα-pulled down immunoprecipitated (IP) material on the *right*. Normal rabbit IgG was used as a negative control antibody (last lane). The promoter region of the gene *Aldh3a*, which does not contain an estrogen response element, was used as a negative control chromatin.

both SPI and E2, two genes, *Dusp1* and *Cebpa*, are known to be regulated by isoflavones. Genistein is a well-described inhibitor of the MAPK phosphatase *Dusp1* (33, 34), which was also reported to be downregulated by equol (4). Expression of the transcription factor *Cebpa* was also found to be reduced following genistein treatment (28, 38). *Dusp1* and *Cebpa*, as well as two other SPI downregulated genes in males, *Aebp1* and *Fgfr1*, are positive regulators of ERK1/2-mediated adipogenesis (21, 28, 38, 39, 61). Along with the observed decrease in serum insulin (59) and body weight (44, 56), the downregulation observed in these genes is consistent with a reduction in mammary gland adipogenesis in SPI-fed rats that has previously been reported in female rats fed SPI throughout early development (56). It is also consistent with the stimulation of osteoblastogenesis, an inversely related process, previously reported by our group in bones from these same animals, since mesenchymal stem cells are multipotential and can differentiate into either adipocytes or osteoblasts (65). We measured mammary gland expression of *Pparg* mRNA, the master transcription factor regulating adipocyte differentiation, and although there was a decrease in mean values associated with the SPI diet, it did not reach statistical significance (data not shown). Moreover, we did not identify any change in mammary gland fat pad area in SPI-fed animals after this short period of feeding. Reduced abdominal fat and mammary adipocyte size have previously been observed in the female mammary gland at PND50 after longer periods of developmental SPI exposure beginning with the dams during gestation (56). Isoflavones and E2 are both recognized to inhibit adipogenesis and reduce fat cell size (8, 28, 36, 38, 56). Reductions in adiposity might be mediated via isoflavone activation of a subset of estrogen receptor-mediated pathways or alternately through other pathways such as inhibition of sterol receptor element binding protein (SREBP-1c)-mediated fatty acid synthesis or activation of the nuclear receptor PPAR-α to increase fatty acid metabolism (44). It is unknown whether the failure to see more significant changes in mammary fat pads in our own study is due to the difference in the age at exposure, duration of treatment, age at death, or a combination of these factors. Epidemiological and animal studies point to a crucial effect of the window of exposure to soy and soy isoflavones on the mammary tumor risk (reviewed in Ref. 32).

Both SPI and E2 induced a decrease in gene and protein expression in ERα. Previous results have shown a similar decrease in ERα in the rat mammary gland with E2 treatment in prepubertal animals (6, 35) and with feeding of a lifelong SPI diet (9). Surprisingly, the decrease in ERα was not associated with an increase in ERβ expression. There was rather a decrease in ERβ expression in males, while no changes were observed in females. These results suggest a negative feedback loop leading to the downregulation of these receptors following chronic stimulation, at least in males (15, 48). Analysis of ERE occupancy in the *Pgr* and *Esr1* genes in the male mammary gland reveals that both E2 and SPI induced increased ERα binding and that SPI also increased ERβ binding. ERβ was recruited to the promoter region of both *Pgr* and *Esr1* by SPI, supporting its role in SPI responses. Increased ERα recruitment was associated with decreased *Esr1* gene expression in both E2 and SPI groups. This suggests the successful recruitment of functional co-repressors to the promoter region of *Esr1*, in association with binding of the ERs. However, differential recruitment of ERβ to the *Esr1* ERE indicates that distinct sets of co-repressors may be implicated in the two responses, as ERβ is known to be involved in the recruitment of a co-repressor complex in *Esr1* (3). In contrast, in the case of ER binding to ERE in the promoter of the progesterone receptor *Pgr* in the male mammary gland, ERα and ERβ were recruited at a similar level in both E2 and SPI. However, upregulation of *Pgr* mRNA was only observed after E2 treatment. We hypothesize that full recruitment of the active transcription complex might be impaired by isoflavone binding to ERE-bound ERs. Differential recruitment of coactivators such as Sp1, or co-repressors such as Fos and Jun, may be involved (41, 42). The exact molecular mechanisms underlying the dissociation between recruitment of ERs to promoters and mRNA expression of estrogen-responsive genes after being fed soy-containing diets is the subject of ongoing studies.

In conclusion, feeding weanling rats with SPI did not lead to a significant morphological change in the developing rat mammary gland of either sex. There were also very few mammary genes whose expression was changed by diets containing SPI as the sole protein source. Only a small subset of ER-regulated genes appeared to also be regulated by SPI, and these were almost all downregulated by both treatments. The observed bias toward downregulation of E2-sensitive genes by SPI may suggest a greater ability to recruit co-repressors than coactivators to EREs in the mammary gland after SPI feeding relative to E2 treatment. Additional detailed analysis of ER-regulated transcriptional complexes on multiple ER target genes are beyond the scope of this study and will be required to further determine the differences in estrogenic potency of E2 and SPI. In addition, more estrogen-responsive organs need to be assessed, notably the gonads, liver, and adipose tissue, to assess the general estrogenicity and safety of SPI feeding during early development. Our results are unsupportive of a proliferative effect of SPI in the developing mammary gland, after feeding during early development.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.R.M., N.S., M.L.B., and J.V. performed experiments; I.R.M., H.G.-A., L.H., M.A.C., and M.J.J.R. analyzed data; I.R.M., K.S., and M.J.J.R. interpreted results of experiments; I.R.M. and M.L.B. prepared figures; I.R.M. drafted manuscript; I.R.M., T.M.B., and M.J.J.R. edited and revised manuscript; I.R.M., T.M.B., and M.J.J.R. approved final version of manuscript; L.H., K.S., T.M.B., and M.J.J.R. conception and design of research.

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