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Effect of chronic exposure to the aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female rats on ovarian gene expression.

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Abstract

The aryl hydrocarbon receptor (AHR) mediates the effects of many endocrine disruptors and contributes to the loss of fertility in polluted environments. Female rats exposed chronically to environmentally relevant doses of the AHR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) across their lifespan experience accelerated reproductive senescence preceded by ovarian endocrine disruption. The purpose of this study was to determine the changes in ovarian gene expression that accompany the loss of ovarian function caused by chronic exposure to TCDD. Beginning in utero, female Sprague Dawley rats received TCDD (1, 5, 50, or 200 ng/kg/wk; n=4 per group) or vehicle weekly throughout their lifespan, and were sacrificed on diestrus just prior to loss of reproductive cyclicity at 11 months of age. Microarray analysis was used to determine differences in ovarian gene expression between control and TCDD-treated (200 ng/kg/wk) animals. To confirm microarray results, real-time PCR was used to assess changes in gene expression among treatment groups. TCDD treatment decreased ($p < 0.05$) proestrus serum estradiol concentrations with no effect on serum progesterone. In ovaries from rats treated with 200 ng/kg/wk TCDD compared to controls, 19 genes of known function were found to be up-regulated, while 31 ovarian genes were found to be down-regulated ≥ 1.5 fold ($p \leq 0.05$). Gene expression of 17 α -hydroxylase decreased following chronic TCDD treatment, suggesting the decrease in estradiol biosynthesis may be a consequence of decreased substrate. Taken together with past studies indicating a lack of effect on hypothalamus or pituitary function, the apparent regulation of key ovarian genes support the hypothesis that chronic TCDD exposure directly affects ovarian function.

Keywords

Aryl hydrocarbon receptor; menopause; ovary; rat

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INTRODUCTION

The aryl hydrocarbon receptor (AHR) is conserved across phyla, mediates the effects of many endocrine disruptors and is postulated to contribute to the loss of fertility in polluted environments [1,2]. The prototypic AHR-specific ligand 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) is the most potent AHR binding toxicant due to its accumulation in the environment, resistance to breakdown and impact on multiple organ systems [2,3]. Short term exposure to high doses of TCDD and similar ligands induces endocrine disruption, endometriosis, teratogenesis and abortion, alters sexual behavior, decreases spermatogenesis and diminishes fertility [1,4–6]. In the rat, a single prepubertal exposure to TCDD has both immediate and persistent effects on female reproductive function, leading to a premature transition to reproductive senescence [4]. However, until recently few past studies have addressed the impact of realistic, life-long activation of the AHR pathway on aging of the female reproductive system.

Previously, we have shown that female rats exposed chronically to environmentally relevant doses of the AHR agonist TCDD across their reproductive lifespan experience a dose-dependent acceleration of reproductive senescence [7]. The mechanisms of premature reproductive senescence during chronic exposure to AHR ligands appear distinct from the effects of acute high dose exposures [4]. In chronically exposed animals, diminished serum estradiol occurs with no differences in number or size distribution of ovarian follicles, and independently of gonadotropin disruption, suggesting TCDD exerts direct effects on ovarian function by endocrine disruption. The purpose of this study was to determine the changes in ovarian gene expression that precede the loss of normal cyclicity cause by chronic exposure to the AHR agonist TCDD.

MATERIALS AND METHODS

Animals

Adult pregnant Sprague Dawley dams (n=15, Charles River Laboratories) were purchased and housed under a 12:12 light:dark photoperiod, controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity. Food (Purina Rat Chow, Ralston Purina Co.) and water were provided ad libitum. TCDD (CAS 1746-01-6; MW, 321.9; purity >99%) was obtained from Cambridge Isotope Laboratories, Inc (Lenexa, KS). All procedures were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Treatment of animals

Pregnant dams were dosed with corn oil vehicle or TCDD (1, 5, 50, 200 ng/kg/wk by gavage) on days 14 and 21 of gestation and days 7 and 14 post-parturition to expose pups gestationally and latationally. Beginning day 21 post-parturition, female pups were directly treated with vehicle or TCDD. The lower doses (1, 5 ng/kg/wk) approximate the estimated cumulative exposure of women in the general population of the United States to dioxins across their reproductive lifespan [8]. The higher doses (50, 200 ng/kg/wk) mimic lifetime exposure of high risk populations to dioxin-like compounds[2] and have been well characterized for other endpoints in previous chronic studies[9]. This causes a loss of reproductive cyclicity by 10–12 months [2,4,7]. Reproductive cycles were monitored by vaginal cytology for 7–10 days each month until the transition to reproductive senescence, as defined by the occurrence of 2 consecutive prolonged (6–9 days) cycles. Cyclicity data and detailed endocrinology from these rats were presented previously [7].

To confirm early changes in the reproductive endocrinology with chronic AHR ligand exposure, normally cycling rats (n=6–8 per group) were cannulated at 9 months (when the

transition to reproductive senescence was first evident in the highest dose group) and blood samples collected at 2200 h on proestrus for measurement of serum progesterone (P₄) and estradiol (E₂). Hormonal data relevant to this microarray analysis are excerpted from the previously published complete hormone profiles of these rats [7].

Serum estradiol and progesterone

Blood was sampled across the estrous cycle following jugular cannulation at 9 months in control and TCDD-treated rats as described previously [7]. Serum concentrations of estradiol and progesterone were measured at the Ligand Assay and Analysis Core in the Center for Research in Reproduction at the University of Virginia using ELISA kits (Estradiol EIA, DSL-10-4300; Progesterone EIA, DSL-10-3900; Diagnostic Systems Laboratories). The assay sensitivity was 7 pg/ml for E₂ and 0.13 ng/ml for P₄. The intra-assay coefficients of variation for E₂ and P₄ were 4.8% and 3.4% [7].

Microarray analysis

Cycling vehicle and dioxin-treated rats were sacrificed on diestrus at 11 months of age and ovaries were dissected and frozen for RNA extraction. Microarray analysis was used to determine differences in gene expression between control and TCDD-treated (200 ng/kg/wk) animals. Total RNA (5 µg; n=3 per group) was isolated using the SV Total RNA Isolation System (Promega), labeled with biotin and hybridized on a Affymetrix Rat 230 v.2.0 high density GeneChip® as described below. This array represents a 31,000 probe set for gene expression level analysis of over 30,000 transcripts that correspond to over 28,000 known genes.

In vitro-transcription biotin labeling—Total RNA was annealed with T7-(dT)24 and reverse transcribed into cDNA using the Superscript Choice System (Invitrogen). The cDNA was phenol extracted, followed by Phase Lock Gel separation (Brinkman Instruments/Eppendorf). The purified cDNA was precipitated and resuspended in DEPC-treated water. In vitro-transcription biotin labeling was conducted using the GeneChip Expression 3-Amplification Labeling Kit (Affymetrix). The RNeasy RNA Purification Mini kit (Qiagen) was used to purify labeled cRNA generated according to manufacturers instructions, and quantified using 260nm/280nm spectrophotometric assay. For fragmentation, 20 µg biotin-labeled cRNA (final concentration 0.5 µg/µl) was added to fragmentation buffer (200mM Tris-Acetate, pH 8.1, 500 mM potassium acetate, 150mM magnesium acetate) and DEPC-treated water to 40 µl final volume. This was incubated at 94 °C for 35 min and the reaction was terminated at 4 °C.

GeneChip hybridization—A hybridization cocktail was composed of 15 µg fragmented cRNA, control biotin-labeled oligo B2 (50pM; Affymetrix), eukaryotic hybridization spike controls (bioB, bioC, bioD, cre; 1.5, 5, 25 and 100 pM respectively), herring sperm DNA (0.1 mg/ml), acetylated BSA (0.5 mg/ml), hybridization buffer, and DEPC-treated water to 300 µl. This mixture was denatured at 99 °C for 5 min prior to applying to GeneChip array for prehybridization at 45 °C for 10 min at 60rpm. The denatured hybridization cocktail was applied to the GeneChip array and hybridized 16 hr at 45 °C and 60 rpm.

Washing and staining—An automated process using the GeneChip Fluidics Station 400 with the standard array format was carried out. Briefly, the first post hybridization wash consisted of 10 cycles of 2 mixes/cycle with wash buffer A (6x SSPE, 0.01% Tween 20) at 30 °C. The second wash consisted of 6 cycles of 15 mixes/cycle with wash buffer B (100mM MES, 0.1M [Na⁺], 0.01% Tween 20) at 50 °C. The probe array was stained in streptavidin-phycoerythrin (SAPE) solution (1x MES stain buffer, 2 mg/ml acetylated BSA, 10 µg/ml SAPE) 5 min at 35 °C. The post-stain wash consisted of 10 cycles of 4 mixes/cycle with wash

buffer A at 30 °C. The second stain was carried out for 5 min at 25 °C in antibody solution (1x MES stain buffer, 2 mg/ml acetylated BSA, 0.1 mg/ml normal goat IgG, 3 µg/ml biotinylated antibody). A third stain was carried out in SAPE solution as described above, followed by a final wash of 15 cycles of 4 mixes/cycle with wash buffer A at 35 °C.

Scanning—An automated process was conducted using the Affymetrix GeneChip Scanner 3000. Briefly, 1x scans were conducted with a pixel value of 3µm and wavelength of 570 nm. Absolute and comparison analysis were conducted using Scaling - All Probe Sets: Target Signal = 500, and the Normalization – User Defined: Scale Factor = 1.

Analysis—The initial data captured by Affymetrix GeneChip Operating Software (GCOS) version 1.4 resulted in a single raw value related to each probe set based on the mean of differences between the intensity of hybridization for each perfect match and the mismatch features for a specific transcript. The data set was exported by the Data Transfer Tool into the library data file by the GCOS software. Data Mining Tool components were utilized to process the initial data quality control assessment and to create sets of expressed probes. A total set of 31,099 probes was divided into ‘present’, ‘marginal’ and ‘absent’ subsets based on the calculated probe detection p-value. Probes present in at least 2 microarray chips out of 3 in treatment versus control were used for evaluation of up-regulated genes, while for down-regulated genes probes present in 2 out of 3 control arrays facilitated the base for comparison. Signal intensities for individual probe sets in the treatment and control triplicates were subsequently compared statistically with significant differences at $p \leq 0.05$. GeneChip data sets from GCOS were transferred into text file inputs for GeneSpring software (Agilent Technologies, Silicon Genetics), normalized per chip and gene and ranked according to fold change for treatment versus control signal intensities.

Real-time RT-PCR

To confirm microarray results, quantitative real-time RT-PCR was used to assess changes in gene expression among treatment groups (control, 1, 5, 50, or 200 ng/kg/wk TCDD; n=4 per group). RNA from ovaries of treated animals and universal rat reference RNA (Stratagene) was reverse transcribed to cDNA using superscript III (Invitrogen) and assayed in duplicate for β -actin, Cyp11a1, forkhead box A2 (FoxA2), forkhead box J1 (FoxJ1), 17- α hydroxylase (Cyp17a1), aromatase (Cyp19a1), LH receptor (LHR), FSH receptor (FSHR), and inhibin beta A (Inhba) gene expression using specific primer and probe sets and TaqMan chemistry [10]. Gene targets were chosen based on microarray results and either their critical role in ovarian steroidogenesis (Cyp17a1, Cyp19a1, LHR, FSHR, Inhba), or their known role in cell survival and aging (FoxA2 and FoxJ1). Cyp11a1 gene expression was examined to confirm AHR activation. While prime and probe sequences are proprietary information, context sequences are provided in Table 1. All assays were pre-validated by Applied Biosystems and used FAM as a reporter dye. Ct values were calculated for each endpoint and corrected for β -actin gene expression. Relative gene expression was calculated using a universal rat cDNA standard curve.

Statistical analysis

Data are presented as mean $\pm$ SEM. Serum concentrations of E₂ and P₄ were compared using one-way analysis of variance (ANOVA). When significant main effects were found, individual means were compared using the Tukey test in *post hoc* analysis. A value of $p \leq 0.05$ was considered significant.

RESULTS

Reproductive endocrinology

At 9 months of age TCDD treatment decreased ($p < 0.05$) proestrus serum E_2 concentrations in a dose-dependent manner with a lowest observable effect at 5 ng/kg/wk (Figure 1A). Serum concentrations of P_4 were unaffected by chronic exposure to TCDD (Figure 1B).

Ovarian gene expression by microarray analysis

Up-regulated genes after chronic TCDD exposure in the ovary are summarized in Table 2 and down-regulated genes are summarized in Table 3. These represent only significantly ($p \leq 0.05$) up- or down-regulated genes with the fold change ≥ 1.5 . Since we have three technical replicates for each, treatment and control, the fold change was calculated from the average signals for treatment and control. Our selection is only on genes present in at least 2 out of 3 replicates. In ovaries from rats treated with 200 TCDD ng/kg/wk compared to controls, 19 genes of known function were found to be up-regulated ≥ 1.5 fold ($p \leq 0.05$), including peroxisomal membrane protein 4, Cyp11b1, lipase, integrin alpha 1, and integrin beta 5. 17α -hydroxylase, aquaporin 9, FoxA2, FoxJ1, and GDF-9 were among the 31 known ovarian genes found to be down-regulated ≥ 1.5 fold ($p \leq 0.05$).

Ovarian gene expression measured by semi-quantitative real-time PCR

To confirm and validate gene expression changes identified by microarray data, real-time PCR analysis was performed targeting Cyp11a1, FoxA2, FoxJ1, and 17α hydroxylase gene expression. While microarray analysis did not indicate a significant change in aromatase, inhibin beta A, FSHR, or LHR gene expression by TCDD treatment, we felt it would be an oversight to dismiss these critical genes in the steroidogenesis pathway.

Ovarian Cyp11a1 gene expression was measured as a biomarker indicating activation of the AHR in response to TCDD. Cyp11a1 gene expression was not detectable in animals treated chronically with vehicle, 1 or 5 ng/kg/wk TCDD (Figure 2). Long-term treatment with 50 ng/kg/wk TCDD induced detectable amounts of Cyp11a1 mRNA, and treatment with 200 ng/kg/wk TCDD increased this about 4-fold.

To determine the mechanistic disruption that underlies the diminished serum estradiol concentrations observed in response to chronic aryl hydrocarbon receptor activation, we chose to focus on critical genes in ovarian steroidogenesis that were affected as indicated by microarray analysis. While no significant differences were detected, most likely due to a high degree of variable expression among the control animals, there was a trend for LHR gene expression to decrease in response to TCDD treatment (Figure 3A). Compared to control animals, chronic TCDD treatment tended to suppress 17α -hydroxylase gene expression in animals treated with 1 or 200 ng/kg/wk ($p = 0.06, 0.09$, respectively), and inhibited 17α -hydroxylase gene expression over 10-fold in animals treated with 5 or 50 ng/kg/wk ($p < 0.05$; Figure 3B). FSHR, aromatase and inhibin beta A gene expression did not differ among treatment groups (data not shown).

FoxA2 gene expression was decreased ($p < 0.05$) following chronic TCDD treatment with 5 or 200 ng/kg/wk compared to control animals, and demonstrated a similar trend when treated with 1 or 50 ng/kg/wk (Figure 4A). Similarly, FoxJ1 expression was decreased ($p < 0.05$) in animals treated long-term with 5, 50, or 200 ng/kg/wk of TCDD compared to control treated animals (Figure 4B).

DISCUSSION

Previous work from our laboratory suggests that chronic exposure to TCDD hastens reproductive aging in females predominantly via local ovarian endocrine disruption [7]. The transition to reproductive senescence often is accompanied by decreased serum estradiol [11]. The purpose of this study was to evaluate the changes in ovarian gene expression that accompany this disruption in ovarian steroidogenesis during premature reproductive senescence. In this experiment chronic TCDD treatment decreased proestrous serum estradiol concentrations without affecting serum progesterone concentrations. This is in agreement with previous studies in rodents and nonhuman primates [12–14]. Additionally, a recent study found a negative association between body burdens of AHR ligands and serum estradiol in pregnant women living in an area of high industrial exposure to dioxins [15]. In the current study, the effects of chronic AHR activation on serum estradiol concentrations were found to precede the transition to reproductive senescence as defined by a disruption of cyclicity [7].

TCDD has been shown to induce Cyp11a1 in vitro and in vivo in the ovary of several species including mouse [16], rat [17], and human [18]. Ovarian Cyp11a1 gene expression was measured as a biomarker indicating activation of the AHR in response to TCDD. We found Cyp11a1 gene expression was detectable only in animals treated chronically with higher doses of TCDD. Although the role of Cyp11a1 in the ovary is not yet known, it could metabolize ovarian estrogens to form catecholestrogens [17]. We previously found no effect of high-dose acute exposure to TCDD on estradiol catabolism in rats, suggesting that TCDD exposure decreases serum estradiol through effects on estrogen biosynthesis [19]. Therefore, we chose to focus on genes known to be involved in the ovarian steroidogenic pathway.

Studies in the rat have shown that TCDD alters ovarian steroid synthesis [17,18]. However, others have reported that short-term treatment with TCDD fails to directly alter ovarian granulosa and thecal-interstitial cell steroidogenesis in vitro [20]. During steroidogenesis in the ovary, cholesterol is converted to pregnenolone by cytochrome P450 side-chain cleavage in theca interna. Pregnenolone is then converted to dehydroepiandrosterone by P450 17 α -hydroxylase. Because serum progesterone concentrations did not differ among our treatment groups, we chose to examine potential rate-limiting enzymes (i.e. 17 α -hydroxylase and aromatase), and the receptors of their stimulators (i.e. LH and FSH receptors), in the conversion of progesterone into estradiol. Dehydroepiandrosterone is converted to androstenedione, which diffuses into granulosa cells and is used as substrate for estradiol synthesis by cytochrome P450 aromatase [21]. In the current study, we found that chronic TCDD treatment caused a decrease in 17 α -hydroxylase gene expression, and while not significant there was a trend for LH receptor gene expression to decrease as well. No differences were found in FSH receptor or aromatase gene expression in response to TCDD treatment. Taken together, this suggests that the decrease in estradiol biosynthesis as a result of chronic AHR activation may be due to a decrease in production of the estradiol precursor, testosterone. However, we cannot rule out the possibility that there was an effect on aromatase enzyme activity independent of gene expression. We must also take into account that some differences in gene expression may be masked by the fact that whole diestrous ovaries were used to evaluate gene expression, as opposed to individual follicles or cell types (i.e. theca interna or granulosa).

In the current microarray analysis, fewer than 100 known genes were found to be significantly affected in the ovary by chronic AHR activation. These may be due to the fact that ovaries were collected at the first signs of the transition to reproductive senescence (i.e. 2 consecutive prolonged estrous cycles). Therefore, genes that appear to be regulated are likely to be important, or at least early, in the transition to reproductive senescence. In addition to the critical genes involved in ovarian steroidogenesis, we also confirm the apparent regulation by TCDD of two transcription factors: FoxA2 and FoxJ1. These genes appeared by both

microarray analysis and qPCR to be decreased by chronic AHR activation. Forkhead box transcription factors are critical regulators of survival and longevity. Mice heterozygous for a mutation in *foxa2* develop an age-related condition associated with a significant loss of dopamine neurons [22]. In a recent study in humans, age was inversely correlated with *foxj1* expression and was suggested to be involved in the age-related changes in cholesterol metabolism [23]. While the roles of FoxA2 and FoxJ1 in the ovary have not been investigated, they are closely related to the FOXO transcription factors which have a central role in cell survival, cancer, and longevity [24,25]. Future studies will investigate the roles of the forkhead transcription factors may play in estradiol biosynthesis and premature reproductive senescence.

In summary, we have previously shown that female rats exposed chronically to environmentally relevant doses of the AHR agonist TCDD across their reproductive lifespan experience a dose-dependent acceleration of reproductive senescence that is preceded by ovarian endocrine disruption. Using microarray analysis and qPCR we have evidence that suggests the decrease in estradiol biosynthesis is due to a down-regulation of genes involved in the estradiol precursor, testosterone. These changes in key ovarian genes support the hypothesis that TCDD exerts direct effects on ovarian function.

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Figure 1. Serum concentrations (means $\pm$ SEM) of estradiol (A.) and progesterone (B.) during proestrus in rats chronically treated with increasing doses of TCDD. Letters denote differences ($p < 0.05$) in estradiol concentrations among treatment groups. Progesterone concentrations did not differ among treatment groups.

Figure 2.

Ovarian Cyp1a1 gene expression among rats chronically exposed to increasing doses of TCDD as measured by quantitative RT-PCR. Asterisk denotes difference ($p < 0.05$) between 50 and 200 ng/kg/wk groups. ND indicates a signal was not detectable.

Figure 3.

Ovarian LH receptor gene (A.) and 17 α -hydroxylase (B.) expression among rats chronically exposed to increasing doses of TCDD as measured by quantitative RT-PCR. LH receptor gene expression did not significantly differ among treatment groups. * denotes the tendency to differ from control animals ($p = 0.06, 0.09$ for 1 and 200 ng/kg/wk treated, respectively). ** denotes difference in gene expression compared to control treated animals ($p < 0.05$).

Figure 4. Ovarian FoxA2 (A.) and FoxJ1 (B.) gene expression among rats chronically exposed to increasing doses of TCDD as measured by quantitative RT-PCR. Letters denote differences ($p < 0.05$) in gene expression among treatment groups.

Table 1

Context sequences of primer-probe sets used for real-time PCR.

Gene name	Context sequence
β -Actin	GCCTTCCTTCCTGGGTATGGAATCC
Cyp1A1	ATTTGAGAAGGGCCACATCCGGGAC
LH receptor	AGATAACGAGACGCTTTATTCCGCC
Cyp17a1 (17 α -hydroxylase)	ATTCCCATGGTTGACGATTTTCCC
Cyp19a1 (Aromatase)	CTTCATGAAAGCTCTGACGGGCCCT
Forkhead box A2	CGAGTTAAAGTATGCTGGGAGCCGT
Forkhead box J1	CACCTGGCAGAATTCCATCCGCCAC
Inhibin β A	TGCCGAGTCAGGCACAGCCAGGAAG
FSH receptor	TGAAGCGGCAAATCTCTGAACTTCA

Table 2

Ovarian genes identified by microarray analysis as being up-regulated by chronic exposure to TCDD (200 ng/kg/wk).

GenBank Assession no.	Gene Name	Common	Fold change	P-value
AI232414	peroxisomal membrane protein 4	Pxmp4	3.4	0.019
NM_012940	cytochrome P450, subfamily 1B, polypeptide 1	Cyp1b1	2.5	0.017
AI406565	small muscle protein, X-linked	Smpx	2.1	0.034
NM_012859	lipase, hormone sensitive	Lipe	2.0	0.009
NM_031009	angiotensin II type-1 receptor	Agtr1	1.9	0.040
NM_030994	integrin alpha 1	Itga1	1.9	0.036
BF397093	cytochrome P450, family 26, subfamily b, polypeptide 1	Cyp26b1	1.8	0.011
BG671530	malate dehydrogenase 1	Mdh1	1.8	0.033
U51153	solute carrier family 13, member 2	Slc13a2	1.7	0.019
NM_053591	dipeptidase 1	Dpep1	1.7	0.014
NM_133602	MAP2 RNA trans-acting protein MARTA1	Marta1	1.7	0.005
AW253995	ER transmembrane protein Dri 42	Dri42	1.6	0.000
AF201331	angiotensin 1 converting enzyme 1	Ace	1.6	0.037
AI177358	peroxisomal Ca-dependent solute carrier-like protein	Pscsl	1.5	0.017
AF336872	integrin, beta 5	Itgb5	1.5	0.048
BI303388	GABA(A) receptor-associated protein like 2	Gabarapl2	1.5	0.049
BF561454	fibrinogen-like 2	Fgl2	1.5	0.013
NM_133528	preimplantation protein 3	Prei3	1.5	0.003
AF177430	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Hs3st1	1.5	0.050

Table 3

Ovarian genes identified by microarray analysis as being down-regulated by chronic exposure to TCDD (200 ng/kg/wk).

Genbank Assession no.	Gene Name	Common	Fold change	P-value
NM_012753	cytochrome P450, subfamily 17	Cyp17	-14.04	0.046
NM_022960	aquaporin 9	Aqp9	-9.7	0.048
NM_053832	forkhead box J1	FoxJ1	-6.1	0.042
NM_012743	forkhead box A2	FoxA2	-4.7	0.038
AI599382	ubiquitin conjugating enzyme	LOC81816	-4.0	0.007
BG371810	HLA-B-associated transcript 1A transmembrane protein with EGF-like and two	Bat1a	-2.9	0.025
BI295864	folliculin-like domains 1	Tmeff1	-2.8	0.048
BF563503	kinesin family member 3a	Kif3a	-2.6	0.011
NM_022857	DNA binding protein (N5)	N5	-2.4	0.045
NM_019332	peptidyl arginine deiminase, type 1	Pdi1	-2.3	0.031
NM_022505	Rhesus blood group	Rh	-2.3	0.043
U08027	glycerol-3-phosphate dehydrogenase 2	Gpd2	-2.0	0.041
J03754	ATPase, Ca++ transporting, plasma membrane 2	Atp2b2	-2.0	0.027
BF522907	Musashi homolog 1(Drosophila)	Msi1h	-1.9	0.046
AA924336	transcription factor MTSG1	Mtsg1	-1.9	0.003
BE101119	parathyroid hormone receptor 1	Pthr1	-1.9	0.026
U31884	dopa decarboxylase	Ddc	-1.8	0.030
AA957449	cytokine inducible kinase	Cnk	-1.8	0.041
NM_022235	mink-related peptide 2	Kcne3	-1.7	0.035
NM_017009	glial fibrillary acidic protein	Gfap	-1.7	0.045
NM_134374	RING finger protein LIRF	Lirf	-1.7	0.018
AI235291	casein kinase 1, alpha 1	Csnk1a1	-1.7	0.031
NM_021672	growth differentiation factor 9	Gdf9	-1.7	0.033
BM385405	ubiquitin specific protease 2	Usp2	-1.6	0.032
NM_012858	lutinizing hormone beta	Lhb	-1.5	0.038
L20468	cerebroglycan	Gpc2	-1.5	0.036
AF094609	fertility related protein WMP1	Wmp1	-1.5	0.037
BE098532	step II splicing factor SLU7	Slu7	-1.5	0.048
NM_021692	Smad5	Madh5	-1.5	0.006
AA944139	inhibitor of DNA binding 4	Idb4	-1.5	0.035
NM_019167	beta-spectrin 3	Spnb3	-1.5	0.019