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Research Article

Dose- and Time-Dependent Transcriptional Response of Ishikawa Cells Exposed to Genistein

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ABSTRACT

To further define the utility of the Ishikawa cells as a reliable *in vitro* model to determine the potential estrogenic activity of chemicals of interest, transcriptional changes induced by genistein (GES) in Ishikawa cells at various doses (10 pM, 1 nM, 100 nM, and 10 μM) and time points (8, 24, and 48 h) were identified using a comprehensive microarray approach. Trend analysis indicated that the expression of 5342 unique genes was modified by GES in a dose- and time-dependent manner ($P \leq 0.0001$). However, the majority of gene expression changes induced in Ishikawa cells were elicited by the highest dose of GES evaluated (10 μM). The GES' estrogenic activity was identified by comparing the Ishikawa cells' response to GES versus 17 α-ethynyl estradiol (EE, at equipotent doses, ie, 10 μM vs 1 μM, respectively) and was defined by changes in the expression of 284 unique genes elicited by GES and EE in the same direction, although the magnitude of the change for some genes was different. Further, comparing the response of the Ishikawa cells exposed to high doses of GES and EE versus the response of the juvenile rat uterus exposed to EE, we identified 66 unique genes which were up- or down regulated in a similar manner *in vivo* as well as *in vitro*. Genistein elicits changes in multiple molecular pathways affecting various biological processes particularly associated with cell organization and biogenesis, regulation of translation, cell proliferation, and intracellular transport; processes also affected by estrogen exposure in the uterus of the rat. These results indicate that Ishikawa cells are capable of generating a biologically relevant estrogenic response and offer an *in vitro* model to assess this mode of action.

Key words: Ishikawa cells; genistein; in vitro; gene expression profiling; microarrays; human uterus.

Currently, a reliable *in vitro* model to determine the potential estrogenic activity of chemicals using a comprehensive mode of action approach (ie, agonistic or antagonistic activity; primary or secondary response) is not available. The establishment of such a model is critical to develop dependable high-throughput screening assays and to minimize animal testing studies for this mode of action. Lately, gene expression profiling has proven to be an effective approach to gather useful data to define chemical specific mode of action. Using a transcriptomics approach to determine the response to a potent and a weak estrogen receptor (ER) agonist, 17 α-ethynyl estradiol (EE) and bisphenol A, respectively, we identified a cell line, Ishikawa cells, capable of generating a robust genomic response to estrogen exposure, which has a high degree of concordance with the response determined *in vivo* (Naciff *et al.*, 2009, 2010).

Ishikawa cells are one of the best characterized human endometrial cell lines currently available. These are cells derived from a well-differentiated adenocarcinoma of the human endometrial epithelium, express functional steroid receptors for estrogen, progesterone, and androgen (Croxtall *et al.*, 1990; Lessey *et al.*, 1996; Lovely *et al.*, 2000; Morishima *et al.*, 2008; Nishida *et al.*, 1985; Nishida, 2002), as well as estrogen-related receptor gamma (ESRRG) (Sun *et al.*, 2014; Yamamoto *et al.*, 2012). As such, Ishikawa cells provide the opportunity to evaluate the effects of chemicals with estrogenic activity on the cellular

responses mediated by these receptors and the response of the endometrial epithelium to estrogen exposure *in vitro*.

Genistein (4',5,7-Trihydroxyisoflavone, GES) is one of the predominant soy isoflavones, and an important component of the regular human diet. This isoflavone has multiple biological activities (reviewed by Polkowski and Mazurek, 2000), including the inhibition of protein tyrosine kinases; topoisomerase II, an enzyme that regulate the over- or under-winding of DNA, which is essential in the separation of entangled daughter DNA strands during replication; and also inhibits enzymes involved in phosphatidylinositol metabolism. Genistein also regulates the signal transduction components Akt, FAK, ErbB-2, and Bcl-2 (Banerjee *et al.*, 2008). Because of its structural similarity, GES has been shown to compete with 17 β-estradiol for ER binding (Kuiper *et al.*, 1998), resulting in agonistic or antagonistic activity, depending on the tissue being evaluated and specific interactions between the ERs and the proteins, coactivators and transcription factors, that associate with them (Leitman *et al.*, 2010). Genistein also arrests the cell cycle, induces apoptosis, has antioxidant activity, antiangiogenic and antimetastatic properties, and has been shown to inhibit carcinogenesis in animal models (Banerjee *et al.*, 2008; Sahin *et al.*, 2011). Various *in vivo* and *in vitro* studies have shown that GES can regulate the expression of multiple genes, whose products are involved in the regulation of multiple biological processes, including cell growth, cell cycle, cell signal transduction, angiogenesis, tumor cell invasion, and metastasis (Di *et al.*, 2012; Li *et al.* 2004; Naciff *et al.*, 2002). Thus, GES is not just capable of affecting the estrogen system but also multiple systems independent of its estrogenic activity and offers the opportunity to use it as a case study to test the robustness of the *in vitro* system we have selected as a surrogate of estrogen-responsive tissue, the Ishikawa cell line.

In order to better understand the estrogenic response of Ishikawa cells, define their value as an *in vitro* alternative to evaluate estrogenicity, and at the same time obtain an insight into the molecular mechanism involved in the response of the human-derived cells to GES, we have determined the gene expression profiles induced by GES at various doses (10 pM, 1 nM, 100 nM, and 10 μM) and time points (8, 24 and 48 h). This genomic response to GES was compared to the response elicited by EE at equipotent doses in the Ishikawa cells as well as in the uterus of the rat, as previously reported (Naciff *et al.*, 2007, 2009).

MATERIALS AND METHODS

Chemicals

Genistein (≥98%, HPLC) was obtained from Sigma Chemical Company (St. Louis, MO).

Cell culture

Tissue culture flasks (catalog no. 430725 and 3506) and plastic disposables were obtained from Corning (Corning Inc.; Corning, NY). Dulbecco's modified Eagle medium (DMEM)/F12 was purchased from Invitrogen (Life technologies; Carlsbad, CA), and the fetal bovine serum (FBS) came from Hyclone (Logan, UT). The Ishikawa cell line was a generous gift from Dr Masato Nishida (Kasumigaura National Hospital, Tsuchiura-shi, Ibaraki-ken, Japan). The cells were routinely maintained in DMEM/F12 supplemented with 10% FBS and antibiotic/antimycotic solution containing 100 units/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen/Gibco Life Technologies, Grand Island, NY) in a 37°C incubator in a humidified atmosphere containing 5% CO_2. As previously described (Naciff *et al.*, 2009), the cells (passage 8, starting with the culture received from Dr Nishida) were synchronized at the G0/G1 cell cycle stage by serum deprivation, in order to optimize the detection of the cells' genomic response after exposure to graded doses of GES. However, since serum provides components essential to maintaining cellular viability and adequate responsiveness, the exposure of Ishikawa cells to GES was done in media supplemented with dextran-coated charcoal (DCC) stripped serum, which contains minimal quantities of steroids. Using this approach, we also ensure that the steroids present in the regular serum would not interfere with the GES response, particularly at low doses. Cells were stripped of endogenous steroids by 48 h culture in phenol red-free DMEM/F12 + 10% DCC-treated FBS (Invitrogen/Biosource Biofluids Cell Culture Products, Life Technologies; Rockville, MD), before the experiments started. After steroid starvation, the cells were exposed to vehicle-control (0.1% ethanol in DCC medium), or to GES at doses of 10^{-11} M (10 pM, very low dose, vL), 10^{-9} M (1 nM, low dose, L), 10^{-7} M (100 nM, high, H), or 10^{-5} M (10 μM, very high, vH) for 8, 24, or 48 h. The doses tested include the GES concentrations that correspond to the IC_{50} (30–50 nM as assayed by Luciferase reporter gene assay with MVLN- or HGELN-cells). The highest dose of GES evaluated is very close to the IC_{50} determined by competitive binding for the recombinant human ERα (~15 μM) and ERβ (~25 μM) (Gutendorf and Westendorf, 2001). For the hERβ, Han *et al.* (2002) determined an IC_{50} of 5×10^{-8} M or 50 nM. Thus, the GES concentrations evaluated in this study should result in significant activation of both ERα and ERβ. The indicated concentrations of GES were prepared in fresh phenol red-free DMEM/F12 + 5% DCC FBS. Five independent cell cultures per time point and dose were used as "biological replicates." Cell viability was evaluated by dye exclusion assay, using 10% trypan blue and quantified by counting viable and dead cells in a hemocytometer.

Expression profiling

Total RNA was extracted from each individual cell culture, "biological replica," using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH), 8, 24, and 48 h after exposure to vehicle (controls) or GES (at the different doses tested). Total RNA was further purified by RNeasy kit (QIAGEN, Valencia, CA). After determining total RNA quality, using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA), 10 μg of total RNA from each sample were converted into double-stranded cDNA using SuperScript Choice system (Invitrogen/GIBCO BRL, Life Technologies Rockville, MD) with an oligo-dT primer containing a T7 RNA polymerase promoter. The double-stranded cDNA was purified by phenol/chloroform extraction and then used for *in vitro* transcription using ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY). Biotin-labeled cRNA was purified by RNeasy kit (QIAGEN), and a total of 20 μg of cRNA were fragmented randomly to ~200 bp at 94°C for 35 min (200 mM Tris-acetate, pH 8.2, 500 mM potassium acetate, and 150 mM magnesium acetate). Samples from 4 replicates of each treatment group with high-quality cRNA were selected and hybridized to Affymetrix Human Genome U133 Plus 2.0 high-density oligonucleotide microarrays (Affymetrix Inc., Santa Clara, CA) for 16 h. These microarrays have 54 613 probe sets which include 38 500 annotated human genes and expressed sequence tags (ESTs). The microarrays were washed and stained by

streptavidin-phycoerythrin to detect bound cRNA. The signal intensity was amplified by second staining with biotin-labeled antistreptavidin antibody and followed by streptavidin-phycoerythrin staining. Fluorescent images were read using the Affymetrix GeneChip Scanner 3000 with Autoloader (Affymetrix Inc., Santa Clara, CA).

Real-time QRT-PCR

In order to corroborate the relative changes in gene expression in selected genes identified by the oligonucleotide microarrays, real-time (kinetic) quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) approach was used, as described previously (Naciff *et al.*, 2002, 2009). Supplementary Table S1 lists the nucleotide sequences for the primers used to evaluate the expression level of the indicated gene transcripts.

Data analysis

Potential interindividual variability was addressed by using independent samples of each dose group ($n = 4$) for analysis. For gene expression analysis, scanned output files of Affymetrix microarrays were visually inspected for hybridization artifacts and then analyzed using the Affymetrix Expression Console Software and MAS5 (Affymetrix Microarray Analysis Suite 5) statistical algorithm to analyze the data (http://www.affymetrix.com/products). Arrays were scaled to an average intensity of 1500 units and analyzed independently. For the entire analysis, each probe set was analyzed as an individual entity, based upon its Affymetrix ID number (Affy ID), regardless of the multiplicity of probe sets representing any given gene product, and were considered as representing an individual gene until the completion of the analysis. Subsequently, all the probe sets (Affy IDs) were annotated and when possible, identified by its corresponding gene symbol. Distinct algorithms made an absolute call, present/marginal/absent, for each transcript, and calculated the average difference between perfect match and mismatch probe pairs (signal value). The mathematical definitions for each algorithm are described in the Affymetrix Expression Console Software and MAS5 User's Guide. Probe sets that were called absent in both the control and treated groups, at any given dose and time, were excluded from further analysis, only for that particular dose and time group. For any given probe set to be designated "present" in the differential expression analysis, such probe set needed to have at least 75% of replicate samples called as present, for that particular set of samples (control or treated at any given dose and time point). This approach further reduces the possibility of including false positives since it removes data that are not reliably detected by the microarrays.

Gene expression values derived from MAS5, hereafter termed "signal," were evaluated for quality based upon both overall measures of the microarray quality and simple outlier detection methods. None of the microarrays were detected as outliers. The signal values were used for a complete statistical analysis to identify treatment and time effects using the approach and statistical tests previously described (Naciff *et al.*, 2009). The expression of probe sets (Affy IDs) for which any of the tests had $P \leq 0.0001$ was interpreted as significantly modified by GES treatment at the time and dose being tested. Fold-change summary values for genes were calculated as a signed ratio of mean signal values, from 4 independent samples for each time and dose point group compared with the appropriate time matched control. After selecting all the significant probe sets for any given time and dose being evaluated, redundancies due to multiple probe sets representing the same gene were eliminated (using the significance criteria, *P* value and fold change determined for each probe set). The probe with the most statistically robust response was selected for each annotated gene. Once selected, the response of this probe set was used in the analysis across dose and time points evaluated, as well as to compare the genomic response of Ishikawa cells exposed to GES, EE (Naciff *et al.*, 2009; GEO Accession: GSE11869), or to the response of the juvenile rat uterus to EE exposure (Naciff *et al.*, 2007; data available upon request).

Biological processes affected by GES exposure in Ishikawa cells

The Gene Ontology (GO) terms associated with these GES-responsive genes were used to identify specific biological processes, or molecular functions, affected by GES treatment following the procedure previously described for the analysis of the effect of EE on these cells (Naciff *et al.*, 2009). This approach allows the identification of statistically over-represented GO terms within the significant group of GES-responsive genes. For the comparison of the Ishikawa cells' response to GES to the response of the rat uterus to estrogen exposure, after identifying the orthologs (using public sources, including HUGO, Entrez Gene, Mouse Genome Informatics, and Rat Genome Database), the annotation was used as a common vocabulary to annotate genes for comparison, and thus, helped to identify gene homologs between the *in vivo* (rat uterus) and the *in vitro* (Ishikawa cell line) data sets. This approach assumes that the products of the estrogen- and GES-responsive genes perform the same molecular functions, take part in the same biological processes and are located in the same cellular component in the 2 different species.

RESULTS

The viability of Ishikawa cells in the media containing 10% DCC-FBS without GES (controls cells) or with the various GES doses tested was determined to ensure that the cell survival was not altered by either treatment. No differences in cell viability ($\geq 95\%$) were observed, at any of the times evaluated (8, 24, or 48 h) in control or GES-treated cells. Further, the transcript profile determined in the 10 pM (vL), or 1 nM (L), GES-treated and the appropriate time-matched control cells was comparable (Fig. 1, and vL and L in Supplementary Fig. S1). Thus, the gene expression changes observed at the different doses and times evaluated were due to the GES treatment and not to differences in cell viability. Further, the gene expression analysis showed similar ratios of present and absent calls in control and GES-treated samples, at the 3 times tested, which also indicated that both control and treated cells were healthy and biologically competent.

Temporal and dose-responsive gene expression changes induced by GES

The responses that chemical exposure induces on a biological system are usually dependent on the dose of the chemical, as well as the time of exposure. In order to better evaluate the genomic response of Ishikawa cells to GES exposure, the gene expression profile elicited by GES at various doses (10 pM, 1 nM, 100 nM, and 10 μM) and time points was determined. The transcript profiles were compared between treatment groups

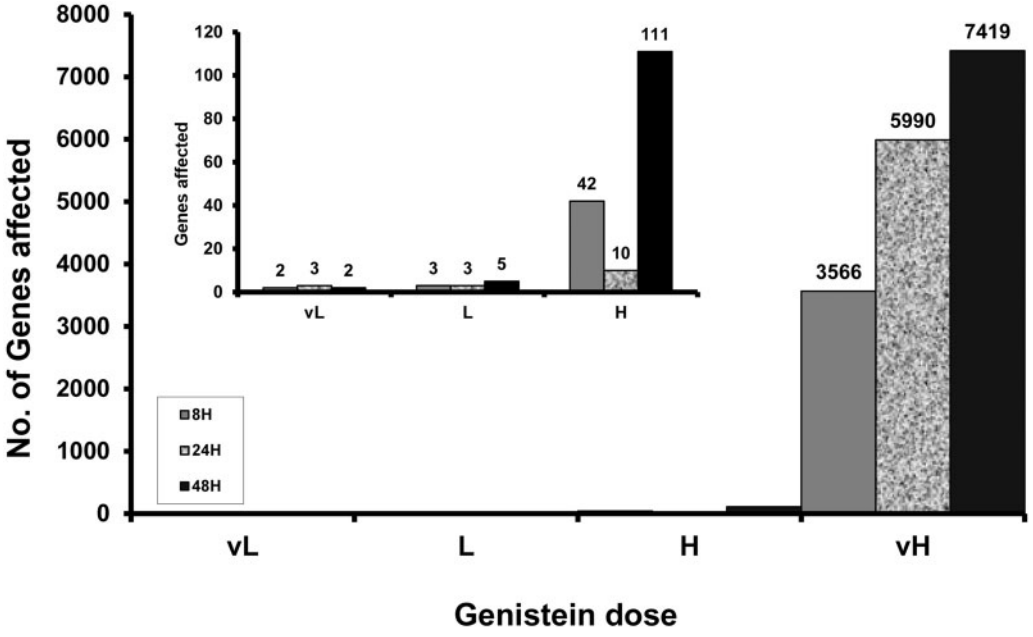

FIG. 1. Temporal and dose-responsive gene expression changes induced by GES in Ishikawa cells. For this analysis, the number of unique genes whose expression was affected by exposure to GES at the different times and doses were selected on the bases of significance ($P \leq 0.0001$, *t* test) and fold change (1.2-fold, up- or down-regulated) compared to the appropriate time-matched control. The insert shows the results of the 3 GES lower doses, at different scale, for better visualization of the results.

(GES at the indicated doses) and time-matched controls (vehicle treated) at 8, 24, and 48 h. For this analysis, the genes whose expression was affected by GES exposure were selected on the basis of significance ($P \leq 0.0001$, *t* test) of this change and fold change (1.2-fold, up- or down-regulated) compared to the appropriate time-matched control. Trend analysis revealed that the expression of 5342 unique genes was modified by exposure to GES ($P \leq 0.0001$). The number of genes whose expression was affected by exposure to GES as a function of exposure time and doses is shown in Figure 1.

The genomic response of the Ishikawa cells to GES exposure is time and dose dependent. Although GES elicited identifiable gene expression changes at each time point and dose evaluated, the largest number of genes affected by GES was detected at 24–48 h after treatment, and most of these changes were induced by the highest dose tested (10μM GES) (Fig. 1, and Supplementary Fig. S1). The exposure of Ishikawa cells to 10 pM (vL), 1 nM (L), 100 nM (H), and 10 μM (vH), GES induced the change in the expression of 7, 11, 163, and 6657 unique genes, respectively, across the different time points evaluated ($P \leq 0.0001$, and fold change 1.2-fold).

A list of all the genes whose expression was modified in Ishikawa cells by 10 pM, and 1 nM, at the indicated times, along with their accession number, gene symbol, and the average fold change induced by GES is shown in Tables 1 and 2, respectively. Given the larger number of genes affected by the 2 highest doses of GES (100 nM and 10 μM), only a selected list of those genes are shown in Tables 3 and 4, respectively. With the purpose of providing a better insight of the potential functional implications for each individual gene affected by GES exposure, a representative GO term from the biological process or the molecular function (when applicable) has been included in these tables. The complete data set is available at GEO (Accession: GSE71717).

The genomic response of Ishikawa cells to low doses (10 pM–1 nM) of GES was extremely limited (Tables 1 and 2), judged not just by the low number of genes affected, but also by the magnitude of the change induced in the individual GES-responsive genes. For example, in cells exposed to 10 pM GES the gene that showed the greatest up-regulation was AF086490, with an average 4.1-fold change, while the gene showing the greatest down-regulation was AK097499, with a −2.4-fold change. When the cells were exposed to 1 nM GES, the most up-regulated genes were MAX and PPP2R5C (GES elicited an increase in the expression of these 2 genes in the mouse uterus; GEO Profiles: GDS982/98993_at/Ppp2r5c, and GDS981/99095_at/Max), with an average 4.2- and 2.3-fold increase, respectively, while the most down-regulated gene was NR_027036 (with still an unknown gene product), with a −2.6-fold decrease. Comparing the genes affected by 10 pM GES to the ones affected by 1 nM GES, at any of the exposure times evaluated, only the up-regulation of CA13 was in common.

In contrast, and in agreement with *in vivo* studies of GES (Naciff *et al.*, 2002), the exposure of Ishikawa cells to higher doses of GES (10 μM) clearly elicited a robust genomic response (Fig. 1 and Table 4). The data showed a marked difference in the magnitude of the response between cells exposed to 10 μM or lower GES doses, judged not just on the number of genes whose expression is significantly changed but also by the magnitude of the change induced on individual genes. For example, the up-regulated gene most responsive to 10 μM GES was PAG1 (GES elicited an increase in the expression of this gene in the mouse uterus; GEO Profiles: GDS982/103685_at/Pag1), with a 59.1-fold increase compared to control, while the down-regulated gene most responsive to this GES dose was SOX5, with a −62.8-fold decrease. This gene encodes a member of the SOX (SRY-related HMG-box) family of transcription factors, some of which are known to be down-regulated by estrogen (see eg, Hunt and Clarke, 1999). However, the comparison of the genomic response of Ishikawa cells to the 2 highest doses evaluated (100 nM and 10 μM) indicates that this system is able to identify genomic responses elicited by GES in a dose dependent fashion.

TABLE 1. Gene Expression Changes Induced in Ishikawa Cells by Exposure to 10 pM GES

NCBI RefSeq	Gene name	Average fold change*			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
NM_001037283	eukaryotic translation initiation factor 3, subunit B	**−2.0**	1.0	−1.2	EIF3B	translational initiation
AK097499	EST, clone TESTI2018035	**−2.4**	−1.5	1.0	—	—
AF086490	EST clone ZD94H12.	−1.4	**4.1**	1.3	—	—
NM_004972	Janus kinase 2	−1.5	**3.6**	1.0	JAK2	activation of MAPKK activity
NM_173855	MORN repeat-containing protein 3	1.1	**1.9**	−1.0	MORN3	—
NM_025010	kelch-like 18 (Drosophila)	−1.2	1.2	**2.7**	KLHL18	protein binding
NM_198584	carbonic anhydrase XIII	1.1	1.0	**1.6**	CA13	one-carbon metabolic process

*Numbers in bold indicate statistically significant change ($P \leq 0.0001$).

TABLE 2. Gene Expression Changes Induced in Ishikawa Cells by Exposure to 1 nM GES

NCBI RefSeq	Gene name	Average fold change[a]			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
NM_001040157	centrosomal protein 44kda (KIAA1712)	**1.5**	1.2	1.3	CEP44	protein ubiquitination
BC028845	EST, clone IMAGE:4796102	**−1.6**	1.1	1.2	—	—
BC010527	EST, clone IMAGE:4151535	**−2.1**	1.1	−1.1	—	—
NM_018306	transmembrane protein 40	1.2	**1.9**	−1.4	TMEM40	—
NM_138804	chromosome 2 open reading frame 65	1.1	**−1.7**	1.2	C2orf65	—
NR_027036	hypothetical LOC100271722	−1.2	**−2.6**	1.0	LOC100271722	—
AK022167	MYC associated factor X	1.8	−1.0	**4.2**	MAX	transcription cofactor activity
NM_001161725	protein phosphatase 2, regulatory subunit B', gamma	1.1	1.1	**2.3**	PPP2R5C	DNA damage response
NM_014966	DEAH (Asp-Glu-Ala-His) box polypeptide 30	1.1	1.1	**2.0**	DHX30	nucleotide binding
NM_002381	matrilin 3	−1.1	1.2	**1.9**	MATN3	extracellular matrix structural constituent protein binding
NM_198584	carbonic anhydrase XIII	1.1	1.1	**1.7**	CA13	one-carbon metabolic process

[a]Numbers in bold indicate significant change ($P \leq 0.0001$).

Comparison of the response to GES at 100 nM versus 10 μM across time, indicated that the expression of 156 genes was modified in a dose-dependent manner. Among these genes are RASGRP1, CCND2, EGR1, G0S2, SPHAR, POLR3G, HEY1, PELI2, SLC40A1, EFNB2, ENC1, KCTD12, PRSS23, FGFR2, ODZ1, and others (Tables 3 and 4).

Functional groupings of GES regulated genes

To better understand the biological relevance of the gene expression changes elicited by GES in Ishikawa cells, the biological terms or functional categories associated with each gene was determined using the GO terms associated with these genes. The probe sets representing ESTs were not included for this analysis since there was no annotation associated with them. In order to assign a unique GO term to individual probe sets identified as showing statistically significant expression changes ($P \leq 0.0001$, *t* test; and at least 1.2-fold change) induced by GES exposure, the selected probe sets from any time and dose group were mapped to their corresponding annotations in the GO. Redundancies due to multiple probe sets representing the same gene were eliminated for this analysis, which resulted in the annotation of 8213 unique probe sets that matched the selection criteria ($P \leq 0.0001$). These probe sets represented 5342 individual genes whose expression showed the most robust response to GES exposure. Each gene was assigned to all possible biologically descriptive GO terms and processed to identify clusters of genes representing unique GO terms. The clusters in the ontology were identified by the statistical significance of the mapping (see Naciff *et al.*, 2009). This approach resulted in the generation of clusters that contain genes which are both functionally related and highly co-regulated by GES exposure and whose dependency on the dose and/or time of exposure can be clearly delimited. This analysis indicated that GES elicited changes in the expression of genes associated with 515 unique GO biological processes and 230 unique GO molecular functions. The clusters associated with the unique GO biological process and molecular functions are shown in the Supplementary Tables S2a and b, respectively. The results of the clustering analysis for the GO biological process and molecular function, where the data of all the genes that were affected by GES exposure, at the different doses and times, had been included without any restriction on *P* value and fold change. Using only the *P* value from the Fisher's exact test to estimate the statistical significance of the clustered annotated genes whose expression was modified by GES, we determined that there was a specific enrichment of GO annotation terms (with the highest number of genes associated with each term) at the 2 highest doses of GES evaluated. For example, the cluster for the GO term "regulation of transcription" includes 1538 unique genes whose expression changed by exposure to GES, with the highest degree of significance found when the cells were exposed to 1 μM and 10 μM GES and mostly within the first 8 h of exposure. A very similar pattern was observed with the GO term "regulation of

TABLE 3. Selected Gene Expression Changes Induced in Ishikawa Cells by Exposure to 100 nM GES

NCBI RefSeq	Gene name	Average fold change[a]			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
NM_001099691	transforming growth factor, alpha	9.0	5.3	12.3	TGFA	activation of MAPK activity
AW014518	ESTs	4.8	3.1	9.8	—	—
NM_022783	DEP domain containing 6	5.1	2.5	5.8	DEPDC6	negative regulation of protein kinase activity
NM_001632	alkaline phosphatase, placental	1.4	2.9	5.5	ALPP	metabolic process
XR_108881	hypothetical LOC100506966	4.8	2.3	4.9	LOC100506966	—
NM_018043	anoctamin 1, calcium activated chloride channel	2.0	1.8	4.2	ANO1	transport
NM_019089	hairy and enhancer of split 2 (Drosophila)	3.2	1.8	3.8	HES2	transcription
NM_031313	alkaline phosphatase, placental-like 2	1.2	1.4	3.2	ALPPL2	metabolic process
NM_014668	growth regulation by estrogen in breast cancer 1	1.8	2.2	3.1	GREB1	—
NM_001099691	transforming growth factor, alpha	2.8	2.0	3.0	TGFA	activation of MAPK activity
NM_004058	calcyphosine	1.1	1.5	2.7	CAPS	intracellular signaling pathway
NM_000565	interleukin 6 receptor	1.0	1.4	2.4	IL6R	hepatic immune response
NM_016518	pipecolic acid oxidase	1.5	1.6	2.4	PIPOX	tetrahydrofolate metabolic process
NM_001099691	transforming growth factor, alpha	2.3	1.4	2.3	TGFA	activation of MAPK activity
NM_000067	carbonic anhydrase II	1.6	1.6	2.2	CA2	kidney development
T68858	ESTs	12.7	1.8	2.2	—	—
NM_031475	espin	1.4	1.8	2.1	ESPN	sensory perception of sound
XR_111050	hypothetical LOC100509635	1.0	1.2	2.0	LOC100509635	—
NM_004740	myosin XVIIIA	1.7	1.6	2.0	MYO18A	DNA metabolic process
NM_001218	carbonic anhydrase XII	1.8	1.4	2.0	CA12	one-carbon metabolic process
NM_001964	early growth response 1	1.7	1.5	1.9	EGR1	regulation of transcription
AI198829	ESTs	1.7	1.3	1.9	—	—
NM_000282	propionyl CoA carboxylase, alpha polypeptide	1.0	1.4	1.9	PCCA	metabolic process
NM_004753	dehydrogenase/reductase (SDR family) member 3	2.4	1.4	1.8	DHRS3	visual perception
NM_014550	caspase recruitment domain family, member 10	1.6	1.5	1.7	CARD10	protein complex assembly
NM_001759	cyclin D2	1.6	1.4	1.7	CCND2	ovarian follicle development
NM_002894	retinoblastoma binding protein 8	1.2	1.2	1.7	RBBP8	cell cycle checkpoint
NM_001128602	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	2.5	1.7	1.5	RASGRP1	signal transduction
NM_015516	tsukushi small leucine rich proteoglycan homolog (Xenopus laevis)	1.6	1.4	1.5	TSKU	protein binding
NM_002467	v-myc myelocytomatosis viral oncogene homolog (avian)	1.6	1.6	1.4	MYC	regulation of transcription
NM_002211	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2.2	1.0	1.2	ITGB1	G1/S transition of mitotic cell cycle
NM_003489	nuclear receptor interacting protein 1	1.6	1.2	1.2	NRIP1	negative regulation of transcription from RNA polymerase II promoter
NM_000291	Phosphoglycerate kinase 1	−1.3	−1.8	1.2	PGK1	glycolysis
NM_004961	gamma-aminobutyric acid (GABA) A receptor, epsilon	−2.4	−1.3	1.2	GABRE	transport
NM_012153	ets homologous factor	1.8	1.1	1.1	EHF	transcription
XR_109165	similar to FLJ16518 protein	−1.5	1.1	1.1	LOC440288	—
AK024158	ESTs	1.1	−3.3	1.0	—	—
NM_001008397	glutathione peroxidase 8 (putative)	−1.5	1.0	−1.1	GPX8	response to oxidative stress
NM_003388	CAP-GLY domain containing linker protein 2	−2.2	1.6	−1.2	CLIP2	protein binding

(continued)

TABLE 3. Continued

NCBI RefSeq	Gene name	Average fold change[a]			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
NM_005529	heparan sulfate proteoglycan 2	−2.3	1.1	−1.4	HSPG2	angiogenesis
NM_021255	pellino homolog 2 (Drosophila)	−1.3	−1.3	−1.5	PELI2	protein binding
NM_000204	complement factor I	1.1	−1.2	−1.5	CFI	proteolysis
NM_021202	tumor protein p53 inducible nuclear protein 2	−1.2	−1.1	−1.5	TP53INP2	protein binding
NM_015419	matrix-remodeling associated 5	−1.2	−1.3	−1.5	MXRA5	protein binding
NM_001130992	retinol binding protein 1, cellular	−1.1	−1.1	−1.5	RBP1	vitamin A metabolic process
NM_000366	tropomyosin 1 (alpha)	−1.2	1.1	−1.6	TPM1	in utero embryonic development
NM_001135733	tumor protein p53 inducible nuclear protein 1	−1.1	−1.2	−1.6	TP53INP1	apoptosis
NM_001168319	endothelin 1	−1.2	−1.2	−1.6	EDN1	skeletal system development
NM_000141	fibroblast growth factor receptor 2	−1.1	−1.3	−1.6	FGFR2	angiogenesis
NM_004093	ephrin-B2	−1.3	−1.2	−1.6	EFNB2	lymph vessel development
NM_001336	cathepsin Z	−1.0	1.1	−1.8	CTSZ	proteolysis
NM_001130845	B-cell CLL/lymphoma 6	−1.2	−1.6	−1.8	BCL6	protein import into nucleus, translocation
NM_007173	Protease, serine, 23	−1.3	−1.1	−1.8	PRSS23	proteolysis
NR_023389	chromosome 9 open reading frame 130	1.0	−1.2	−1.8	C9orf130	—
NM_001163278	odz, odd Oz/ten-m homolog 1(Drosophila)	−1.1	−1.2	−1.9	ODZ1	immune response

[a]Gene expression of listed genes was significantly changed at least at one time point.

nucleobase, nucleoside, nucleotide and nucleic acid metabolism," which at 8 h (and at 10 μM GES) included 1577 unique genes. The biological processes that were particularly associated with the response to the GES exposure were protein modification, macromolecule metabolism, cellular metabolism, biopolymer metabolism, primary metabolism, cell proliferation, cell differentiation, cell migration, cell communication, cell cycle, and cell organization and biogenesis. While the molecular functions that were strongly associated with the GES-induced response were protein binding activity, transcription factor binding, nucleotide binding, transcription regulator activity, RNA binding activity, ligase activity, and catalytic activity among others.

The genes responsive to GES exposure, at the different doses and times, were also mapped against a set of 202 canonical pathway maps from the Kyoto Encyclopedia of Genes and Genomes. These pathways represent current knowledge on the molecular interaction and reaction networks for metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, and some human diseases (http://www.genome.jp/kegg/). From the metabolic pathways mapped, 136 contained a considerable number of genes whose expression was modified by GES exposure. The metabolic pathways that were particularly enriched with these genes include signal transduction, cell communication, cell growth and death, cell cycle, and translation, among others. In most of the cases, the expression of the majority of genes that mapped to the indicated pathways was affected mostly at relatively high concentrations of GES exposure (Supplementary Table S2c).

Comparison of the genomic response of Ishikawa cells to GES and EE exposure

As indicated, GES has multiple biological functions; however, only some are dependent on its ability to interact with the estrogen system, while others are completely independent of it. In order to better define the estrogenic activities of GES, we compared the genomic response of Ishikawa cells to the exposure to EE, a potent ER agonist (Naciff *et al.*, 2009) under a similar experimental protocol to the one used with GES, and importantly at equivalent concentrations (1 and 10 μM, respectively). Since these 2 sets of data were obtained under similar experimental conditions, and EE has a strong estrogenic activity (and presumably the only biological activity that it has), these data could be used to define the transcriptional response of these cells elicited just by the common estrogenic activity of these 2 chemicals. This comparison also could allow for the identification of the biological activity elicited by GES that is not associated with the estrogenic response; however, these results are out of the scope of this article and are not presented here. Since the most robust response to GES was observed primarily at the highest dose tested, in this comparison we only included the responses of the uterine cell line to 10 μM GES and 1 μM EE, which are equipotent doses, at 8, 24, and 48 h. In this exercise, the genes included were those that showed a robust response to each chemical, based on P value ($P \leq 0.0001$) and fold change (1.2 up- or down-regulated) at each indicated time point. This comparison showed that the expression of 554 unique genes was affected by GES and EE at any given time. For a better comparison of the data, the schematic comparison of the response of the most significantly changed genes, at the different doses and time points, is shown in Supplementary Figure S2. The change in the expression of only 337 unique genes occurred in the same direction, although with a different magnitude for either chemical. A selected group of these genes is listed in Table 5. Many of these genes affected in a similar way (up- or down-regulated) by both GES and EE encode proteins associated with multiple biological functions, such as FOS, PTEN, TGFA, EGR1, FGFR2, IGFBP3, and SOX4, among others. The response of some of these genes was more robust to either GES or EE exposure, based upon the magnitude of the change of their expression.

TABLE 4. Selected Gene Expression Changes Induced in Ishikawa Cells by Exposure to 10 μM GES

NCBI RefSeq	Gene name	Average fold change[a]			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
NM_018440	phosphoprotein associated with glycosphingolipid microdomains 1	11.2	24.0	59.1	PAG1	signal transduction
NM_001195053	DNA-damage-inducible transcript 3	5.8	20.3	38.3	DDIT3	response to amphetamine
NM_014398	lysosomal-associated membrane protein 3	2.0	7.9	30.1	LAMP3	cell proliferation
NM_031479	inhibin, beta E	2.2	11.8	20.3	INHBE	growth
NM_001657	amphiregulin	2.9	6.6	17.2	AREG	epidermal growth factor receptor signaling pathway
NM_001030287	activating transcription factor 3	4.1	9.9	14.1	ATF3	gluconeogenesis
NM_004864	growth differentiation factor 15	2.6	18.0	11.3	GDF15	signal transduction
NM_001142776	ChaC, cation transport regulator homolog 1 (E. coli)	5.4	9.0	11.2	CHAC1	apoptosis
NM_012328	DnaJ (Hsp40) homolog, subfamily B, member 9	2.8	5.7	10.7	DNAJB9	protein folding
NM_001924	growth arrest and DNA-damage-inducible, alpha	2.8	6.1	8.2	GADD45A	regulation of cyclin-dependent protein kinase activity
NM_001161572	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	2.3	3.9	7.7	MAFF	in utero embryonic development
NM_001142853	hairy and enhancer of split 6 (Drosophila)	2.9	6.4	6.9	HES6	transcription
NM_032772	zinc finger protein 503	1.7	2.7	6.5	ZNF503	transcription
NM_001040152	paternally expressed 10	2.7	3.6	6.3	PEG10	apoptosis
NM_032523	oxysterol binding protein-like 6	1.5	3.9	6.0	OSBPL6	transport
NM_021158	tribbles homolog 3 (Drosophila)	1.5	4.9	6.0	TRIB3	transcription
NM_001136528	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.9	3.9	5.9	SERPINE2	multicellular organismal development
NM_003842	tumor necrosis factor receptor superfamily, member 10b	1.3	4.2	5.7	TNFRSF10B	apoptosis
NM_019891	ERO1-like beta (S. cerevisiae)	2.3	3.9	5.5	ERO1LB	protein folding
NM_001018073	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	3.3	8.4	5.3	PCK2	gluconeogenesis
NR_028272	nuclear paraspeckle assembly transcript 1 (non-protein coding)	3.6	6.1	5.3	NEAT1	protein binding
NM_004582	Rab geranylgeranyltransferase, beta subunit	2.7	4.5	5.2	RABGGTB	protein modification process
NM_002357	MAX dimerization protein 1	3.0	3.7	5.2	MXD1	transcription
NM_001999	fibrillin 2	2.1	4.3	5.0	FBN2	anatomical structure morphogenesis
NM_001008564	nucleoporin like 1	2.5	3.0	4.9	NUPL1	transport
NM_001102559	phosphatidic acid phosphatase type 2 domain containing 1B	1.8	4.0	4.8	PPAPDC1B	catalytic activity
NM_003921	B-cell CLL/lymphoma 10	2.6	4.4	4.8	BCL10	neural tube closure
NM_178012	tubulin, beta 2B	2.4	4.1	4.8	TUBB2B	neuron migration
NM_001037535	sex comb on midleg-like 1 (Drosophila)	2.8	2.8	4.7	SCML1	transcription
NM_001193493	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.6	3.9	4.7	SLC1A4	transport
NM_001178075	asparagine synthetase (glutamine-hydrolyzing)	2.4	5.3	4.6	ASNS	liver development
NM_031459	sestrin 2	3.4	4.3	4.5	SESN2	cell cycle arrest
NM_004417	dual specificity phosphatase 1	1.8	2.5	4.5	DUSP1	protein dephosphorylation
NM_017593	BMP2 inducible kinase	1.7	3.2	4.4	BMP2K	protein phosphorylation
NM_001098402	zinc finger protein 295	1.5	3.0	4.3	ZNF295	transcription
NM_001135099	transmembrane protease, serine 2	−1.8	−3.0	−5.9	TMPRSS2	proteolysis
NM_005733	kinesin family member 20A	−2.1	−3.8	−6.0	KIF20A	transport
NM_004932	cadherin 6, type 2, K-cadherin (fetal kidney)	−1.4	−3.3	−6.0	CDH6	cell adhesion
NM_002526	5'-nucleotidase, ecto (CD73)	−2.3	−2.7	−6.8	NT5E	purine nucleotide biosynthetic process
NM_001447	FAT tumor suppressor homolog 2 (Drosophila)	−2.8	−2.8	−7.1	FAT2	cell adhesion
NM_018658	potassium inwardly rectifying channel, subfamily J, member 16	−2.4	−7.2	−7.5	KCNJ16	transport
NM_004625	wingless-type MMTV integration site family, member 7A	−1.7	−2.7	−8.1	WNT7A	embryonic axis specification
NM_001168319	endothelin 1	−4.1	−6.7	−8.3	EDN1	skeletal system development
NM_005030	polo-like kinase 1	−3.3	−5.7	−8.7	PLK1	mitotic prometaphase
NM_004962	growth differentiation factor 10	−2.7	−6.9	−8.8	GDF10	skeletal system development
NM_001001395	LIM domain only 3 (rhombotin-like 2)	−2.8	−6.6	−8.9	LMO3	transcription
NM_005472		−6.6	−7.8	−9.4	KCNE3	transport

(continued)

TABLE 4. Continued

NCBI RefSeq	Gene name	Average fold change[a]			Gene acronym	Biological process GO term
		8 h	24 h	48 h		
	potassium voltage-gated channel, Isk-related family, member 3					
NM_001079874	vav 3 guanine nucleotide exchange factor	−3.3	−5.4	−9.8	VAV3	angiogenesis
NM_018952	homeobox B6	−2.1	−3.4	−9.9	HOXB6	transcription
NM_178013	proline rich membrane anchor 1	−2.6	−5.8	−10.1	PRIMA1	neurotransmitter catabolic process
NM_001164749	neuronal PAS domain protein 3	−2.7	−6.6	−10.2	NPAS3	transcription
NM_000104	cytochrome P450, family 1, subfamily B, polypeptide 1	−4.9	−10.7	−10.3	CYP1B1	cellular aromatic compound metabolic process
NM_001164464	tripartite motif-containing 43	−2.5	−4.1	−10.6	TRIM43	protein binding
NM_001548	interferon-induced protein with tetratricopeptide repeats 1	−1.8	−3.6	−10.9	IFIT1	protein binding
NM_012293	peroxidasin homolog (Drosophila)	−3.1	−3.3	−11.9	PXDN	immune response
NM_032487	actin related protein M1	−2.6	−11.7	−13.3	ARPM1	protein binding
NM_014722	family with sequence similarity 65, member B	−7.4	−9.8	−14.0	FAM65B	multicellular organismal development
NM_005345	heat shock 70kDa protein 1A	−8.1	−30.7	−15.4	HSPA1A	mRNA catabolic process
NM_020805	kelch-like 14 (Drosophila)	−3.3	−6.6	−15.7	KLHL14	protein binding
NM_001956	endothelin 2	−2.7	−9.9	−18.0	EDN2	prostaglandin biosynthetic process
NM_005494	DnaJ (Hsp40) homolog, subfamily B, member 6	−1.9	−11.9	−18.1	DNAJB6	protein folding
NM_001040002	mesenchyme homeobox 1	−3.0	−13.4	−22.5	MEOX1	somite specification
NM_001463	frizzled-related protein	−2.5	−22.4	−26.9	FRZB	skeletal system development
AK055877	hypothetical protein LOC151438	−2.7	−15.9	−32.9	LOC151438	—
NM_007350	pleckstrin homology-like domain, family A, member 1	−7.2	−18.4	−34.8	PHLDA1	apoptosis
NM_006940	SRY (sex determining region Y)-box 5	−2.7	−20.9	−62.8	SOX5	in utero embryonic development

[a]Gene expression of listed genes was significantly changed at all time points.

Nonetheless, the change in the expression of these genes is brought about by the common estrogenic activity of the 2 chemicals.

In vivo versus in vitro data comparison

In order to better assess the value of transcription profiling to screen chemicals for potential estrogenic activity in our *in vitro* system, we compared the transcriptional response of Ishikawa cells to GES exposure with the response of the juvenile rat uterus to EE exposure (Naciff *et al.*, 2007), at the same time points and equipotent doses. It is important to note that this was not just an *in vitro* versus *in vivo* comparison but also an interspecies comparison (human vs rat). Thus, this approach allows the assessment of the value of using the transcriptional response to chemical exposure in interspecies extrapolation too. The main weakness of this comparison resides in the fact that our *in vitro* approach only evaluates the response of a single cell type, representing part of the endometrial response, while the uterus' response is the integrated response of this organ, which encompasses multiple cell types. For this comparison, only the genomic response of Ishikawa cells elicited by the highest dose of GES tested (10 μM) was used and compared to the response of the juvenile rat uterus after exposure to a single dose of EE (10 μg/kg), at equivalent time points (8, 24, and 48 h). The probe sets representing ESTs whose expression was significantly regulated in both *in vivo* as well as *in vitro* by EE (rat) or GES (Ishikawa cells) exposure, respectively, were not included for this analysis, since there was no annotation associated with them which makes the identification of the corresponding homologs between these 2 species difficult. Further, in this analysis, the selection criterion for any given gene was that its response was statistically significant ($P \leq 0.0001$, *t* test). This comparison had shown that the change in the expression of 576 unique genes was identical in the direction of the change, although the magnitude of the change of some genes was different in Ishikawa cells versus the rat uterus. Of these 576 common genes, 574 of them showed a change in their expression level of at least 1.5-fold (up or down-regulated) for at least one time point *in vivo* and *in vitro*, and this change was significant in both systems ($P \leq 0.0001$, *t* test). A representative list of these genes is presented in Table 6. In the Ishikawa cells, the genes that showed the most robust response to GES exposure included NGFR, SPKIB, CUZD1, SARS, ATP1A1, ASNS, SDKN1A, TCEA1, FOS and KLF4 (up-regulated); and ABCG5, METTL7A, KCNK2, NT5E, STS, IGFBP3, EDN1, ALDH1A1, STC1, FGFR2, and SOX4 (down-regulated). While the genes that were the most responsive to EE exposure in the juvenile rat uterus included CUZD1, FOS, NGFR, HYOU1, EHHADH, PKIB, INSIG1, ASNS, KLF4, and CDKN1A (up-regulated); and ATP1A2, AMIGO2, LEPR, METTL7A, MAP2K6, ALDH1A1, SLC15A2, IGFBP3, SLC15A2, and SOX4 (down-regulated). Using the annotation available for these genes responsive to GES and EE, it was determined that the response of Ishikawa cells as well as the juvenile rat uterus involved similar cellular processes, including regulation of transcription, signal transduction, cell proliferation, cell growth, cell differentiation and tissue remodeling, transport, and metabolism.

To further define the identity of the genes with the most robust response to estrogenic exposure and potentially those which are biologically most relevant, the genes that responded in the same direction to both GES and EE in Ishikawa cells, and the homologs that respond to EE in the juvenile rat uterus (Naciff *et al.*, 2007, 2009) were identified. For this analysis, only the probe sets that represented unique and annotated genes

TABLE 5. Selected Gene Expression Changes Induced by GES and EE in Ishikawa Cells

Probe ID	Gene acronym	GES (10 μM)			EE[a] (1 μM)			Biological process GO term
		8 h	24 h	48 h	8 h	24 h	48 h	
36711_at	MAFF	2.3	3.9	7.7	3.8	4.6	4.7	in utero embryonic development
238430_x_at	SLFN5	3.4	4.1	6.9	1.8	1.4	4.3	cell differentiation
229228_at	CREB5	−1.1	1.9	5.5	1.3	4.0	3.4	transcription
212094_at	PEG10	2.1	3.2	4.7	1.5	2.0	3.1	apoptosis
226982_at	ELL2	1.7	2.1	4.0	1.9	3.5	3.1	transcription
209189_at	FOS	1.3	3.7	3.8	1.8	2.2	2.5	conditioned taste aversion
209457_at	DUSP5	1.5	2.2	3.5	1.2	1.0	2.3	protein dephosphorylation
228181_at	SLC30A1	3.1	4.0	3.2	1.5	2.1	2.1	in utero embryonic development
225056_at	SIPA1L2	1.1	2.4	3.2	1.2	1.5	2.0	regulation signal transduction
204286_s_at	PMAIP1	2.5	3.0	2.8	1.1	1.1	1.9	protein binding
238694_at	DGKE	1.1	1.4	2.5	1.9	2.2	1.9	diacylglycerol kinase activity
205590_at	RASGRP1	3.3	2.2	2.4	1.4	1.7	1.8	signal transduction
226771_at	ATP8B2	−1.5	1.8	2.2	1.5	1.6	1.8	ATP biosynthetic process
200953_s_at	CCND2	1.5	2.1	2.2	1.6	1.7	1.8	ovarian follicle development
226419_s_at	FLJ44342	1.1	1.7	2.1	−1.0	1.3	1.7	—
205016_at	TGFA	1.8	1.7	2.0	1.6	1.9	1.7	activation of MAPK activity
227856_at	C4orf32	1.8	1.8	2.0	1.4	1.8	1.7	—
201694_s_at	EGR1	−1.7	−1.0	1.9	1.1	1.2	1.7	regulation of transcription
225796_at	PXK	1.4	1.8	1.9	1.2	1.3	1.7	protein phosphorylation
227425_at	REPS2	1.1	1.8	1.9	1.5	2.0	1.7	protein complex assembly
203344_s_at	RBBP8	1.0	1.4	1.9	−1.4	1.2	1.7	cell cycle checkpoint
1554997_a_at	PTGS2	1.5	2.0	1.8	1.0	1.4	1.6	prostaglandin biosynthetic process
230917_at	ESTs	1.9	1.9	1.8	1.1	1.4	1.6	—
226283_at	POC1B	1.4	1.8	1.7	3.9	2.4	1.6	cell projection organization
202923_s_at	GCLC	1.3	1.7	1.6	2.6	2.6	1.6	cysteine metabolic process
229656_s_at	EML6	−1.7	−1.2	1.6	1.9	2.0	1.5	
213524_s_at	G0S2	1.5	2.8	1.6	1.2	1.4	1.5	cell cycle
204053_x_at	PTEN	1.2	1.3	1.6	1.2	1.3	1.5	protein phosphatase activity
225972_at	TMEM64	1.1	1.3	1.5	2.8	3.2	1.5	—
200849_s_at	AHCYL1	1.1	1.7	1.5	1.0	1.3	1.5	one-carbon metabolic process
212593_s_at	PDCD4	1.1	2.5	1.4	1.2	1.4	1.5	apoptosis
217143_s_at	TRD@	−1.6	−2.2	−5.1	−1.2	−1.4	−2.0	immune response
220994_s_at	STXBP6	−1.4	−2.6	−5.4	−1.5	−2.0	−2.0	vesicle-mediated transport
206089_at	NELL1	−2.1	−2.5	−5.4	−1.6	−2.2	−2.0	induction of apoptosis
209596_at	MXRA5	−1.3	−3.6	−5.5	−1.3	−1.7	−2.0	protein binding
204627_s_at	ITGB3	1.1	−1.7	−5.6	−1.2	−1.2	−2.0	receptor activity
228949_at	WLS	−1.2	−3.1	−5.8	−2.5	−2.9	−2.1	signal transducer activity
244353_s_at	SLC2A12	−2.3	−2.8	−5.9	−1.2	−1.6	−2.1	transport
213456_at	SOSTDC1	−2.4	−4.8	−5.9	−1.4	−1.9	−2.1	pattern specification process
211401_s_at	FGFR2	−1.8	−3.6	−6.0	−1.5	−1.9	−2.1	angiogenesis
1555724_s_at	TAGLN	1.1	−1.2	−6.0	−1.6	−1.9	−2.1	muscle organ development
232656_at	ESTs	−1.1	−2.0	−6.4	−1.3	−2.0	−2.2	—
226553_at	TMPRSS2	−2.6	−4.6	−6.7	−1.1	−1.5	−2.2	proteolysis
210165_at	DNASE1	1.5	−1.1	−7.2	−1.5	−1.5	−2.2	DNA catabolic process
218736_s_at	PALMD	−2.4	−4.0	−7.4	−2.4	−3.3	−2.2	regulation of cell shape
218960_at	TMPRSS4	−1.3	−1.8	−7.6	−1.2	−1.9	−2.2	proteolysis
222802_at	EDN1	−4.1	−6.9	−7.8	−1.2	−1.7	−2.2	skeletal system development
228104_at	PLXNA4	−1.5	−4.6	−8.0	−1.5	−2.4	−2.3	signal transduction
212143_s_at	IGFBP3	−1.1	−2.7	−8.4	−1.4	−2.2	−2.3	regulation of cell growth
214803_at	CDH6	−1.3	−4.6	−8.5	−1.5	−1.9	−2.3	cell adhesion
204424_s_at	LMO3	−2.8	−6.6	−8.9	−1.3	−1.4	−2.5	transcription
205728_at	ODZ1	−1.1	−5.3	−8.9	−1.4	−1.9	−2.5	immune response
208084_at	ITGB6	1.1	−2.1	−9.0	−1.5	−2.0	−2.5	inflammatory response
224221_s_at	VAV3	−3.3	−5.4	−9.8	−1.6	−2.2	−2.6	angiogenesis
230087_at	PRIMA1	−2.6	−5.8	−10.1	−1.5	−2.1	−2.8	protein binding
235092_at	ESTs	−2.2	−5.9	−10.6	−1.1	−2.5	−2.9	—
216191_s_at	TRDV3	−3.2	−4.2	−10.9	−1.6	−2.2	−3.0	receptor activity
236098_at	C17orf110	−1.3	−4.7	−11.4	−1.5	−1.4	−3.1	—
212013_at	PXDN	−3.1	−3.3	−11.9	−1.2	−1.6	−3.2	immune response
204777_s_at	MAL	−1.1	−4.6	−14.0	−2.2	−2.4	−3.3	membrane raft polarization

(continued)

TABLE 5. Continued

Probe ID	Gene acronym	GES (10 μM)			EE[a] (1 μM)			Biological process GO term
		8 h	24 h	48 h	8 h	24 h	48 h	
200799_at	HSPA1A	−8.1	−30.7	−15.4	−1.6	−2.9	−3.4	mRNA catabolic process
205680_at	MMP10	−1.3	−3.9	−15.7	−1.4	−2.2	−3.4	proteolysis
228377_at	KLHL14	−3.3	−6.6	−15.7	−1.4	−1.9	−3.8	protein binding
206758_at	EDN2	−2.7	−9.9	−18.0	−1.6	−2.7	−3.8	receptor binding
236163_at	LIX1	−1.2	−3.5	−18.6	−1.1	−1.1	−3.9	—
205619_s_at	MEOX1	−3.0	−13.4	−22.5	−2.7	−5.3	−4.0	somite specification
203698_s_at	FRZB	−2.5	−22.4	−26.9	−1.5	−1.8	−4.0	skeletal system development
218963_s_at	KRT23	−1.3	−6.1	−29.4	−1.2	−3.0	−4.6	structural molecule activity
225842_at	PHLDA1	−7.2	−18.4	−34.8	−2.4	−2.3	−4.8	apoptosis

[a]The reliability of the microarray data from these samples has been independently corroborated by QRT-PCR.

were included. The selection criteria also encompassed the unique genes responsive to the highest dose tested of GES and EE in Ishikawa cells (10 and 1 μM, respectively), the significance of the change induced ($P \leq 0.0001$, *t* test; treated vs appropriate time-matched control) and a fold change (up- or down-regulated) by at least 20%, at any given time point (8, 24, or 48 h). Under these criteria, 42 unique genes were identified as being regulated by GES and EE in Ishikawa cells and by EE in the juvenile rat uterus, these genes are listed in Table 7. Among these genes are some that have been previously described as responsive to estrogen exposure in various biological systems, such as FGFR2, EGR1, KLF4, MYC, FOS, IGFBP3, NDRG2, and SOX4 among others. The concordance of the transcriptional changes elicited by estrogen (EE) exposure of the juvenile rat uterus with the changes elicited by GES and EE in the Ishikawa cells was also shown by the timing of the response of individual genes. For example, one of the early responsive genes was MYC, which is upregulated by estrogen. In agreement with this early response, the expression of MYC was increased by both GES and EE in Ishikawa cells, as well as by EE in the juvenile rat uterus, with a peak in such response within the first 8 h of exposure in the 2 systems. A similar pattern was followed by other early response genes, including FOS and EGR1, further supporting the validity of the Ishikawa cells as a biologically relevant model to screen chemicals for estrogenic activity. This notion was reinforced by the identification of genes previously known to be regulated by estrogen, such as KLF4, FOS, MYC, EGR1, IGFBP3, and SOX4 among others, within this common set of genes whose expression was affected by GES and EE *in vitro* and EE *in vivo* (Table 7).

Quantitative RT-PCR

The reliability of the microarray data was independently corroborated by QRT-PCR analysis of selected genes in samples from the same batch of RNA, from each biological replica, used for microarray analysis, at the different times, and GES doses evaluated. The group of genes evaluated by QRT-PCR included up- and down-regulated genes. The relative expression level of CKB, EGR1, KLF4, IGFBP3, and TAGLN was similar to that determined by microarray analysis (Supplementary Table S3). The expression changes in the indicated genes induced by GES exposure, as measured by microarray analysis or QRT-PCR, showed the same trend with time and dose responsiveness. These data clearly support the reliability of the gene expression changes identified by microarray analysis in Ishikawa cells exposed to GES.

The reliability of the microarray data from the rat uterine-derived samples, as well as from Ishikawa cells exposed to EE, has also been independently corroborated by QRT-PCR analysis of selected genes (Naciff *et al.*, 2007, 2009).

DISCUSSION

In this study, we have assessed the transcriptional response of a human endometrial cancer cell line, Ishikawa cells, to physiologically relevant concentrations of GES, and thus these data can be used to better understand the biological activity of GES *per se*. The concentrations of this phytoestrogen here evaluated fall in the range of concentrations present in systemic circulation of humans consuming a diet containing soy and/or soy derived-products and other legumes. The concentrations of total GES (free + conjugates) in serum from humans which diet includes this phytoestrogen range from <1 to ~5 μM (0.27–1.35 μg/ml) (Klein and King, 2007; Setchell *et al.*, 1997). Moreover, we have compared the transcriptional response of these cells to GES to the one elicited by a pure estrogen agonist, EE, in these cells as well as in an *in vivo* model (the uterus of juvenile rats) to better define the identity of the gene expression changes elicited by the estrogenic activity of GES. Our results indicate that the Ishikawa cells are sensitive to phytoestrogen exposure and modify their transcriptional profile in a dose and time-sensitive manner. Our results do not substantiate a biphasic dose-response relationship in the transcriptional changes elicited in the Ishikawa cells by GES exposure. It has been proposed that some endocrine disrupters with estrogenic activity show a nonmonotonic (biphasic) dose response. For example, Shioda *et al.* (2013) evaluated the transcriptional response of MCF-7 cells after 48 h of exposure to various chemicals with estrogenic activity and observed nonmonotonic responses (biphasic) for some of these chemicals. Although the GES dose range covered in the present study overlaps the dose range evaluated by Shioda *et al.* (50 nM to 16 μM), our results do not indicate a biphasic response, even when we only compare the transcriptional data obtained in the Ishikawa cells at 48 h (Fig. 1). This discrepancy could be the result of the differences on the statistical stringency we have used in our analysis ($P \leq 0.0001$, *t* test), the cell type evaluated, or other reasons.

Although the majority of gene expression changes induced in Ishikawa cells were elicited by the highest dose of GES evaluated (10 μM), the limited genomic response of these cells exposed to lower doses is indicative of the specificity as well as of the monotonicity of such response. The data obtained in the Ishikawa cells indicate that low doses of GES are able to elicit robust changes at the transcriptome level of a very select number of genes. From our previous work with these cells (Naciff *et al.*,

TABLE 6. Selected Gene Expression Changes Induced by GES in Ishikawa CELLS and EE in the Rat Uterus

Probe ID	Gene acronym	GES (10 μM)			EE[a] (10 μM)			Biological process GO term
		8 h	24 h	48 h	8 h	24 h	48 h	
205858_AT	NGFR	1.3	19.3	23.7	11.5	3.4	−1.4	signal transducer activity; apoptosis
231120_X_AT	PKIB	2.0	8.4	16.8	3.5	1.5	−1.1	regulation of protein kinase activity
220275_AT	CUZD1	1.1	2.3	10.8	4.6	3.5	44.6	cell adhesion
231894_AT	SARS	1.7	4.4	6.1	2.0	1.5	1.3	gene expression
236623_AT	ATP1A1	1.8	11.2	5.8	2.4	1.4	1.1	protein binding
205047_S_AT	ASNS	2.4	5.3	4.6	3.2	1.6	1.1	asparagine biosynthetic process
202284_S_AT	CDKN1A	1.5	3.6	4.3	3.1	2.1	1.7	regulation of progression through cell cycle
236375_AT	TCEA1	1.6	3.3	3.9	1.5	1.3	−1.1	gene expression
209189_AT	FOS	1.3	3.7	3.8	16.1	2.8	3.1	regulation of transcription
202234_S_AT	SLC16A1	1.7	2.6	3.8	2.1	1.6	1.0	organic anion transport
239451_AT	HSP90B1	2.3	2.1	3.7	2.0	1.9	−1.3	protein binding
238886_AT	TMED10	1.9	2.7	3.3	1.6	1.5	1.2	ER to Golgi vesicle-mediated transport
202655_AT	ARMET	1.2	2.6	3.3	2.7	2.8	−1.1	growth factor activity
221841_S_AT	KLF4	2.2	2.5	3.1	3.2	2.9	1.1	regulation of transcription, DNA-dependent
224953_AT	YIPF5	1.9	2.7	3.0	1.5	1.7	1.0	ER to Golgi vesicle-mediated transport
201096_S_AT	ARF4	1.0	2.0	2.8	1.6	1.7	1.1	ER to Golgi vesicle-mediated transport
200969_AT	SERP1	1.3	2.1	2.8	2.2	2.0	1.2	protein amino acid glycosylation
208499_S_AT	DNAJC3	1.2	2.2	2.7	3.0	3.4	1.3	protein folding
200629_AT	WARS	1.2	1.9	2.7	2.0	1.7	−1.0	tRNA aminoacylation for protein translation
242725_AT	KDELR2	1.6	2.8	2.7	1.5	1.9	1.1	receptor activity
235013_AT	SLC31A1	1.1	1.9	2.7	1.4	2.5	1.4	ion transport
201626_AT	INSIG1	2.2	2.6	2.5	3.2	3.1	1.1	lipid metabolic process
239251_AT	RTN4	1.8	1.4	2.5	1.4	1.9	1.4	angiogenesi
241775_AT	SCFD1	2.9	1.8	2.5	1.6	1.9	−1.1	protein binding
218615_S_AT	TMEM39A	1.3	2.0	2.5	1.9	1.5	−1.1	—
201000_AT	AARS	1.3	2.9	2.5	2.2	2.0	−1.0	protein translation
244700_AT	SEC61B	1.3	1.6	2.5	1.5	2.3	1.1	protein targeting
217869_AT	HSD17B12	1.3	1.7	2.4	1.8	1.8	1.1	steroid biosynthetic process
200825_S_AT	HYOU1	1.3	1.8	2.4	4.2	4.4	−1.1	protein folding
205222_AT	EHHADH	1.4	2.4	2.4	4.1	4.0	2.9	3-hydroxyacyl-CoA dehydrogenase activity
205968_AT	KCNS3	1.5	2.1	2.4	2.2	1.6	1.1	potassium ion transport
211593_S_AT	MAST2	1.2	2.3	2.2	2.0	1.4	1.1	protein amino acid phosphorylation
225435_AT	SSR1	1.3	1.8	2.2	2.4	3.0	−1.1	positive regulation of cell proliferation
224780_AT	RBM17	1.3	1.5	2.1	1.6	1.3	1.1	RNA splicing
222108_AT	AMIGO2	−2.1	−1.9	−2.9	−7.5	−2.7	−1.7	cell adhesion
202986_AT	ARNT2	−1.9	−2.7	−3.0	−2.0	−1.8	1.1	DNA binding; response to hypoxia
203845_AT	PCAF	−1.5	−2.3	−3.0	−1.7	−1.2	−1.1	transcription cofactor activity
235518_AT	SLC8A1	−1.2	−2.3	−3.0	−2.9	−1.6	−1.5	ion transport
212494_AT	TENC1	−1.8	−2.1	−3.0	−1.9	−1.8	−1.8	intracellular signaling cascade
205698_S_AT	MAP2K6	−1.3	−2.1	−3.1	−3.9	−1.3	−1.1	activation of MAPK activity
204115_AT	GNG11	−1.1	−2.0	−3.5	−2.8	−1.4	−1.1	signal transduction
203157_S_AT	GLS	−1.1	−2.5	−3.6	−2.1	−2.0	−1.4	glutamine metabolic process
1558460_AT	ABCC5	1.0	−1.6	−3.6	−1.8	−1.8	−1.1	ATP binding; transport
229441_AT	PRSS23	−1.4	−2.0	−3.7	−1.3	−2.3	−1.1	proteolysis
203296_S_AT	ATP1A2	−1.1	−2.9	−3.7	−15.6	−4.4	−1.3	potassium ion transport
241963_AT	ZNF704	−1.0	−1.5	−3.8	−2.6	−1.8	−1.4	zinc ion binding
209114_AT	TSPAN1	1.1	−1.3	−3.8	−1.9	−2.6	1.1	cell motility; cell proliferation
209867_S_AT	LPHN3	−4.5	−8.4	−3.8	−2.7	−2.0	−1.6	signal transduction
206117_AT	TPM1	1.0	−1.3	−3.9	1.1	−2.5	−1.5	cell motility
201035_S_AT	HADH	1.1	−1.8	−4.0	−2.4	−1.4	1.1	lipid metabolic process
201416_AT	SOX4	−1.4	−3.4	−4.1	−2.7	−2.5	1.0	regulation of transcription
211354_S_AT	LEPR	1.1	−1.4	−4.2	−4.6	−3.2	−1.1	signal transduction
214761_AT	ZNF423	−1.0	−2.9	−4.3	−1.9	−1.5	−1.3	nucleic acid binding
227529_S_AT	AKAP12	−1.0	−2.0	−4.3	−1.5	−1.8	−1.3	signal transduction
1569054_AT	SLC1A3	1.0	−2.5	−4.5	−2.8	−2.2	−1.5	transmembrane transporter activity
205710_AT	LRP2	1.0	−2.6	−4.9	−3.0	−3.2	−2.6	protein amino acid glycosylation
205316_AT	SLC15A2	−1.0	−2.2	−4.9	−3.1	−3.4	−2.1	transporter activit
203639_S_AT	FGFR2	−2.3	−3.3	−5.0	−2.5	−1.7	−1.2	cell growth; protein kinase activity
212032_S_AT	PTOV1	−1.3	−3.4	−5.2	−1.5	−1.2	−1.0	tRNA aminoacylation for protein translation
226847_AT	FST	−8.3	−8.0	−5.2	−2.6	−4.2	−2.5	regulation of transcription

(continued)

TABLE 6. Continued

Probe ID	Gene acronym	GES (10 μM)			EE[a] (10 μM)			Biological process GO term
		8 h	24 h	48 h	8 h	24 h	48 h	
206089_AT	NELL1	−2.1	−2.5	−5.4	−2.3	−1.4	1.1	cell adhesion; kinase activity
204596_S_AT	STC1	−1.8	−2.2	−5.8	−1.6	−2.0	−1.6	signal transduction
212224_AT	ALDH1A1	−1.0	−1.7	−7.9	−3.6	−2.5	1.1	aldehyde metabolic process
218995_S_AT	EDN1	−4.1	−6.7	−8.3	−1.2	−1.9	1.1	receptor binding
212143_S_AT	IGFBP3	−1.1	−2.7	−8.4	−3.1	−5.3	−1.5	regulation of cell growth
203767_S_AT	STS	1.1	−2.1	−8.8	−1.9	−1.8	−1.1	steryl-sulfatase activity
1553995_A_AT	NT5E	−1.6	−3.3	−9.3	−2.3	−2.7	−1.2	DNA metabolic process
210261_AT	KCNK2	−1.7	−3.0	−11.8	−3.1	−1.6	−1.3	ion transport
207761_S_AT	METTL7A	1.0	−3.3	−12.1	−4.1	−2.3	−1.0	methyltransferase activity
220383_AT	ABCG5	−1.0	−3.6	−12.3	−1.5	−2.7	−2.1	transport

[a]Average fold change on the expression of genes from the rat uterus from animals exposed to a single dose of 10 mg EE/kg, at the indicated times. The reliability of the microarray data from these samples has been independently corroborated by QRT-PCR.

2009, 2010), we know that this *in vitro* system is capable of generating a biological or pharmacological response to estrogens of various potencies. We have demonstrated that Ishikawa cells are capable of generating a robust genomic response after exposure to low doses of EE (1 pM, 100 pM, or 10 nM; Naciff *et al.*, 2009), it is reasonable to believe that this *in vitro* system has the biological capacity to respond to low doses of any estrogenic chemical. Thus, the limited genomic response elicited by low doses of GES in Ishikawa cells is not likely due to a lack the biological apparatus to produce such a response. Further, this response correlates with the response observed in other systems. For example, we determined that in cells exposed to 10 pM GES, the gene that showed the greatest up-regulation was AF086490, with an average 4.1-fold increase, while the gene showing the greatest down-regulation was AK097499, with a −2.4-fold change. Increase in the expression level of AF086490 has been observed in the human endometrium, particularly in the secretory phase (GEO; GDS2052/1561644_x_at/AF086490), while the expression of AK097499 also in the human endometrium is the lowest during the secretory phase (GEO; GDS2052/1562777_at/AK097499), when circulating 17β-estradiol (E2) levels are relatively high. These similarities between the Ishikawa cells' response to low dosed of GES and the human endometrium exposed to physiological levels of E2 strengthen the relevance of the Ishikawa cells as a model system to evaluate biologically relevant responses to chemicals with estrogenic activity. Further, the data from Konstantakopoulos *et al.* (2006), although limited, are in agreement with our results. These authors evaluated the response of the Ishikawa cells to a 2-h exposure period to GES at concentrations of 0.5, 5, 50, and 500 μM using a custom made cDNA microarray. The majority of gene expression changes were observed at the supraphysiological concentrations of 50 and 500 μM GES, and few were also observed with 5 μM GES. However, in cells exposed to 0.5 μM GES, Konstantakopoulos *et al.* determined that the expression of only 2 genes was modified (dual specificity phosphatase 1, upregulated; and TATA binding-protein-related factor, which was down-regulated). These authors determined that the cells exposed to 5 μM GES responded with changes in the expression of gene whose products are involved in multiple cellular pathways such as Ras signaling, MAPK, transcription, apoptosis, cell-cycle regulation, proliferation, cell migration and adhesion, antioxidation, and signal transduction. These expression changes are more in line with the gene expression changes that we have determined in the Ishikawa cells exposed to 10 μM GES for 8 or 24 h (Supplementary Tables S2a–c).

Recently, Becker *et al.* (2015) proposed an exposure:activity profiling method for interpreting high-throughput screening data for estrogenic activity using the results for the *in vitro* assays implemented by the Environmental Protection Agency's ToxCast program and the interagency Tox21 consortium. These authors concluded that for any given assay based on measurement of different events along the ER activity pathway for determining potential ER interaction with xenobiotics, the most reliable measure of potency is the AC50, the concentration of the chemical being evaluated that elicits 50% of maximum activity. The AC50 values for the different assays where GES (CAS no. 446-72-0) is active are found between 0.1 and 10 μM concentration (http://actor.epa.gov/dashboard/#chemical/446-72-0). This is also true for other chemicals with estrogenic activity (Judson *et al.*, 2015). These results are in agreement with our results, since the most robust transcriptional response elicited by GES in the Ishikawa cells was obtained when the cells were exposed to concentrations of 10 μM GES.

Using the annotation available for the genes whose expression was modified according to the time of exposure, we determine that response of the Ishikawa cells to GES exposure is progressive and results in changes in specific pathways at different times (see Supplementary Tables S2a–c, for a comprehensive list of the pathways affected by GES). For example, within the first 8 h of exposure, the pathways most affected are related to cell cycle control, transcriptional regulation, cytoarchitectural control, and regulation of macromolecule biosynthetic process. After 24 h of exposure to GES, the cells display the most changes in pathway related to signal transduction, protein folding, and cell cycle control. By 48 h of exposure to GES, the pathways that showed the highest modification are related to cytoskeleton remodeling, cell adhesion, and apoptosis.

The cellular pathway-specific effects of some of the transcriptional changes elicited by GES exposure in the Ishikawa cells that we have identified have been also observed in other more limited studies where GES exposure has been included. For example, Boehme *et al.* (2009) evaluated the transcriptional response of a clone of the Ishikawa cell line, obtained from the European Collection of cell Cultures (ECACC, catalog no. 99040201), which they named Ishikawa plus cells, after 24 h of exposure to 2 doses of GES (200 nM and 1 μM). As in our study, these authors determined that the low GES dose caused changes in the expression of very few genes, while at the higher dose, GES elicited changes in the expression of genes whose products are related to cell proliferation and differentiation, transcriptional regulation, immune response, cell signaling, and

TABLE 7. Gene Expression Changes Induced by Exposure to GES and EE in Ishikawa Cells and EE in the Rat Uterus

Probe ID	Gene acronym	GES (10 μM)			EE (1 μM)[a]			EE rat uterus (10 μM)[b]			Biological process GO term
		8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h	
212224_AT	ALDH1A1	−1.0	−1.7	−7.9	−1.3	−1.7	−1.2	−3.6	−2.5	1.1	dehydrogenase activity
202986_AT	ARNT2	−1.9	−2.7	−3.0	−1.3	−1.6	−2.2	−2.0	−1.8	1.1	response to hypoxia
205047_S_AT	ASNS	2.4	5.3	4.6	1.0	1.2	1.3	3.2	1.6	1.1	asparagine biosynthetic process
240983_S_AT	CARS	1.1	1.5	1.7	−1.0	1.1	1.2	3.1	2.4	−1.3	nucleotide binding
209286_AT	CDC42EP3	−1.7	−1.3	−1.1	−1.1	−1.2	−1.4	−1.4	−1.8	−1.3	signal transduction
209101_AT	CTGF	−1.9	1.2	−1.4	1.2	−1.9	−1.6	−2.5	−4.0	−1.1	signal transduction
215537_X_AT	DDAH2	−1.2	−1.6	−3.2	−1.4	−1.3	−1.2	−2.7	−2.3	−1.2	hydrolase activity
209457_AT	DUSP5	1.5	2.2	3.5	1.5	1.6	1.3	1.5	−1.1	1.3	protein dephosphorylation
218995_S_AT	EDN1	−2.0	−2.5	−4.0	−1.1	−1.4	−1.4	−1.2	−1.9	1.1	skeletal system development
227404_S_AT	EGR1	−1.7	−1.0	1.9	2.7	2.1	1.4	2.0	−1.5	−1.0	transcription
211401_S_AT	FGFR2	−1.8	−3.6	−6.0	−1.5	−1.8	−4.0	−2.5	−1.7	−1.2	angiogenesis
209189_AT	FOS	1.3	3.7	3.8	2.6	2.6	1.6	16.1	2.8	3.1	conditioned taste aversion
235256_S_AT	GALM	1.1	−1.4	−1.7	−1.0	−1.6	−1.1	−2.1	−1.4	1.0	isomerase activity
203108_AT	GPRC5A	−1.0	−1.1	−2.5	−1.1	−1.4	−1.7	1.3	−1.2	−1.6	signal transduction
212641_AT	HIVEP2	−2.2	−2.0	−1.3	−1.5	−1.2	−1.4	−1.3	−2.0	−1.0	regulation of transcription
212143_S_AT	IGFBP3	−1.1	−2.7	−8.4	−1.3	−1.3	−1.8	−3.1	−5.3	−1.5	regulation of cell growth
205945_AT	IL6R	−1.3	−1.1	1.5	1.8	2.8	3.6	4.7	1.9	2.9	cytokine receptor activity
201625_S_AT	INSIG1	1.7	2.8	2.6	1.0	1.7	1.2	3.2	3.1	1.1	lipid metabolic process
221841_S_AT	KLF4	2.2	2.5	3.1	1.1	1.5	1.4	3.2	2.9	1.1	transcription
202193_AT	LIMK2	−1.1	−2.1	−3.2	−1.4	−1.7	−1.7	−1.4	−1.7	−1.3	protein phosphorylation
205710_AT	LRP2	1.0	−2.6	−4.9	−1.2	−1.3	−2.0	−3.0	−3.2	−2.6	protein glycosylation
204058_AT	ME1	−1.1	1.3	2.4	−1.1	1.2	1.3	1.5	2.1	1.2	oxidoreductase activity
202431_S_AT	MYC	1.4	−1.1	−2.5	1.7	1.7	1.1	1.7	1.1	−1.0	regulation of transcription
209757_S_AT	MYCN	−1.8	−2.3	−4.3	−1.6	−1.7	−1.5	−1.5	1.0	3.7	transcription
206453_S_AT	NDRG2	1.1	−1.3	−2.1	−1.2	−1.5	−1.7	−4.2	−1.4	1.1	signal transduction
206089_AT	NELL1	−2.1	−2.5	−5.4	−2.7	−5.3	−4.0	−2.3	−1.4	1.1	protein binding
203845_AT	PCAF	−1.5	−2.3	−3.0	−1.5	−1.6	−1.3	−1.7	−1.2	−1.1	chromatin remodeling
202458_AT	PRSS23	−1.4	−2.0	−3.7	−1.2	−1.4	−1.6	−1.3	−2.3	−1.1	proteolysis
205194_AT	PSPH	1.2	1.5	1.4	1.0	1.1	1.2	1.9	1.7	−1.0	phosphatase activity
205128_X_AT	PTGS1	−1.1	−1.3	−1.8	−1.1	−1.4	−1.7	−2.3	−2.1	1.0	prostaglandin biosynthetic process
225796_AT	PXK	1.6	1.4	1.5	1.2	1.1	1.5	−1.1	−1.5	1.3	protein phosphorylation
210051_AT	RAPGEF3	−1.2	−1.1	−2.3	−1.1	−1.5	−1.1	−1.2	−1.6	−1.2	regulation of protein phosphorylation
219142_AT	RASL11B	−1.4	−2.6	−2.1	1.1	−1.6	−1.5	−1.4	−1.6	−1.3	signal transduction
203423_AT	RBP1	1.0	−1.1	−1.5	−1.2	−1.6	−1.3	−2.5	−1.5	−1.0	retinol-binding protein
231894_AT	SARS	1.7	4.4	6.1	1.0	1.1	1.4	2.0	1.5	1.3	translation
205933_AT	SETBP1	−1.7	−2.0	−2.0	−1.0	−1.3	−1.4	−1.6	−1.8	−1.5	protein binding
205799_S_AT	SLC3A1	−1.6	−3.4	−4.3	−1.4	−1.6	−1.5	−1.4	−1.6	−1.4	transporter activity
205921_S_AT	SLC6A6	−1.4	−1.7	−4.4	1.1	1.2	−1.4	−1.7	−1.3	1.1	symporter activity
240120_AT	SORBS2	−2.4	−2.8	−4.0	−1.1	−1.1	−3.8	−1.4	−1.8	1.3	signal transduction
213668_S_AT	SOX4	1.2	−2.3	−4.9	−1.5	−1.7	−1.7	−6.2	−2.8	1.2	pro-B cell differentiation
238688_AT	TPM1	1.0	−1.3	−3.9	−1.1	−1.6	−1.8	1.1	−2.5	−1.5	muscle contraction
209114_AT	TSPAN1	1.1	−1.3	−3.8	−1.1	−1.5	−1.5	−1.9	−2.6	1.1	signal transduction

[a]The reliability of the microarray data from these samples has been independently corroborated by QRT-PCR.
[b]Average fold change on the expression of genes from the rat uterus from animals exposed to a single dose of 10 mg EE/kg, at the indicated times. The reliability of the microarray data from these samples has been independently corroborated by QRT-PCR.

intracellular transport. These authors compared the gene expression changes elicited by GES to the ones produced by diethylstilbestrol (DES, a potent estrogen) exposure and determine a high degree of similarity between the 2 transcriptional profiles, which they attribute to the estrogenicity of both chemicals.

Using a customized DNA microarray, Ise *et al.* (2005) evaluated the response of 172 estrogen responsive genes in MCF7 cells (human breast adenocarcinoma cell line) exposed to various phytoestrogens, including GES (10 μM), for 3 days. Although the specific gene expression changes elicited by GES in this study cannot be compared directly with our results, given the cellular and experimental differences, Ise *et al.* determined that GES modified the expression of genes whose products are implicated in the regulation of multiple signal transduction pathways such as FOS, JUN, ATF3, among others. Regenbrecht *et al.* (2008) evaluated the transcriptional response of primary glioblastoma, rhabdomyosarcoma, hepatocellular carcinoma, and human embryonic carcinoma cells (NCCIT) to 50 μM GES after 48 h of exposure. These authors determined that cancer cells treated with GES undergo cell cycle arrest at different checkpoints. This arrest was associated with a decrease in the mRNA levels of core regulatory genes, PBK, BUB1, and CDC20. In contrast, human NCCIT cells showed over-expression of GADD45 A and G, as well as down-regulation of OCT4, and NANOG protein. Furthermore, GES induced the expression of apoptotic and antimigratory proteins p53 and p38 in all cell lines, and also up-regulated steady-state levels of both Cyclin A and B. Of these genes, in our experiments only the over-expression of GADD45

A was observed in the Ishikawa cells exposed to 10 μM GES, at the 3 times (Table 4). However, at the cellular pathway level, there is a clear effect of GES on cell cycle control, cell growth, and division.

As indicated, to better define the estrogenicity of GES, we compared the expression profile of the Ishikawa cells exposed to GES and to EE, a potent ER agonist (Naciff *et al.*, 2009) under a similar experimental protocol and at equipotent doses (10 and 1 μM, respectively) at 8, 24, and 48 h. This comparison showed that the expression of 554 unique genes was affected by GES and EE at any given time. However, the change in the expression of only 337 unique genes occurred in the same direction, although with a different magnitude for either chemical. Many of these genes, affected in a similar fashion (up- or down-regulated) by both GES and EE, encode proteins associated with multiple biological functions and have been associated with an estrogenic response in various systems, such as FOS, PTEN, TGFA, EGR1, FGFR2, IGFBP3, SOX4, among others. The response of some of these genes was more robust to either GES or EE exposure, based upon the magnitude of the change of their expression.

Further, the comparison of the transcriptional response of the Ishikawa cells to GES (*in vitro* system) with the response of the juvenile rat uterus to EE exposure (*in vivo* system) (Naciff *et al.*, 2007), at the same time points and equipotent doses, showed that the change in the expression of 576 unique genes was identical in the direction of the change, although the magnitude of the change of some genes was different in Ishikawa cells versus the rat uterus. Of these 576 common genes, 574 of them showed a change in their expression level of at least 1.5-fold (up or down-regulated) for at least one time point *in vivo* and *in vitro*, and this change was significant in both systems ($P \leq 0.0001$, *t* test). Among these genes, once again there were some of the well-known estrogen-responsive genes including NGFR, FOS, KLF4, IGFBP3, FGFR2, and SOX4 (Naciff *et al.* 2007, 2009). Using the annotation available for these genes responsive to GES (human) and EE (rat), we determined that the response of Ishikawa cells as well as the juvenile rat uterus involved similar cellular processes, including regulation of transcription, signal transduction, cell proliferation, cell growth, cell differentiation and tissue remodeling, transport, and metabolism. The concordance of the genomic response to estrogen (EE) exposure of the juvenile rat uterus with the one elicited by GES and EE in the Ishikawa cells is also observed in the timing of the response of individual genes. For example, one of the early responsive genes was MYC, which is up-regulated by estrogen. In agreement with this early response, the expression of MYC was increased by both GES and EE in Ishikawa cells, as well as by EE in the juvenile rat uterus, with a peak response occurring within the first 8 h of exposure in the 2 systems. A similar pattern was followed by other early genes, including FOS and EGR1, among others (Table 7). These similarities in the response of both the Ishikawa cells and the juvenile rat uterus attest to the robustness of the transcriptional response of these 2 systems to the exposure to chemicals with estrogenic activity. These data further support the usefulness of the Ishikawa cells to investigate the estrogenic potential of chemicals of interest. Further, the transcriptional changes brought about by GES exposure in the Ishikawa cells are also concordant with gene expression changes observed by Punyadeera *et al.* (2005) in uncultured human endometrium collected during menstruation and the late-proliferative phase of the menstrual cycle, as well as after 24 h incubation (cultured as explants) in the presence of 17 β-estradiol (E2). These authors demonstrated that in the human endometrium, E2 (1 nM) regulates the expression of SOX4, IGFBP3, CYCA1, CD44, SCGB1A1, SCGB2A1, PTGS2, FGF9 and FGF18, FLOT1, CA2, RPL27A, and GK among other genes. The exposure of the Ishikawa cells to GES resulted in changes in the expression of these genes, in the same direction, which adds support to the robustness of the Ishikawa cells in displaying estrogenic responsiveness similar to the human endometrium.

Some of the genes listed in Table 7 could be used to define a transcriptome estrogenic fingerprint. However, in order to better define the "optimal" set of genes that could represent an estrogenic fingerprint, which could be suitable for the evaluation of unknown compounds, it is necessary to determine the transcriptional profile of more chemicals, including those with known estrogenic activity, as well as chemicals known to be nonestrogenic, both in the Ishikawa cell *in vitro* system, as well as in a suitable *in vivo* system.

It has to be emphasized that the transcriptional response of the Ishikawa cells to GES exposure also involves changes in the expression of multiple genes not previously associated with an estrogenic response, and most probably completely independent of the estrogenicity inherent to GES, but also involved with the regulation of specific cellular pathways and process sensitive to GES. These gene expression changes are not identified in this article; however, the data are available for further analysis, which could be guided to identify these genes (GEO: GSE71717).

Our data also can be used to better understand the biological activities attributed to GES. The ingestion of relatively high concentrations of GES, mostly from soy-derived food products and nutritional supplements, has been proposed as being protective against various cancers, although the mechanism through which GES exerts these effects has not been determined, it has been assumed that changes in the expression of specific GES-responsive genes is a necessary step. Although the comprehensive analysis of the specific gene expression changes associated with the anticarcinogenic activity of GES are not within the scope of our work, we have pointed out some of the most robust gene expression changes elicited by GES that could associated with this activity (Supplementary Material). However, further research is definitively necessary to determine the molecular mechanisms associated with the proposed anticarcinogenic activity of GES.

Access to robust and biologically relevant *in vitro* systems to assess the estrogenic potential of chemicals of interest is needed to address the needs of the chemical industry both for product innovation as well as for regulatory compliance. The results presented here further strengthen the potential use of the Ishikawa cells as a relevant *in vitro* system to assess the estrogenic potential of chemicals of interest, using a transcript profiling approach. The use of these cells for gene expression analysis after chemical exposure allows for the potential development of mechanism-based assessments that are more predictive of the potential human response to chemical exposure. This *in vitro* model, when used to define transcriptional profiles associated with chemical exposure, fits in the new paradigm in toxicology necessary to improve human and environmental health risk assessment, delineated by the National Academy of Sciences report Toxicity testing in the 21st century (NRC, 2007). This paradigm requires a shift from the reliance on observing apical toxicity endpoints in animal models toward testing systems (preferable human-relevant cells or tissues) that evaluate perturbations of specific biological pathways (toxicity pathways). Furthermore, the biological competence of the Ishikawa cells makes them a reliable system to address the endocrine disruptive capabilities for industrial chemicals, chief among

them the ability to disrupt the estrogen system (US-EPA, 2015), using a toxicogenomics approach. The integrity of the estrogen system is critical for normal physiology of the human body, at different life stages, and its dysregulation by chemical exposure could result in detrimental effects. Therefore, it is imperative that the potential to disrupt the estrogen system be evaluated for any chemical placed in commerce that is anticipated to have significant human exposure. The robustness of the Ishikawa cells' response to the exposure to environmentally relevant concentrations of chemicals with estrogenic activity (see also Boehme *et al.*, 2009; Naciff *et al.*, 2009, 2010) supports its use as a reliable first tier *in vitro* method designed to screen chemicals with the potential to interact with components of the estrogen systems. The simplicity and robustness of the Ishikawa cell system allows for the implementation of a high-throughput approach for chemical testing, a critical need for the Endocrine Disruptor Screening Program (US-EPA, 2015).

In summary, we have demonstrated that GES, at physiologically attainable concentrations, can regulate the expression of numerous genes in Ishikawa cells of which many are directly or indirectly regulated by estrogen. These genes encode products which have various cellular functions and are part of multiple cellular pathways affecting cell proliferation, differentiation, development, transcriptional regulation, and transport processes among others. The evaluation of potential endocrine disruptor properties of chemicals of interests is a priority in the risk assessment process, within this mode of action, the most common effect is on the estrogen system. The data presented herein demonstrate that the Ishikawa cells can serve as a valuable tool to screen chemicals for estrogenic (or antiestrogenic) properties. In addition, the use of these cells can provide information on the specific molecular mechanisms driving this activity which may lead to desired or undesired outcomes and allow the exploration of specific cellular pathways affected by chemical exposure. This *in vitro* system is amenable for implementing assays in a large scale, in a high-throughput mode and to allow testing multiple chemicals under equivalent conditions in parallel and, at the very least, can be used to prioritize chemicals of interest for *in vivo* testing.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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