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ALTERED GENE EXPRESSION IN THE BRAIN AND OVARIES OF ZEBRAFISH (*DANIO RERIO*) EXPOSED TO THE AROMATASE INHIBITOR FADROZOLE: MICROARRAY ANALYSIS AND HYPOTHESIS GENERATION

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Abstract—As part of a research effort examining system-wide responses of the hypothalamic–pituitary–gonadal (HPG) axis in fish to endocrine-active chemicals (EACs) with different modes of action, zebrafish (*Danio rerio*) were exposed to 25 or 100 μg/L of the aromatase inhibitor fadrozole for 24, 48, or 96 h. Global transcriptional response in brain and ovarian tissue of fish exposed to 25 μg/L of fadrozole was compared to that in control fish using a commercially available, 22,000-gene oligonucleotide microarray. Transcripts altered in brain were functionally linked to differentiation, development, DNA replication, and cell cycle. Additionally, multiple genes associated with the one-carbon pool by folate pathway (KEGG 00670) were significantly up-regulated. Transcripts altered in ovary were functionally linked to cell–cell adhesion, extracellular matrix, vasculogenesis, and development. Promoter motif analysis identified GATA-binding factor 2, Ikaros 2, alcohol dehydrogenase gene regulator 1, myoblast-determining factor, and several heat shock factors as being associated with coexpressed gene clusters that were differentially expressed following exposure to fadrozole. Based on the transcriptional changes observed, it was hypothesized that fadrozole elicits neurodegenerative stress in brain tissue and that fish cope with this stress through proliferation of radial glial cells. Additionally, it was hypothesized that changes of gene expression in the ovary of fadrozole-exposed zebrafish reflect disruption of oocyte maturation and ovulation because of impaired vitellogenesis. These hypotheses and others derived from the microarray results provide a foundation for future studies aimed at understanding responses of the HPG axis to EACs and other chemical stressors.

Keywords—Transcriptomics Fish Endocrine disruption Neurotoxicity Reproduction

INTRODUCTION

Endocrine-active chemicals (EACs) in the environment are a concern for both human and ecological health. Although EACs have been the subject of intensive study, methods for detecting exposure to EACs and predicting the effects and risks associated with those exposures remain fairly limited. This is particularly true for EACs that act through modes of action other than direct binding to the estrogen receptor. As part of the U.S. Environmental Protection Agency (EPA) computational toxicology initiative [1], a program of research was developed to examine the system-wide responses of the hypothalamic–pituitary–gonadal (HPG) axis in fish to EACs that act through a variety of molecular initiating events [2]. Key objectives of the present research are to identify new molecular biomarkers of exposure for various classes of EACs, to establish functional links between molecular biomarker responses and whole-organism outcomes, and to use the knowledge generated to inform extrapolation of toxicological effects across endpoints, species, and chemicals. The current study represents one portion of this overall research effort and focuses on aromatase inhibition as a mode of action.

Cytochrome P450 aromatase is an enzyme that catalyzes conversion of C19 androgens (e.g., testosterone or androstenedione) to C18 estrogens (e.g., 17β-estradiol or estrone) [3]. Regulation of aromatase expression and activity plays a critical role in maintaining the balance between circulating estrogens and androgens that mediate sexual differentiation, phenotypic and behavioral sexual dimorphism, and reproduction in fish and other vertebrates. A variety of environmental contaminants, including a number of fungicides, certain polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls, some organotins, benzo[*a*]pyrene, and DDT and its metabolites, have been shown to inhibit aromatase activity in vitro [4–6]. Thus, a need exists to understand the system-wide effects of aromatase inhibition on the HPG axis and how those effects can result in impaired reproduction and/or development in fish and other vertebrates. Additionally, identification of novel molecular biomarkers of exposure to aromatase inhibitors could aid in assessment of the prevalence and distribution of aromatase-inhibiting contaminants in the environment.

A previous study by Ankley et al. [7] examined the effects of a pharmaceutical aromatase inhibitor, fadrozole, in a fathead minnow (*Pimephales promelas*) reproduction test. Although fadrozole is not an ecologically relevant chemical, it is a useful model compound for studying this mode of action. Ankley et al. [7] established that in vivo inhibition of aromatase activity caused significant reductions in circulating plasma 17β-estra-

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diol and vitellogenin concentrations, reduction of vitellogenin uptake into developing oocytes, and ultimately, concentration-dependent reduction in fecundity. Previous work by Villeneuve et al. [8] demonstrated that fadrozole inhibits both brain and ovarian aromatase activity and that the expression of the two aromatase isoforms (CYP19a1a, the predominant isoform in ovary, and CYP19a1b, the predominant isoform in brain [9]) was affected differently in female fathead minnows exposed to fadrozole for 7 d [8]. Expression of *cyp19a1a*, which is thought to be regulated primarily via cAMP-mediated signal transduction, was up-regulated, whereas that of *cyp19a1b*, which has estrogen-response elements in its promoter region, was down-regulated [8,10]. Together, these studies demonstrated the potential for aromatase inhibitors to disrupt fish reproduction, established a chain of responses across multiple biological levels of organization that serves as a plausible toxicity pathway for the apical effects, and suggested molecular mechanisms through which the organisms may adapt to the stressor to maintain homeostatic balance.

These studies, though informative, were narrow in focus and relied on hypothesis-driven, supervised analysis of specific endpoints. They did not examine the broader spectrum of fadrozole's effects on the HPG axis, both direct and indirect, in an unsupervised or discovery-driven manner. The aim of the present study was to conduct an unsupervised analysis of the impacts of an aromatase inhibitor on tissues of the fish reproductive axis using high-density oligonucleotide microarrays. Zebrafish (*Danio rerio*) were exposed to fadrozole for up to 96 h, and global changes in gene-specific mRNA transcript abundance in brain and ovarian tissue were examined. Initial analysis of results from this work identified a 50-gene classifier, or fingerprint, that could distinguish an aromatase inhibitor from a potent estrogen or androgen receptor agonist [11]. A more detailed analysis of the specific transcripts altered and their potential biological significance is reported in the present study. Differentially expressed genes were identified. Statistically enriched gene ontology (GO) categories were determined for the five sex/tissue/time conditions examined. Promoter motifs associated with coexpressed gene clusters were elucidated. Several specific targets were examined in more detail using real-time quantitative polymerase chain reaction (QPCR). Finally, annotation information and technical literature associated with differentially expressed genes were used to draw inferences regarding the potential relevance of specific responses relative to aromatase inhibition. Together, these lines of analyses were used to identify endpoints and testable hypotheses that can be examined in future studies.

MATERIALS AND METHODS

Fadrozole exposure

Fadrozole was provided by Novartis. Reproductively mature adult zebrafish (*D. rerio*; ab wild-type strain; age, five months) were obtained from an on-site culture unit at the U.S. EPA National Health and Environmental Effects Research Laboratory. Three days before the initiation of exposure, fish were randomly loaded into 12 tanks, each receiving a continuous flow of Lake Superior (USA) water pumped from approximately 200 m offshore of the U.S. EPA laboratory at a depth of 20 m (mesh size, 1 μm; flow rate, 45 ml/min), at a density of 10 males and 10 females per tank and allowed to acclimate to exposure-like holding conditions. A continuous flow (45 ml/min) of fadrozole, dissolved in Lake Superior water without use of carrier solvents (n = 4 tanks at 100 μg/L and 4 tanks at 25 μg/L, nominal) or Lake Superior water only (control; n = 4 tanks) was delivered to 12 other tanks (no fish), and fadrozole concentrations were allowed to equilibrate and stabilize over a 48-h period. Exposure concentrations were selected based on previous fathead minnow studies with the chemical [7,8] but were set somewhat higher than before because of the shorter exposure duration and previous experience suggesting that zebrafish are less sensitive to some chemicals compared with fathead minnows.

To initiate the test, the zebrafish were transferred from acclimation tanks to exposure tanks. Transfer of fish was staggered by replicate such that the one replicate tank from each treatment group received fish at 8:30 AM, with subsequent replicates from each treatment group receiving fish at 9:30, 10:30, and 11:30 AM, respectively. After 24 h (±30 min) and 48 h (±30 min) of exposure, two males and two females were sampled from each replicate tank in the same staggered order that fish had been added to the tanks. Fish were killed in a buffered solution of tricaine methanesulfonate (Finquel; Argent). Blood was collected with a heparinized microhematocrit tube, and plasma was separated by centrifugation (3 min at 15,000 *g*) and stored at −80°C for later proteomic analyses (not discussed). Gonads, livers, and brains (including the pituitary) were removed using dissection tools washed with RNAseZap® (Ambion) between each sample and immediately transferred to preweighed microcentrifuge tubes containing RNAlater® (Sigma). After 96 h (±60 min), four males and four females were sampled from each tank (in staggered order by replicate). Two males and two females per tank were sampled as described for 24 and 48 h, whereas gonad, liver, and brain samples from the other two males and two females were snap-frozen in liquid nitrogen rather than preserved in RNAlater. All samples preserved in RNAlater were stored frozen at −20°C until extracted. All snap-frozen tissue samples were stored at −80°C until analyzed. The extra two males and two females were allocated to each exposure tank to generate the desired balanced sample sizes in cases where a fish was inadvertently mis-sexed based on external morphology or died. All laboratory procedures involving animals were reviewed and approved by the Mid-Continent Ecology Division's Institutional Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Fadrozole concentrations in each exposure tank were monitored over the course of the experiment. Water samples (2 ml) were collected from each tank 1 h before the addition of fish and approximately 1 h before sample collection on each sampling date. Fadrozole concentrations in the water samples were determined by high-pressure liquid chromatography with diode-array detection as previously described [7].

Microarray analysis

Given resource limitations, it was not feasible to conduct microarray analyses for all sex, tissue, time point, and exposure concentration combinations tested. Using the design described by Wang et al. [12], however, we were able to compare control and low-fadrozole (25 μg/L, nominal) treatments for five separate conditions: 48-h female brains, 96-h female brains, 96-h male brains, 48-h ovaries, and 96-h ovaries. The RNA extracted from the appropriate tissue of one control fish and one fadrozole (25 μg/L)-treated fish was labeled with cyanine 3 (control) or cyanine 5 (25 μg/L) and hybridized to each microarray (G2518A; Agilent Technologies). Five microarrays

representing five separate biological replicates (fish) from each treatment (control and 25 μg/L) were analyzed for each time/sex/tissue condition noted above. Wherever possible, female brains and ovaries collected from the same fish were used for the microarray analyses, and at least one fish from each replicate tank was included (*Supporting Information,* Table S1; http://dx.doi.org/10.1897/08-653.S1). In addition to the 25 microarrays hybridized with experimental samples, an additional three microarrays were used for dye-swaps (hybridized with cyanine 5–labeled RNA from a control fish and cyanine 3–labeled RNA from a fish treated with 25 μg/L) to assess gene-specific dye bias. For detailed description of RNA extraction and purification, labeling and hybridization procedures, microarray scanning, and a comprehensive evaluation of data quality, see Wang et al. [12]. All microarray data described in the present study were submitted to the U.S. National Institute of Environmental Health Sciences Chemical Effects in Biological Systems knowledgebase (http://cebs.niehs.nih.gov/cebs-browser/cebsHome.do; investigation accession no. 010-00002-0001-000-4; study accession no. 010-00002-0002-000-4).

Differentially expressed genes

To identify differentially expressed genes, output from Agilent's feature extraction software for individual microarray scanned images was filtered based on feature saturation, uniformity, pixel population consistency, and signal to noise ratio using procedures described in the Agilent Technologies Training Manual (Ver 4.5, May 2006). Genes that remained after filtering each file were combined, and those for which a signal was present on at least 15 of 59 microarrays (those from the fadrozole experiment and two additional independent experiments) [11,12] were compiled into a list of 18,252 genes or features that were analyzed for differential expression in the samples from control versus fadrozole-treated fish. The gene list was then imported into GeneSpring GX 7.3.1 software (Agilent Technologies) as the starting point for all subsequent microarray analysis. Data normalization was conducted in GeneSpring according to the procedures recommended for Agilent two-color microarrays (i.e., using Agilent FE Enhanced Import normalizations; GeneSpring GX 7.3.1). Lists of genes that were differentially expressed between the fadrozole (25 μg/L)-treated and control fish samples were generated using a one-class *t* test and a nonpermutation-based Benjamini and Hochberg false-discovery rate. The unbalanced incomplete block design [12] used for the present study has the advantage of increasing the total number of experimental samples that can be analyzed with a given number of microarrays but does not provide independent measures of the relative expression of a given gene in the control and treated sample, as would be generated using a common reference sample design. Instead, it yields a single log ratio of the expression in the fadrozole-treated sample over that in the control sample (cyanine 5 to cyanine 3 ratio). Consequently, a statistical test based on five separate biological replicates from two treatment groups would have reduced degrees of freedom, increased variance (i.e., the variability of the control sample from its population mean is effectively multiplied by the variability of the treated sample from its population mean in generating a ratio), and reduced power compared to a reference design. For this reason, the statistical false-discovery rate [13] was set to 0.15 or less for defining differentially expressed genes, and the *p* value associated with each gene also was used as a relative indicator of the statistical confidence of the response.

Functional analysis: Gene ontologies

The representation of differentially expressed genes within defined GO categories [14] was compared to the overall representation of genes within that GO category on the microarray to determine whether fadrozole treatment enriched their occurrence relative to what might be expected from chance alone. Determination of statistically significant enrichment was based on a hypergeometric test without multiple test correction as calculated using GeneSpring GX 7.3.1 software ($p < 0.05$). Enriched GO terms were ranked by *p* value, and the 20 most significant terms (lowest *p* values) were used to focus analysis of biological processes impacted by fadrozole. The hierarchical and nonmutually exclusive nature of GO terms assigned to a given gene or genes was considered in the analysis, and when the same gene expression changes drove the statistical significance of multiple GO terms or categories, some redundant terms were eliminated from the list of 20 terms. Enrichment was examined in all three major GO categories (e.g., biological process, cellular component, and molecular function). However, only biological process results are reported here, because they were the most informative category in the context of the present study. The primary GO analysis for hypothesis generation was conducted at the tissue level, with all genes differentially expressed in either brain or ovary (regardless of time point and, in the case of brain, sex) compiled into a single list. Enriched GO categories associated with each time/sex/tissue condition were examined primarily to investigate whether notable time- or sex-related differences existed in the profiles of differentially expressed genes within a tissue.

Promoter motif analysis

Differentially expressed genes, combined from all sex/tissue/time conditions, were clustered using consensus K-means clustering (TM4 MeV; http://www.tm4.org/mev.html), with K determined based on figure of merit. Promoters (transcription factor–binding motifs) corresponding to sequences found in several high-confidence coexpressed gene clusters were identified based on zebrafish assembly version 7 (Zv7, released July 13, 2007; http://www.ensembl.org/Danio_rerio/index.html). Two algorithms, CisModule [15] and BioProspector [16], were used to identify potential common motifs. Output motifs were then combined as a single sequence file and searched against the TRANSFAC® database (http://motif.genome.jp) to identify specific promoter motifs.

Real-time QPCR

Eleven genes were selected as targets for follow-up investigation using QPCR (*Supporting Information,* Table S2; http://dx.doi.org/10.1897/08-653.S1). Genes were selected based on inspection of the expression profiles using Genespring GX 7.3.1 (e.g., magnitude of log ratio, trend as a function of time, and consistency across replicates), robustness of the response across conditions, evidence in the literature suggesting regulation by estradiol and/or modulation of aromatase expression or activity, and/or known role in HPG-axis regulation [17]. Gene-specific primers (*Supporting Information,* Table S2) were designed based on published zebrafish sequences using PrimerExpress® software (Applied Biosystems). Five biological replicates consisting of RNA extracted from the tissues of different fish from each of the three treatment groups (0 [control], 25, and 100 μg/L) were analyzed for each time/sex/tissue condition, and microarray results were used to

guide the selection of the specific conditions examined (*Supporting Information,* Table S1). Total RNA samples analyzed by QPCR were aliquots of the same samples prepared for microarray analysis but obtained before labeling. Reverse transcription and Dynamo Sybrgreen™ (Bio-Rad) QPCR reactions were conducted using methods and reaction conditions detailed previously [18]. Primer specificity was determined through melting-curve analysis, and quantity values were calculated using the $2^{\Delta\Delta CT}$ method using 18S rRNA as a normalizer [12,18]. Normality of the QPCR data was tested using a Kolmogorov–Smirnov test, and Levene's test was used to evaluate homogeneity of variance. When the QPCR data met parametric assumptions, one-way analysis of variance was used to test for differences across fadrozole concentrations, with data grouped by time point. When data did not conform to parametric assumptions, a nonparametric Kruskall–Wallis test was used to examine for differences across fadrozole concentrations. Statistical differences between the three treatments, at each time point, were determined using Duncan's multiple-range test or Dunn's test. Unless otherwise noted, differences were considered to be significant at $p < 0.05$. All statistical analyses except Dunn's test were conducted using Statistica® 8 (StatSoft); Dunn's test was conducted using GraphPad® Instat (Ver 3.01; GraphPad Software).

RESULTS

Exposure verification, survival, and morphometrics

Over the course of the experiment, fadrozole concentrations measured in the exposure tanks were close to nominal. Fadrozole was not detected in any control tanks (method detection limit, 10 μg/L). Fadrozole concentrations in the nominal 25 μg/L treatment ranged from 29.3 ± 2.4 (mean ± standard deviation) to 30.5 ± 0.6 μg/L over the duration of the exposure, and no significant differences were found among replicate tanks within the treatment group. The mean concentrations in the nominal 100 μg/L treatment ranged from 101.3 ± 1.0 μg/L after 23 h to 89.3 ± 9.91 μg/L after 95 h, and no significant differences in concentration were found among replicate tanks.

No mortalities occurred during the exposure period. Some statistically significant differences were found in the body weight and gonadosomatic index among the groups of females sampled (for details, see *Supporting Information,* Table S3; http://dx.doi.org/10.1897/08-653.S1); however, those differences were not between the 25 μg/L group and control group from the 48- and 96-h time points and, thus, were not expected to confound microarray-based analyses. Among the 96 females sampled for the present study, 55 (57.3%) had ovulated, as indicated by the release of eggs on application of gentle pressure to the abdomen at the time of sample collection. The incidence of ovulated eggs appeared to be randomly distributed over treatments and time points and was not correlated with the differences in female mass or gonadosomatic index observed. No significant differences were found in male body wet weight (526 ± 77 mg), testes mass (10.5 ± 4.0 mg), or gonadosomatic index (1.98% ± 0.64%) among treatments.

Differentially expressed genes

Using a one-class *t* test with the false-discovery rate set at 0.15 or less, the number of differentially expressed genes for each of the five conditions analyzed ranged from 201 in the brains of females exposed to 25 μg/L for 48 h to 778 in the ovaries of females exposed for the same duration (Table 1 and *Supporting Information,* Tables S4 to S8; http://dx.doi.org/10.1897/08-653.S1). Across the five conditions examined, up-regulated genes accounted for 89.3 to 100% of the differentially expressed genes (Table 1). Down-regulated genes accounted for just 0 to 4.4% of those differentially expressed in brain and approximately 10% of those differentially expressed in ovary (Table 1). Whereas the results suggest a possible bias toward the detection of up-regulated genes over down-regulated genes in this experiment, no clear evidence of such a bias was found, and other microarray experiments analyzed in conjunction with this experiment [11,12] did not have a similar skew toward detection of up-regulated differentially expressed genes (D.C. Bencic and A.D. Biales, unpublished results). In general, greater numbers of differentially expressed genes were detected in ovary than in whole-brain samples (Table 1).

Table 1. Number of differentially expressed genes identified for each condition (time point, sex, and tissue) using a one-class *t* test with nonpermutation-based Benjamini and Hochberg false-discovery rate (FDR) ≤ 0.15[a]

One-class *t* test	48-h F brain	96-h F brain	96-h M brain	48-h F ovary	96-h F ovary
Total	201	590	515	778	609
Up-regulated	201	564	497	698	544
Down-regulated	0	26	18	80	65

[a] F = female; M = male.

Real-time QPCR

Expression of *cyp19a1a, fshr* (follicle-stimulating hormone receptor), *npyryb* (neuropeptide Y receptor YB), *txnip* (thioredoxin-interacting protein), *sox9b* (sry box-containing gene 9b), and *prox1* (prospero-related homeobox gene 1) in ovary was examined for all concentrations and time points included in the present study (Fig. 1 and *Supporting Information,* Table S1). Based on QPCR results, *cyp19a1a* was significantly up-regulated in the 100 μg/L treatment group at all time points. Transcripts for *fshr, npyyrb*, and *prox1* were significantly more abundant in the ovaries of fish exposed to 100 μg/L for either 48 or 96 h than in time-matched controls. Expression of *sox9b* in ovary was significantly elevated only after exposure to 100 μg/L for 96 h, whereas that of *txnip* was significantly elevated after 48 h, but not after 96 h, of exposure. Expression of *zgc:64022* (ras-like estrogen-regulated growth inhibitor), examined in the 48-h ovaries, was significantly up-regulated in the 100 μg/L treatment group.

The full concentration and time series of expression in female brain was examined for three genes, *cyp19a1b, star* (steroidogenic acute regulatory protein), and *sox9b*, using QPCR, whereas expression of *hsd17b3* (hydroxysteroid dehydrogenase 17b3), *gng1* (G protein gamma), and *zgc:60422* was examined for all concentrations but at selected time points only (Fig. 2 and *Supporting Information,* Table S1). In general, the response profiles in brain were more complex than in ovary. All six genes showed a similar profile at the 96-h time point, with mean transcript abundance (normalized to 18S rRNA) decreased (significant for *cyp19a1b* and *hsd17b3* only) in fish exposed to 25 μg/L, whereas mean transcript abundance generally was increased in fish exposed to 100 μg/L (*gng1* being the one exception). Among the genes examined in female brain using QPCR, only *sox9b* was significantly modulated at a time point earlier than 96 h (Fig. 2).

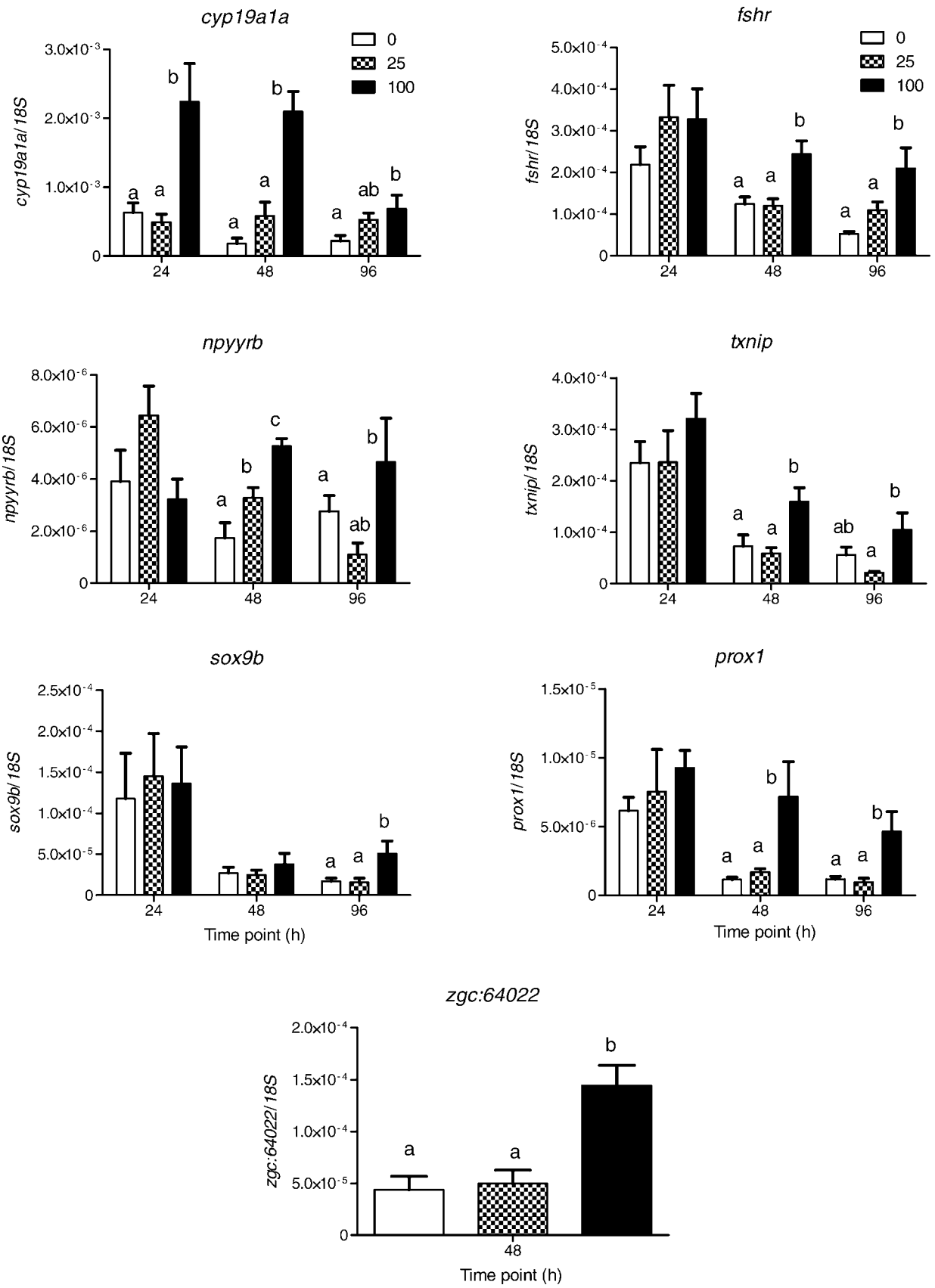

Fig. 1. Relative abundance of *cyp19a1a* (aromatase A), *fshr* (follicle-stimulating hormone receptor), *npyryb* (neuropeptide Y receptor YB), *txnip* (thioredoxin-interacting protein), *sox9b* (sry box-containing gene 9b), *prox1* (prospero-related homeobox gene 1), and *zgc:64022* (a ras-like estrogen-regulated growth inhibitor) transcripts in the ovarian tissue of zebrafish (*Danio rerio*) exposed to 0, 25, or 100 μg/L of fadrozole for 24, 48, or 96 h as determined by quantitative real-time polymerase chain reaction. Gene-specific transcript abundance was normalized to the abundance of 18S ribosomal RNA detected in each sample. Data presented as the mean ± standard error. Different letters indicate statistically significant differences among treatments at a given time point ($p < 0.05$).

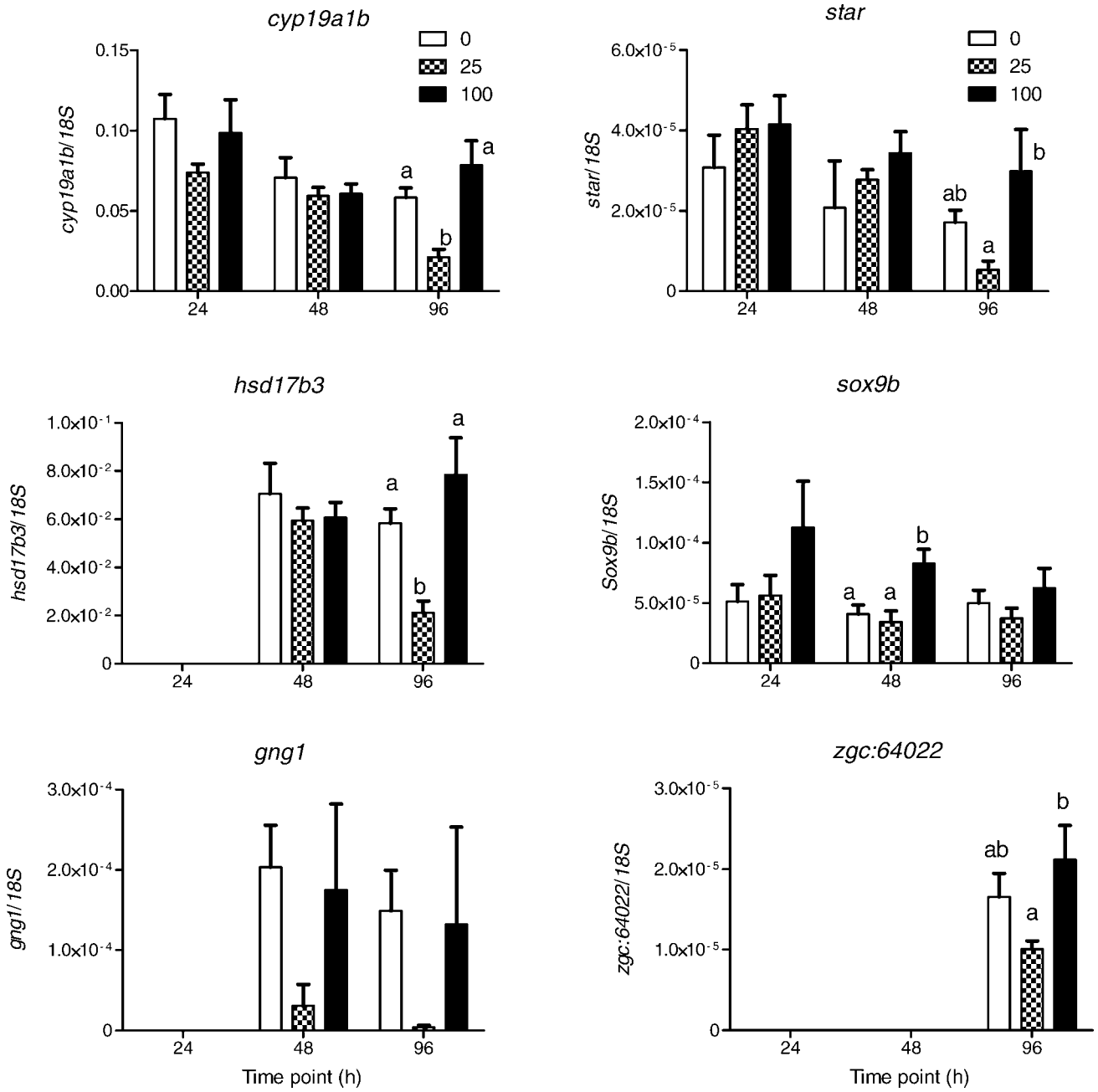

Fig. 2. Relative abundance of *cyp19a1b* (aromatase B), *star* (steroidogenic acute regulatory protein), *hsd17b3* (hydroxysteroid dehydrogenase 17b3), *sox9b* (sry box-containing gene 9b), *gng1* (G protein gamma), and *zgc:64022* (a ras-like estrogen-regulated growth inhibitor) transcripts in the brain tissue of female zebrafish (*Danio rerio*) exposed to 0, 25, or 100 μg/L of fadrozole for 24, 48, or 96 h as determined by quantitative real-time polymerase chain reaction. Gene-specific transcript abundance was normalized to the abundance of 18S ribosomal RNA detected in each sample. Data are presented as the mean ± standard error. Different letters indicate statistically significant differences among treatments at a given time point ($p < 0.05$).

Expression of *sox9b* and *zgc:64022* in the brains of male zebrafish also was examined using QPCR (*Supporting Information,* Fig. S1; http://dx.doi.org/10.1897/08-653.S2). Although overall trends were similar for the QPCR and microarray results, neither gene was detected as statistically significantly up-regulated by QPCR.

Wang et al. [12] previously demonstrated good agreement between the microarray results and QPCR data for more than a dozen genes selected based on their distinct responses to three different endocrine-disrupting chemicals. Overall, the current study also found reasonable agreement between the results of QPCR and microarray analyses for the selected targets. Whereas variability, statistical significance, and absolute fold-change differed between methods, the same trends usually were observed. However, exceptions were noted. For example, at the 96-h time point, QPCR results suggested a nonsignificant down-regulation of *npyryb* expression in the brains of females exposed to 25 μg/L, whereas microarray analysis indicated up-regulation. Similarly, QPCR results for *sox9b* and *zgc:60422* generally suggested either no change or slight (nonsignificant) down-regulation in the brains or ovaries of females exposed to 25 μg/L, whereas microarray analysis indicated up-regulation. Interestingly, in all three cases, significant up-regulation was detected by QPCR, but in animals from the 100 μg/L

Table 2. Top 20 (lowest *p* value) enriched gene ontology (GO) categories associated with genes differentially expressed in the brains of male or female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 or 96 h

Category	Genes in category	% Genes in category	Genes in list in category	% Genes in list in category	*p*
Regulation of biological process, cell cycle					
GO:79: Regulation of cyclin-dependent protein kinase activity	10	0.106	5	1.004	8.03E−05
GO:45859: Regulation of protein kinase activity	33	0.349	6	1.205	0.0068
GO:7049: Cell cycle	275	2.908	24	4.819	0.0102
GO:910: Cytokinesis	91	0.962	11	2.209	0.0080
Development					
GO:9790: Embryonic development	228	2.411	22	4.418	0.0044
GO:7569: Cell aging	3	0.0317	2	0.402	0.0080
GO:48731: System development	370	3.913	31	6.225	0.0069
GO:7420: Brain development	81	0.857	11	2.209	0.0033
Physiological process/organismal physiological process					
GO:30299: Cholesterol absorption	3	0.0317	2	0.402	0.0080
Physiological process/cellular physiological process					
GO:6825: Copper ion transport	8	0.0846	3	0.602	0.0066
Physiological process/metabolism					
GO:8610: Lipid biosynthesis	69	0.73	11	2.209	0.0009
GO:6725: Aromatic compound metabolism	75	0.793	10	2.008	0.0057
GO:6563: L-Serine metabolism	18	0.19	5	1.004	0.0019
GO:9069: Serine family amino acid metabolism	29	0.307	6	1.205	0.0034
GO:9070: Serine family amino acid biosynthesis	17	0.18	4	0.803	0.0105
GO:6260: DNA replication	79	0.835	14	2.811	5.54E−05
GO:6259: DNA metabolism	324	3.426	28	5.622	0.0066
GO:6636: Fatty acid desaturation	9	0.0952	4	0.803	0.0008
GO:42402: Biogenic amine catabolism	4	0.0423	2	0.402	0.0155
Cell differentiation					
GO:10001: Glial cell differentiation	4	0.0423	2	0.402	0.0155

rather than the 25 μg/L group. The cause of the discrepancy between methods for these particular genes and conditions is unclear.

Functional analysis: Gene ontologies

At the time these analyses were conducted, approximately 35% of the probes on the zebrafish microarray were annotated with one or more GO terms describing gene function within the general category of biological process. Among the 1,861 genes (probes) classified as differentially expressed in the present study, 718 (38.6%) were annotated with biological process GO terms. That included 41% of those differentially expressed in brain and 35% of those differentially expressed in ovary. This annotated subset of genes was used for functional analyses.

The top 20 GO categories significantly enriched (p = 0.000055–0.0155) in brain were organized into six groups (Table 2). Four enriched categories were associated with genes involved in cell-cycle regulation, and a second group of four enriched categories was related to development. Differential expression of two probes for caveolin 1 suggested alterations in cholesterol absorption, whereas three genes coding for proteins with a role in copper ion transport indicated significant enrichment within that category. Nine of the enriched categories were organized under the general physiological process of metabolism. These included genes involved in DNA replication and metabolism, serine family amino acid metabolism, fatty acid and lipid metabolism, and biogenic amine metabolism. Finally, differential expression of two genes represented a statistical enrichment of those on the array known to be associated with glial cell differentiation.

When analysis of GO enrichment was restricted to each time/sex condition analyzed for brain, sex-dependent differences in functional grouping were evident. The dominant GO terms among the 20 most significant biological processes associated with genes differentially expressed in the brain of females exposed for 48 or 96 h were related to cell cycle, cell division, and DNA replication processes (*Supporting Information,* Tables S9 and S10; http://dx.doi.org/10.1897/08-653.S1). In contrast, GO terms associated with responses to stress, biotic stimuli, immune and defense response, and cell proliferation were dominant among the 20 most significantly enriched processes in the brain of males exposed for 96 h (*Supporting Information,* Table S11; http://dx.doi.org/10.1897/08-653.S1). Lipid- and fatty acid–related GO terms were enriched for females exposed for 96 h, but not for 48 h, whereas transcriptional changes associated with L-serine metabolism were prominent at both time points (*Supporting Information,* Tables S9 and S10). No specific GO terms were present among the top 20 enriched processes in brain for all three time/sex conditions examined.

The top 20 enriched biological process GO terms associated with fadrozole-induced gene expression changes in ovary (p = 0.0000037–0.0071) were quite different from those for brain. They were organized into three broad categories (Table 3). The first group pertained to cellular processes, including cell adhesion processes, driven by changes in expression of cadherins, fibulins, procollagens, and certain growth factors, as well as vascular development signaled by changes in the vascular endothelial growth factor (VEGF) and neuropilin expression. A second group of enriched GO terms described physiological processes primarily associated with ion transport. That group was primarily composed of genes coding for collagens and procollagens. Finally, a third group of enriched

Table 3. Top 20 (lowest *p* value) enriched gene ontology (GO) categories associated with genes differentially expressed in the ovaries of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 or 96 h[a]

Category	Genes in category	% Genes in category	Genes in list in category	% Genes in list in category	*p*
Cellular process					
GO:48010: VEGF receptor signaling pathway	9	0.0952	5	0.967	5.03E−05
GO:7156: Homophilic cell adhesion	34	0.36	9	1.741	6.24E−05
GO:16337: Cell–cell adhesion	42	0.444	9	1.741	0.000359
GO:1570: Vasculogenesis	9	0.0952	5	0.967	5.03E−05
GO:7155: Cell adhesion	164	1.734	19	3.675	0.00153
Physiological process					
GO:6817: Phosphate transport	38	0.402	11	2.128	3.66E−06
GO:15698: Inorganic anion transport	61	0.645	13	2.515	1.96E−05
GO:6752: Group transfer coenzyme metabolism	72	0.761	10	1.934	0.00551
GO:6820: Anion transport	78	0.825	14	2.708	7.18E−05
GO:6811: Ion transport	629	6.652	53	10.25	0.000965
GO:50874: Organismal physiological process	451	4.769	41	7.93	0.000869
Development					
GO:16331: Morphogenesis of embryonic epithelium	23	0.243	5	0.967	0.0071
GO:9880: Embryonic pattern specification	50	0.529	8	1.547	0.00532
GO:9887: Organ morphogenesis	220	2.327	24	4.642	0.000929
GO:1502: Cartilage condensation	7	0.074	3	0.58	0.00482
GO:48513: Organ development	475	5.023	50	9.671	5.10E−06
GO:7389: Pattern specification	169	1.787	19	3.675	0.00217
GO:9653: Morphogenesis	382	4.04	33	6.383	0.00596
GO:9790: Embryonic development	228	2.411	22	4.255	0.00673
GO:7275: Development	1,254	13.26	94	18.18	0.000686

[a] VEGF = vascular endothelial growth factor.

biological process GO terms was related to development, including morphogenesis and pattern specification. The GO terms related to cell cycle, serine amino acid metabolism, and lipid metabolism, which were associated with gene expression changes in female brain, were not among the top 20 biological processes associated with genes differentially expressed in ovary.

Within ovary, the prominent enriched GO categories were largely conserved among the two time points examined. Samples collected at both 48 and 96 h had changes in gene expression associated with cell adhesion, development, and ion transport (*Supporting Information,* Tables S12 and S13; http://dx.doi.org/10.1897/08-653.S1). The most prominent difference was that GO terms related to vasculogenesis and VEGF signaling were not among the biological process GO terms significantly enriched in 96-h ovary samples. Instead, an enrichment of gene expression changes was associated with sensory perception, detection of light stimulus, and so on, driven by changes in the expression of four different opsin genes as well as certain G proteins, nerve growth factors, and other genes associated with the retinal photoreceptor layer (e.g., *zgc:56548*) (*Supporting Information,* Table S13). Cholesterol absorption was among the top 20 enriched GO terms at both time points. This was driven by changes in the expression of two probes for caveolin 1, however, and the term dropped out of the top 20 when the gene lists from both time points were combined (Table 3).

Promoter motifs of differentially expressed gene clusters

Using two separate algorithms, 32 promoter motifs were identified as potentially associated with coexpressed clusters of genes that were significantly differentially expressed in fadrozole-exposed zebrafish (Table 4). BioProspector identified 26 motifs, whereas CisModule identified 13 motifs. Seven motifs were identified using both algorithms, including cap signal for transcription initiation, GATA-binding factor 2, Ikaros 2, alcohol dehydrogenase gene regulator 1, heat shock factor (*Drosophila*), heat shock factor (yeast), and myoblast-determining factor (Table 4), adding greater confidence in those particular results. Among the motifs identified by BioProspector only, GATA-binding factor 1 was the motif with the greatest confidence score. Five different GATA binding–related motifs were identified, providing additional weight of evidence for an effect the expression of genes with GATA-binding motifs.

DISCUSSION

Real-time QPCR

From the hundreds of genes identified as differentially expressed based on microarray analysis, QPCR was used to try to develop a more complete understanding of the time- and/or concentration-dependence of a handful of transcriptional responses of particular interest. For example, fadrozole-induced changes in the expression of *cyp19a1a* were examined previously in the ovaries of female fathead minnows exposed for 7 d [8]. Based on the results of that study, a concentration- and time-dependent compensatory up-regulation of *cyp19a1a* transcripts in zebrafish was expected. Real-time QPCR-based examinations of the entire time-course and concentration-response profile were consistent with that expectation, suggesting that ovarian induction of *cyp19a1a* is a common compensatory response to aromatase inhibition in multiple fish species. Similarly, based on evidence that binding of follicle-stimulating hormone to follicle-stimulating hormone receptor tends to support vitellogenesis and preovulatory follicle growth, at least in fish species that spawn annually [19], it was hypothesized that *fshr* expression may be induced as part of a compensatory response to impaired vitellogenesis associated with aromatase inhibition [7,17]. This hypothesis also was

Table 4. Promoter motifs associated with differentially expressed gene clusters identified using two separate algorithms, BioProspector [16] and CisModule [15]

Algorithm[a]	Score[b]
BioProspector motifs	
GATA-binding factor 1	39
Cap signal for transcription initiation	29
Deformed	27
Activator of nitrogen-regulated genes	22
GATA-binding factor 2	22
CCAAT/enhancer-binding protein	14
Broad-complex Z3	12
Ikaros 2	7
3′-Part of bipartite RAV1-binding site, interacting with AP2 domain	4
Alcohol dehydrogenase gene regulator 1	3
Heat shock factor 2	3
Transcriptional repressor CDP	3
Activator protein 2	2
Clox	2
Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2	2
Bicoid	1
DeltaEF1	1
GATA-binding site	1
GATA-binding factor 3	1
Heat shock factor (*Drosophila*)	1
Heat shock factor (yeast)	1
Maize activator P of flavonoid biosynthetic genes	1
Myoblast-determining factor	1
N-Myc	1
Sex-determining region Y gene product	1
Yeast repressor/activator protein 1	1
CisModule motifs	
Cap signal for transcription initiation	46
Alcohol dehydrogenase gene regulator 1	14
Activator protein 4	3
GATA-binding factor 2	3
Myoblast-determining factor	3
Heat shock factor (*Drosophila*)	2
Heat shock factor (yeast)	2
Maternal gene product	2
TCF11/KCR-F1/Nrf1 homodimers	2
Aspergillus stunted protein	1
c-Ets-1(p54)	1
Dof2-single zinc finger transcription factor	1
Ikaros 2	1

[a] Common motifs identified using both algorithms are shown in boldface.
[b] Score indicates the relative degree of confidence in the motif identification. A higher score indicates greater confidence.

supported by the QPCR analysis as well as microarray analysis. Thus, these results provided increased confidence in the ability to use a systems model of the teleost HPG axis [17] to predict certain molecular responses associated with exposure to aromatase inhibitors.

Other results modify a previous understanding of HPG-axis regulation. For example, binding of neuropeptide Y to neuropeptide Y receptors is thought to be involved in the regulation of gonadotropin secretion by the brain and pituitary [17,20]. Furthermore, *npyryb* expression was has been shown to be altered in the telencephalon of zebrafish exposed to a potent estrogen, ethynylestradiol, for 21 d [21]. However, both microarray and QPCR results from this study suggested an impact on *npyryb* expression in the ovary of fadrozole-exposed zebrafish. Based on microarray results, expression in whole brain was not affected. Neuropeptide Y transcripts have been detected in fish ovarian tissue (see, e.g., [22] and, in human ovarian tumor cells, [23]), but to our knowledge, a role for neuropeptide Y receptors in the ovary is not well understood.

Unsupervised analysis of the microarray data helped to identify several genes that were differentially expressed among multiple conditions tested in the present study as well as in other studies that examined effects of either strong estrogens or aromatase inhibitors on global gene expression. The robust nature of these specific transcriptional responses across different time points, tissues, and even studies suggests they might be useful markers of exposure to, or effects of, chemicals that modulate circulating estrogen concentrations. These genes included those coding for the transcription factor *sox9b*, an endogenous inhibitor of thioredoxin *txnip*, and a ras-like estrogen-regulated growth inhibitor, *zgc:64022.* All three targets were differentially expressed in fadrozole-treated fish in four of the five time/sex/tissue conditions analyzed by microarray (48-h female brain being the exception). Additionally, *sox9b* was among the genes up-regulated in the telencephalon of zebrafish exposed to ethynylestradiol [21]. Teleost aromatase genes (*cyp19a1a* and *cyp19a1b*) have been reported to contain multiple sry/sox-binding sites in their promoter regions [9]. Expression of *txnip* was altered in MCF-7aro cells treated with aromatase inhibitors or tamoxifen [24]. A goldfish transcript homologous to *zgc:64022* was up-regulated in the brain of goldfish exposed to ethynylestradiol for 15 d [25]. In the present study, QPCR results for these genes did not suggest significant effects at the 24-h time point. Nor did QPCR-based analysis detect the statistically significant effects in fish exposed to 25 μg/L. However, the data indicated significant modulation of ovarian expression of all three of these gene targets as a result of exposure to 100 μg/L. Together, gene expression data from the present study, as well as previous reports, provide weight of evidence for estrogen-related regulation of these genes.

Unlike the genes discussed above, *prox1* was identified as up-regulated in the ovary, but not in the brain, of fadrozole-exposed zebrafish. PROX1 is an important regulator of angiogenesis, particularly lymphangiogenesis, and also has the ability to repress the transcriptional activity of liver receptor homologue 1 (*lrh-1*), a gene involved in brain aromatase activation and gonadtrope regulation, and of *ff1b*, the zebrafish homologue of SF-1 [26–31]. Real-time QPCR results confirmed the fadrozole-induced up-regulation of ovarian *prox1* expression. Thus, *prox1* transcription may have potential as a marker of exposure to and/or effects of aromatase inhibitors, particularly if a functional linkage to apical outcomes can be described.

Targets for expanded QPCR-based analysis in brain tissue were identified using a rationale similar to that used to select specific ovary targets. The genes s*tar, cyp19a1b*, and *hsd17b3* code for steroidogenic enzymes or transport proteins that play a role in local estrogen synthesis in the brain [32]. Microarray results indicated up-regulation of *star* (at 48 h) and *hsd17b3* (at 96 h) in female brain but did not indicate an effect on *cyp19a1b*, in contrast with previous studies showing down-regulation of *cyp19a1b* in fadrozole-exposed fathead minnows [8,33]. As noted above, *sox9b* and *zgc:64022* were differentially expressed in both brain and ovarian tissue and were modulated in other studies with strong estrogens or aromatase inhibitors. We also examined expression of the gene coding for G protein gamma subunit 1 (*gng1*), which was one of the few genes identified as being down-regulated in fadrozole-exposed fish. Neither *cyp19a1b, star*, nor *sox9b* was affected

in the brains of female zebrafish exposed for just 24 h. At the 48-h time point, the direction of fold-change relative to controls, determined by QPCR, was in agreement with the microarray results, but no statistically significant differences were detected. At 96 h, all the genes examined using QPCR showed a similar, V-shaped concentration–response profile. Whereas 18S exhibited the opposite profile (i.e., a Λ-shaped concentration–response profile at the 96-h time point; data not shown), normalization to 18S only partially accounted for the common trend observed. Thus, the QPCR results suggest that in brain, the transcriptional responses to 100 μg/L may have been quite different from responses to 25 μg/L. These differences were not only in the traditional sense of concentration-dependent magnitude of alteration but also in the time course of the responses and/or the overall nature and mechanism of response to the stressor. Such results reinforce the need for caution in using a few snapshot views to understand the overall response of a biological system in multiple dimensions defined by chemical concentration, duration of exposure, and tissues/targets within the organism [34]. Additionally, they help to highlight the fact that although fadrozole was selected as a relatively specific inhibitor of aromatase activity, the chemical may have other actions beyond direct aromatase inhibition, particularly at higher concentrations. Potential markers or mechanisms associated with aromatase inhibition in the present study should be confirmed through investigations with other aromatase inhibitors.

Promoter motifs

Promoter motifs can be thought of as a common pattern of binding sites for a specific type of transcription factor. It is hypothesized that genes regulated by the same transcription factor or factors should have similar response profiles in space and time as a function of exposure to a stressor. Thus, analysis of common promoter motifs for genes empirically identified as coexpressed can be used to elucidate some of the primary transcription factors activated or repressed in response to a chemical exposure.

Using two separate algorithms, seven common promoter motifs that were associated with coexpressed clusters of genes that were differentially expressed in response to fadrozole exposure were identified (Table 4). Two of these, as well as one additional motif identified by BioProspector, were heat shock factor motifs. Induction of heat shock proteins has long been considered to be a classic feature of organism response to stress [35,36], so it was not surprising to see heat shock factor motifs associated with transcriptional responses to fadrozole exposure. The motif *cap signal for transcription initiation* is similarly nonspecific.

Some of the promoter motifs, however, point more specifically to fadrozole's mode of action and/or specific gene functions. For example, the common identification of the GATA-binding factor 2 motif, as well as identification of four other GATA-related motifs using BioProspector, could be more directly related to fadrozole's specific mode of action. The GATA-2– and GATA-4–binding motifs have been reported to be present in the promoter region of the zebrafish aromatase genes [9,37]. Aromatase, StAR, and inhibin α, all of which were detected as differentially expressed in the present study, but were not identified as part of the K-mean coexpression clusters used for the motif analysis (*Supporting Information,* Table S15; http://dx.doi.org/10.1897/08-653.S1), have been shown to contain GATA-binding motifs and are modulated by GATA-binding factors [38]. The GATA motifs, particularly GATA-1, -2, and -3, are involved in the regulation of hematopoiesis [39], suggesting this as a potential function altered by fadrozole exposure. Notably, Ikaros motifs have been referred to as master hematopoietic switches [40]. In lamprey, expression of Ikaros-like transcription factors was reported to be greatest in the ovary and testis based on reverse-transcription polymerase chain reaction and in situ hybridization [41], suggesting a potentially important role for Ikaros transcription factors in regulating reproductive processes as well. The significance of alcohol dehydrogenase gene regulator 1 and myoblast-determining factor as additional motifs associated with genes modulated by fadrozole is not clear. However, the promoter motifs identified in this study provide a basis for hypothesis formulation and testing. Genes known to contain these motifs in their promoter regions are potential targets for modulation following exposure to fadrozole. Future investigations should help to elucidate whether these transcription factors are involved in molecular response to other aromatase inhibitors specifically or to other chemicals that elicit similar physiological effects (e.g., impaired estradiol synthesis and/or vitellogenesis) more generally.

Hypothesis generation: Transcriptional changes in brain

Gene expression changes observed in the brain of fadrozole-treated zebrafish were suggestive of a nascent cell proliferation response, particularly in females. Gene ontology analysis revealed an enrichment of differentially expressed genes associated with cell cycle, cytokinesis, DNA metabolism and replication, and cyclin-dependent protein kinase activity, all of which would be consistent with increased cell division. Furthermore, five genes coding for enzymes involved in the one-carbon pool by folate pathway (of 25 enzymes in the pathway; KEGG 00670) were up-regulated in the brain of fadrozole-exposed fish (Fig. 3). The role of folate as a critical coenzyme involved in cell division is well established [42]. Folate deficiency and resulting limitations in thymidylate synthesis result in impaired DNA synthesis, whereas overproduction of thymidylate may contribute to uncontrolled cell division and tumor formation [42]. Up-regulation of genes coding for thymidylate synthase (*tyms*), dihydrofolate reductase (*dhfr*), serine hydroxymethyltransferase (*shmt1*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*mtr*), and phosphoribosylglycinamide formyltransferase (*gart*) all could potentially increase the available pool of tetrahydrofolate and/or thymidylate to support DNA synthesis and cell division (Fig. 3). A role for aromatase/estrogen-mediated modulation of the folate pathway is supported by previous work in which transcripts of three genes in this pathway (*tyms, dhfr*, and methylenetetrahydrofolate reductase [*mthfr*]) were down-regulated in biopsy specimens of human breast tumors treated with the aromatase inhibitor letrozole [43]. Along with expression changes suggestive of nascent cell division, a number of transcription factors (e.g., homeobox B5a, hairy-related 5, forkhead box b1, and achaete-scute complex 1b) known to be involved in regulation of brain development were up-regulated in fadrozole-exposed females. As a whole, this profile of gene expression suggests that fadrozole treatment resulted in, or may lead to, cell proliferation and/or brain remodeling.

Estradiol is known to have neurotrophic, neuroprotective, and organizational effects in the brain, and it has been hypothesized that locally produced estrogens may be key modulators of lifelong neurogenesis in fish [44–46]. Given that

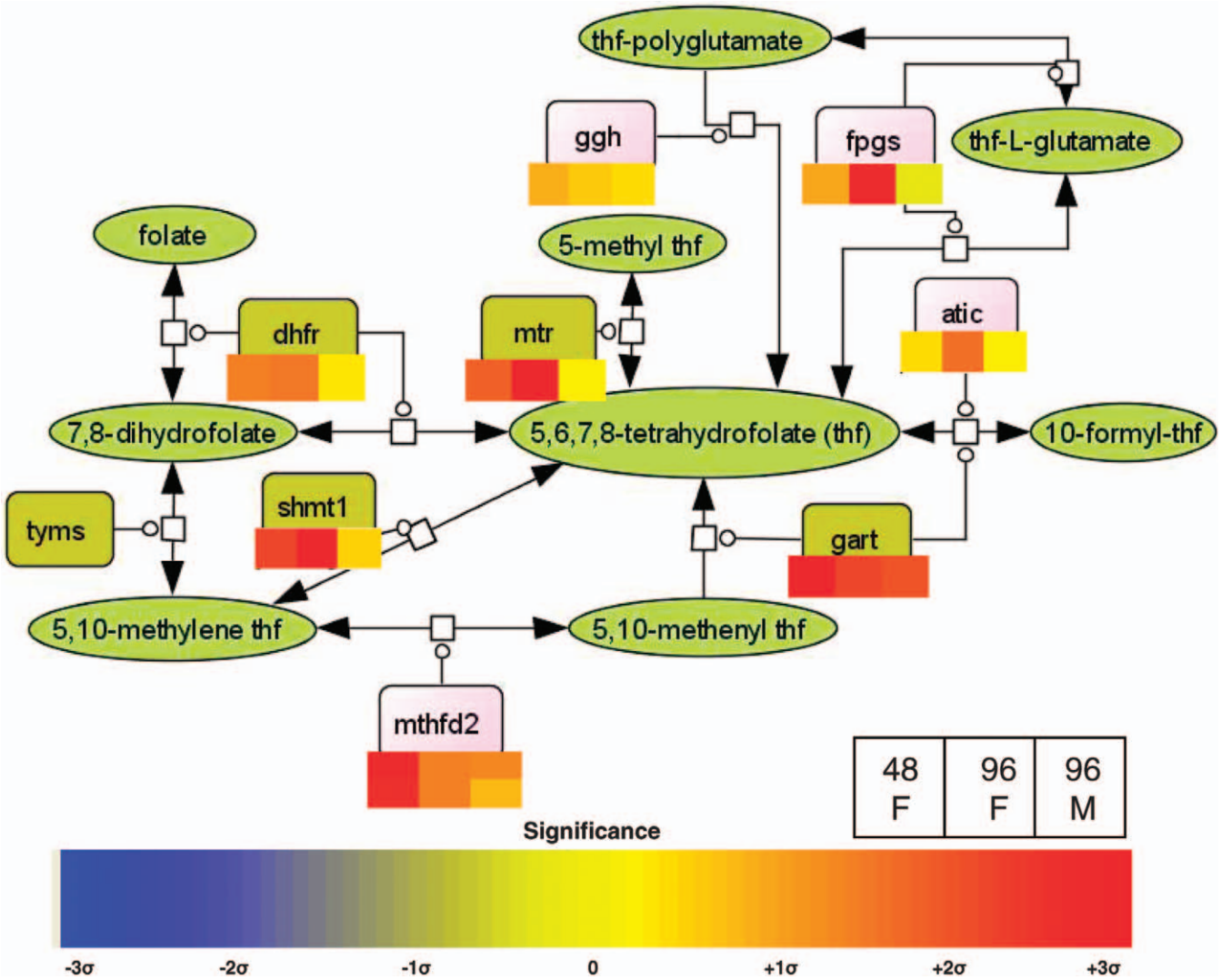

Fig. 3. Portion of the one-carbon pool for folate pathway (KEGG 00670) showing genes coding for enzymes in the pathway that were significantly up-regulated in the brains of zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole. Boxes represent enzymes in the pathway. Ovals identify metabolic reaction reactants/products. Arrows (→, ↔) indicate reactions; a ball and stick (— ○) indicates catalysis. Of 25 enzymes in the KEGG pathway, only nine with corresponding probes on the microarray, and the associated reactions, are shown. Green indicates the corresponding gene was significantly affected by fadrozole exposure, pink indicates lack of significant modulation. The color bar next to each box indicates the significance of up- or down-regulation for each condition tested (e.g., 48-h female, 96-h female, and 96-h male). atic = 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase; dhfr = dihydrofolate reductase; fpgs = folylpolyglutamate synthase; gart = phosphoribosylglycinamide formyltransferase; ggh = gamma-glutamyl hydrolase; mthfd2 = methylenetetrahydrofolate reductase; mtr = 5-methyltetrahydrofolate-homocysteine methyltransferase; shmt1 = serine hydroxymethyltransferase; thf = tetrahydrofolate; tyms = thymidylate synthase.

fadrozole has been shown to inhibit aromatase activity in the brain of fathead minnows [7,8], neurodegeneration and an overall reduction in cell division/proliferation in brain as a result of reduced local production of estradiol might be hypothesized. In the present study, however, gene expression associated with cell division/proliferation was up-regulated. Furthermore, specific gene expression changes have been identified as part of a common mechanism used by nervous tissue to cope with neurodegenerative conditions [46]. Expression of *star*, which has been shown to be up-regulated in the brain following excitotoxic injury and in aged animals [47], was up-regulated in the brain of females after 48 h of fadrozole exposure (based on microarray results; *Supporting Information,* Table S4). The gene coding for cytochrome P450c17 (*cyp17a1*; *zgc:66494*) was up-regulated in the brain of females after 96 h of exposure (*Supporting Information,* Table S5). Cytochrome P450 17a1 is a steroidogenic enzyme involved in the synthesis of progesterone and progesterone derivatives, which have been shown to promote myelin formation and repair of injured nerves [46]. Glutamate transporter and insulin-like growth factor-I, both of which have been reported to modulate brain damage [46], also were up-regulated in female and male brain, respectively, after 96 h of fadrozole exposure. Gene ontology–based analysis of the expression changes in male brain suggested an overall profile of expression changes associated with response to stress, wounding, and/or pathogens, as well as up-regulation of regulators of cell proliferation (*Supporting Information,* Table S11). Although somewhat different from the GO profile for females, the responses as a whole suggest a common theme of potential compensation to neurodegenerative stress resulting from inhibition of local estrogen synthesis.

Thus, microarray-based analysis of the gene expression changes in the brain of zebrafish exposed to fadrozole suggests a number of hypotheses. First, it was hypothesized that many of the transcriptional changes observed reflect an attempt by the organism to compensate for the neurodegenerative stress associated with reduced estradiol production in the brain. Furthermore, it was hypothesized that part of that compensatory response involves increased proliferation of radial glial cells, which have been shown to be the primary sites of *cyp19a1b* gene expression and aromatase B immunoreactivity within the teleost brain [44]. Expression of *cyp19a1b*, the primary isoform of aromatase expressed in fish brain, appears to be estrogen-dependent [48]. Therefore, proliferation of the *cyp19a1b*-expressing cell type may be the most expedient mechanism for the fish to compensate for the stressor. The hypotheses derived from this microarray study could be tested by examining expression of several of the up-regulated genes in more intensive time-course experiments with fadrozole that incorporate a recovery period following exposure. Additionally, histological analysis of brain tissue to test for signs of neurodegeneration and radial glial cell proliferation over the course of the exposure could be very informative.

Hypothesis generation: Transcriptional changes in ovary

Many of the well-annotated genes up-regulated in the ovaries of fadrozole-exposed zebrafish play a role in preovulatory growth and development of ovarian follicles in mammals (Fig. 4). For example, in mammals, preovulatory follicle development is accompanied by angiogenesis—specifically, the formation of capillary networks that nourish the rapidly growing follicles [49]. A number of genes that code for proteins involved in promoting angiogenesis and/or lymphangiogenesis were up-regulated in fadrozole-exposed zebrafish. The gene coding for a well-known angiogenic factor, VEGF, was significantly up-regulated in the ovaries of fish exposed for 48 h (*Supporting Information,* Table S14; http://dx.doi.org/10.1897/08-653.S1). In mammals, VEGF is expressed by granulosa cells and is thought to regulate angiogenesis in the theca cell layer of the developing follicle [49–51]. As in mammals, the theca cell layer of zebrafish follicles is highly vascularized, and theca cells increase in size and numbers throughout the follicular growth stages [52]. Neuropilins that bind VEGF and have hormonally regulated expression during the rat reproductive cycle [53] also were up-regulated in fadrozole-exposed zebrafish ovaries (Fig. 4 and *Supporting Information,* Table S14; Fig. 4). Nerve growth factor, which has been shown to induce *fshr* expression, estradiol production, and *vegf* expression in rat ovary [54,55], was up-regulated in zebrafish ovary after both 48 and 96 h of exposure (*Supporting Information,* Table S14). Similarly, *prox1* was up-regulated in ovary at both time points (*Supporting Information,* Table S14). As noted above, PROX1 is an important regulator of angiogenesis and also is involved in regulation of the reproductive axis [26–31]. Up-regulation of five different homeodomain transcription factors (hox and hhex) and a mid-kine–related growth factor (*Supporting Information,* Table S14) also point toward an angiogenic response [31,56]. Overall, the gene expression profile suggests that up-regulation of proangiogenic factors constituted one component of ovarian response to fadrozole exposure.

A second process that seemed to be influenced by fadrozole was cell–cell contact and/or communication. In addition to vascular development, follicle development in mammals requires close cell–cell contact to facilitate intercellular communication and follicular structural integrity [57,58]. Gene ontology terms related to cell adhesion were prominent among the enriched GO categories associated with ovary (Table 3). In particular, at least five different cadherin genes were up-regulated, as was fibulin 4 (Fig. 4 and *Supporting Information,* Table S14). Cadherins are among the proteins known to play an important role in cell–cell adhesion and various aspects of tissue development [57,59]. The gene coding for ephrin b1, which is expressed in granulosa cells and is involved in cell–cell recognition as well as angiogenesis [60], was up-regulated after 96 h of exposure to fadrozole (*Supporting Information,* Table S14). Additionally, notch 3 and jagged, which help to regulate the formation of cell–cell contacts, including cadherin-mediated junctions, cell matrix interactions, and other aspects of morphogenesis [61,62], were differentially expressed in the ovaries of fadrozole-exposed zebrafish (*Supporting Information,* Table S14). Thus, both GO analysis and examination of individual gene expression changes indicate greater expression of cell adhesion– and cell–cell communication–related genes in fadrozole-treated fish than in controls.

Examination of the differentially expressed genes comprising the ion transport–related GO terms that were enriched in the ovary of fadrozole-treated fish revealed that the majority of genes were collagens (e.g., procollagens 1α2, Vα2, and 9α2; collagen types 1α1 and 1α3) (*Supporting Information,* Table S14). Collagens are key components of extracellular matrix and connective tissue, and changes in extracellular matrix are associated with ovarian follicle development [63,64]. Extracellular matrix components, including type I collagen, also have been shown to modulate granulosa cell proliferation, shape, and steroidogenic activity [64]. Thus, the increased transcription of collagen-related genes may reflect changes in extracellular matrix associated with zebrafish ovarian cell differentiation and/or follicle development.

In addition to those discussed above, the function of a number of other differentially expressed genes could be associated with follicle development. For example, Rap1 is a key member of one of three different signaling cascades that mediate the effects of follicle-stimulating hormone on granulosa cell differentiation [65]. Genes coding for two isoforms of Rap1 were differentially expressed (*Supporting Information,* Table S14). Noggin and gremlin (both up-regulated in the ovary of fadrozole-exposed zebrafish) are antagonists of bone morphogenetic proteins, which are members of the transforming growth factor β superfamily that are involved in ovarian follicle development [66–68]. Quattro and gelsolin (*Supporting Information,* Table S14) help to regulate movements of the actin cytoskeleton to support growth and morphogenesis [69–71]. Transcripts for insulin-like growth factor–binding protein 5 (IGFBP5) were up-regulated in the ovaries of fadrozole-exposed zebrafish. In rainbow trout, IGFBP5 showed limited up-regulation during the postvitellogenic phase of follicle development but down-regulation during final oocyte maturation [72]. As a whole, both the GO-based analysis and the examination of individual gene expression changes point toward a pattern of up-regulated gene expression that would be consistent with early follicle development.

Conversely, relatively few changes in gene expression would commonly be associated with final oocyte maturation and/or ovulation. Ovulation typically involves proteolysis of extracellular matrix mediated by matrix metalloproteinases, vascular permeabilization accompanied by activation of co-

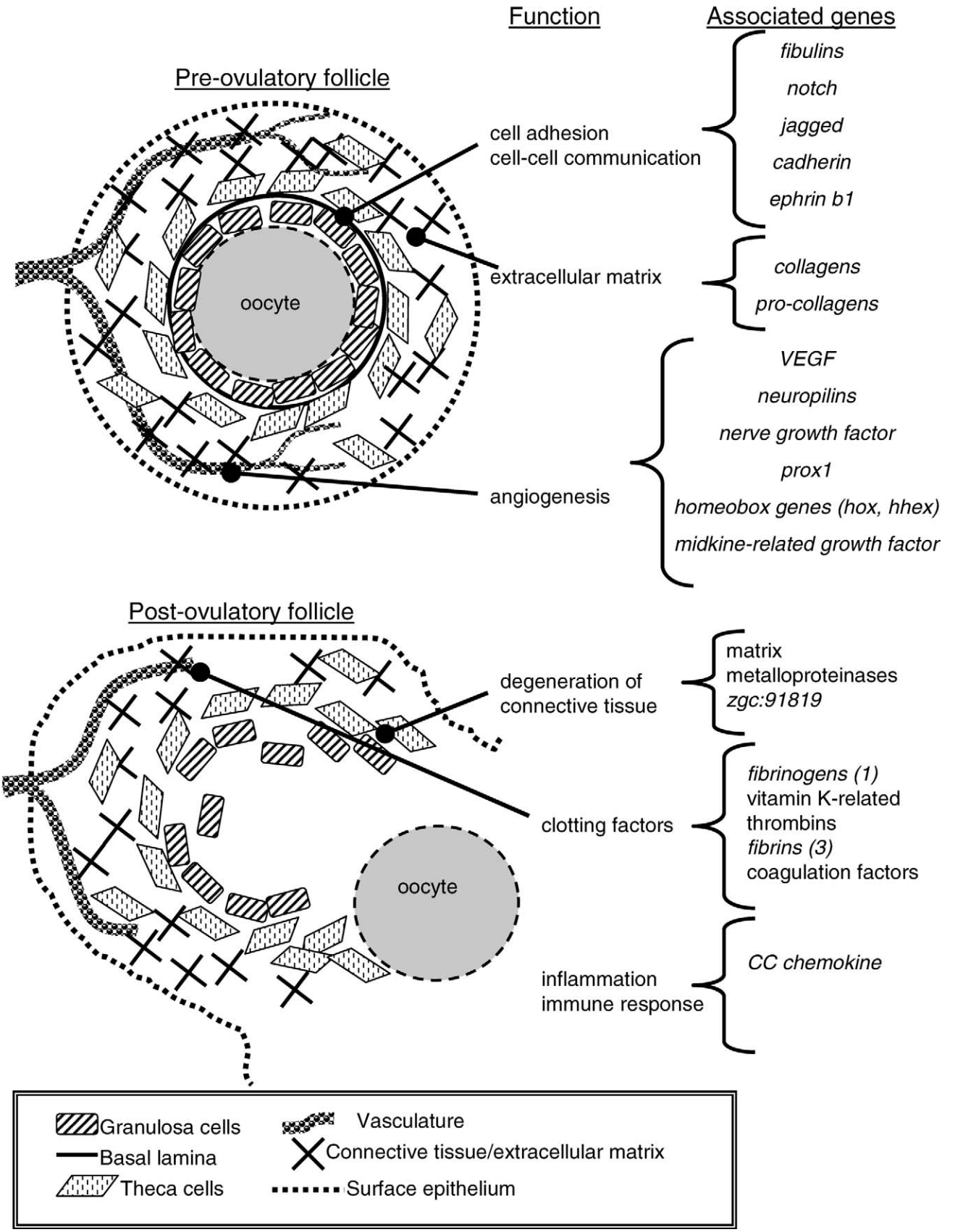

Fig. 4. Cartoon representation of some major features of preovulatory versus postovulatory follicles and genes associated with function related to each stage. Italics indicate genes or classes of genes that were detected as up-regulated in the ovaries of zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole. Other genes/gene classes were representative of the associated function but were not necessarily affected in the experiment. Relative sizes of cells, vasculature, and so on are not necessarily to scale. VEGF = vascular endothelial growth factor.

agulation cascades and clotting factors, influx of immune cells, and ultimately, follicular rupture as the oocyte is released [73–77] (Fig. 4). No GO terms related to these processes were among the significantly enriched categories for ovary (Table 3 and *Supporting Information,* Tables S11 and S12). A couple of individual gene expression changes would be consistent with the profile described above. For example, up-regulation of *zgc:91819* (probe id A_15_P114118), which codes for a protein involved in collagen breakdown, would be consistent with proteolysis of extracellular matrix. Additionally, fibrinogen γ polypeptide (*zgc:56023*; probe id A_15_P121055) was up-regulated in ovary. A search of the annotation information available for the genes differentially expressed in ovary revealed that just 1 of 15 fibrinogen-related genes, 0 of 7 vitamin K-related genes, 0 of 33 coagulation-related genes, 0 of 7 thrombin-related genes, and 3 of 15 fibrin-related genes were among those altered in fadrozole-treated fish. None of the 17 genes on the array annotated as metalloproteinases were altered in ovary, and of 121 genes annotated as having immune-related functions, only six were differentially expressed in ovary. At least one of those, a CC chemokine (probe id A_15_P118283), has been reported to play a role in ovulation [78]. On the whole, the differential gene expression in the ovary seems to be associated predominantly with preovulatory stages of follicle development.

Based on the expression profile observed, it was hypothesized that many of the changes in gene expression observed in the ovaries of fadrozole-exposed fish may be associated with disruptions in final maturation and ovulation resulting from impaired vitellogenesis. Previous studies with fathead minnows demonstrated that exposure to fadrozole could significantly reduce circulating plasma vitellogenin concentrations in females in as little as 24 h [33]. Following 21 d of exposure, reduced fecundity by fadrozole-exposed fish could be clearly linked to a reduction in mature and postovulatory follicles and an increase in the number of preovulatory atretic follicles [7]. It seems unlikely that 48 to 96 h of exposure resulted in histological changes of the same magnitude and severity as those observed in the 21-d fathead minnow study. Nonetheless, compared to control animals, the fadrozole-exposed fish may have had considerably more follicles stalled at early developmental stages (e.g., stages I–III [52]) because of reduced vitellogenin uptake. Thus, it was hypothesized that the profile of differential gene expression observed is indicative of deterred follicle development as a result of fadrozole exposure. If these hypotheses are correct, other chemical stressors that reduce circulating vitellogenin concentrations in females should produce similar changes in gene expression.

Future directions

The results of the present study are not definitive in a traditional sense; rather, they provide an empirical foundation on which to base future investigation. Numerous other hypotheses and interesting results that were not addressed here could be discussed and explored. Data are available to the scientific community in the National Institute of Environmental Health Sciences, Chemical Effects in Biological Systems Knowledgebase (study accession no. 010-00002-0002-000-4), and other investigators are invited to explore them in detail. Subsequent testing of the novel hypotheses derived from these and similar microarray data should lead to a more comprehensive understanding about the response of biological systems to endocrine disruptors and other chemical stressors. In the near term, such research may seem to be academic. However, it is envisioned that longer term, broader elucidation regarding mechanisms of toxicity, facilitated by the application of genomic, bioinformatic, computational, and systems biology approaches to toxicology, should lead to significant innovations in toxicity testing and substantive improvements in predictive ecological risk assessment [79]. Additionally, some of the specific transcriptional responses identified through this unsupervised analysis may have potential utility as biomarkers of exposure and/or effect. Thus, additional efforts to examine the sensitivity, specificity, and persistence of those putative biomarker responses and their functional relationship to adverse outcomes could provide nearer term benefits to the field.

SUPPORTING INFORMATION

Table S1. Overview of samples analyzed by microarray and real-time polymerase chain reaction (QPCR).

Table S2. Sequence accession numbers and primer sequences for genes analyzed using quantitative real-time polymerase chain reaction.

Table S3. Female wet weight, gonad weight, and gonadosomatic index (mean ± standard deviation) by treatment for the 24-, 48-, and 96-h sample groups.

Table S4. Genes identified as significantly differentially expressed in the brain of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 h.

Table S5. Genes identified as significantly differentially expressed in the brain of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S6. Genes identified as significantly differentially expressed in the brain of male zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S7. Genes identified as significantly differentially expressed in the ovary of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 h.

Table S8. Genes identified as significantly differentially expressed in the ovary of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S9. Top 20 (lowest *p* value) enriched gene ontology categories associated with genes differentially expressed in the brain of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 h.

Table S10. Top 20 (lowest *p* value) enriched gene ontology categories associated with genes differentially expressed in the brain of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S11. Top 20 (lowest *p* value) enriched gene ontology categories associated with genes differentially expressed in the brain of male zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S12. Top 20 (lowest *p* value) enriched gene ontology categories associated with genes differentially expressed in the ovaries of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 48 h.

Table S13. Top 20 (lowest *p* value) enriched gene ontology categories associated with genes differentially expressed in the ovaries of female zebrafish (*Danio rerio*) exposed to 25 μg/L of fadrozole for 96 h.

Table S14. Genes differentially expressed in ovary that are associated with preovulatory follicle development.

Table S15. Coexpressed gene clusters used for promoter motif analysis.

All tables found at DOI: 10.1897/08-653.S1 (351 KB PDF).

Fig. S1. Relative abundance of *sox9b* (sry box-containing gene 9b) and *zgc:64022* (a ras-like estrogen-regulated growth inhibitor) transcripts in the brain tissue of male zebrafish (*Danio rerio*) exposed to 0, 25, or 100 μg/L of fadrozole for 96 h, as determined by quantitative real-time polymerase chain reaction (QPCR). Gene-specific transcript abundance was normalized to the abundance of 18S ribosomal RNA detected in each sample. Data are presented as the mean ± standard error.

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REFERENCES

1. Kavlock R, Ankley GT, Collette T, Francis E, Hammerstrom K, Fowle J, Tilson H, Toth G, Schmieder P, Veith GD, Weber E, Wolf DC, Young D. 2005. Computational toxicology: Framework, partnerships, and program development. *Reprod Toxicol* 19:265–280.
2. Ankley GT, Bencic D, Breen MS, Collette TW, Connolly RB, Denslow ND, Edwards SW, Ekman DR, Jensen KM, Lazorchak JM, Martinović D, Miller DH, Perkins EJ, Orlando EF, Villeneuve DL, Wang R-L, Watanabe KH. 2009. Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanisms of action. *Aquat Toxicol* 92: 168–178.
3. Miller W. 1988. Molecular biology of steroid hormone synthesis. *Endocr Rev* 9:295–318.
4. Drenth H-J, Bouwman C, Seinen W, van den Berg M. 1998. Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. *Toxicol Appl Pharmacol* 148:50–55.
5. Vinggaard A, Hnida C, Breinholt V, Larsen J. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. *Toxicol In Vitro* 14:227–234.
6. Sanderson JT. 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicol Sci* 94:3–21.
7. Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol Sci* 67:121–130.
8. Villeneuve DL, Knoebl I, Kahl MD, Jensen KM, Hammermeister DE, Greene KJ, Blake LS, Ankley GT. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 76:353–368.
9. Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. *J Steroid Biochem Mol Biol* 79:305–314.
10. Kazeto Y, Ijiri S, Place A, Zohar Y, Trant J. 2001. The 5′-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochem Biophys Res Commun* 288:503–508.
11. Wang RL, Bencic D, Biales A, Lattier D, Kostich M, Villeneuve D, Ankley G, Lazorchak J, Toth G. 2008. DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. *Environ Toxicol Chem* 27:664–675.
12. Wang RL, Biales A, Bencic D, Lattier D, Kostich M, Villeneuve D, Ankley G, Lazorchak J, Toth G. 2008. DNA microarray application in ecotoxicology: Experimental design, microarray scanning, and factors affecting transcriptional profiles in a small fish species. *Environ Toxicol Chem* 27:652–663.
13. Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A. 2005. False-discovery rate, sensitivity and sample size for microarray studies. *Bioinformatics* 21:3017–3024.
14. Gene Ontology Consortium. 2006. The Gene Ontology (GO) project in 2006. *Nucleic Acids Res* 34(Database issue):D322–D326.
15. Zhou Q, Wong WH. 2004. CisModule: De novo discovery of *cis*-regulatory modules by hierarchical mixture modeling. *Proc Natl Acad Sci U S A* 101:12114–12119.
16. Liu X, Brutlag DL, Liu JS. 2001. BioProspector: Discovering conserved DNA motifs in upstream regulatory regions of coexpressed genes. *Pacific Symposium on Biocomputing* 6:127–138.
17. Villeneuve DL, Larkin P, Knoebl I, Miracle AL, Kahl MD, Jensen KM, Makynen EA, Durhan EJ, Carter BJ, Denslow ND, Ankley GT. 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain–pituitary–gonadal axis. *Environ Sci Technol* 41:321–330.
18. Biales AD, Bencic DC, Flick RW, Lazorchak J, Lattier DL. 2007. Quantification and associated variability of induced vitellogenin gene transcripts in fathead minnow (*Pimephales promelas*) by quantitative real-time polymerase chain reaction assay. *Environ Toxicol Chem* 26:287–296.
19. Yaron Z. 1995. Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture* 129:49–73.
20. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Seran B. 2003. Regulation of fish gonadotropins. *Int Rev Cytol* 225:131–185.
21. Martyniuk CJ, Gerrie ER, Popesku JT, Ekker M, Trudeau VL. 2007. Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17α-ethinylestradiol. *Aquat Toxicol* 84:38–49.
22. Leonard JB, Waldbieser GC, Silverstein JT. 2001. Neuropeptide Y sequence and messenger RNA distribution in channel catfish (*Ictalurus punctatus*). *Mar Biotechnol* 3:111–118.
23. Korner M, Waser B, Reubi JC. 2004. Neuropeptide Y receptor expression in human primary ovarian neoplasms. *Lab Invest* 84: 71–80.
24. Itoh T, Karlsberg K, Kijima I, Yuan YC, Smith D, Ye J, Chen S. 2005. Letrozole-, anastrozole-, and tamoxifen-responsive genes in MCF-7aro cells: A microarray approach. *Mol Cancer Res* 3: 203–218.
25. Martyniuk CJ, Xiong H, Crump K, Chiu S, Sardana R, Nadler A, Gerrie ER, Xia X, Trudeau VL. 2006. Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17α-ethinylestradiol. *Physiol Genomics* 27: 328–336.
26. Liu YW, Gao W, Teh HL, Tan JH, Chan WK. 2003. Prox1 is a novel coregulator of Ff1b and is involved in the embryonic development of the zebra fish interrenal primordium. *Mol Cell Biol* 23:7243–7255.
27. Zheng W, Yang J, Jiang Q, He Z, Halvorson LM. 2007. Liver receptor homologue-1 regulates gonadotrope function. *J Mol Endocrinol* 38:207–219.
28. Makinen T, Norrmen C, Petrova TV. 2007. Molecular mechanisms of lymphatic vascular development. *Cell Mol Life Sci* 64:1915–1929.
29. Ohmuro-Matsuyama Y, Okubo K, Matsuda M, Ijiri S, Wang D, Guan G, Suzuki T, Matsuyama M, Morohoshi K, Nagahama Y. 2007. Liver receptor homologue-1 (LRH-1) activates the promoter of brain aromatase (*cyp19a2*) in a teleost fish, the medaka, *Oryzias latipes*. *Mol Reprod Dev* 74:1065–1071.
30. Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M, Oliver G. 2002. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 225:351–357.
31. Douville JM, Wigle JT. 2007. Regulation and function of homeodomain proteins in the embryonic and adult vascular systems. *Can J Physiol Pharmacol* 85:55–65.
32. Callard GV, Kruger A, Betka M. 1995. The goldfish as a model for studying neuroestrogen synthesis, localization, and action in the brain and visual system. *Environ Health Perspect* 103(Suppl 7):51–57.
33. Villeneuve DL, Knoebl I, Larkin P, Miracle AL, Carter BJ, Denslow ND, Ankley GT. 2008. Altered gene expression in the brain and liver of female fathead minnows *Pimephales promelas* Rafinesque exposed to fadrozole. *J Fish Biol* 72:1–60.
34. Neumann NF, Galvez F. 2002. DNA microarrays and toxicogenomics: Applications for ecotoxicology? *Biotechnol Adv* 20:391–419.
35. Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK. 2002. Heat shock protein genes and their functional significance in fish. *Gene* 295:173–183.
36. Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu Rev Physiol* 61:243–282.

37. Tong SK, Chung BC. 2003. Analysis of zebrafish cyp19 promoters. *J Steroid Biochem Mol Biol* 86:381–386.
38. Tremblay JJ, Viger RS. 2001. GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. *Endocrinology* 142:977–986.
39. Viger RS, Guittot SM, Anttonen M, Wilson DB, Heikinheimo M. 2008. Role of the GATA family of transcription factors in endocrine development, function, and disease. *Mol Endocrinol* 22: 781–798.
40. Hansen JD, Strassburger P, Du Pasquier L. 1997. Conservation of a master hematopoietic switch gene during vertebrate evolution: Isolation and characterization of Ikaros from teleost and amphibian species. *Eur J Immunol* 27:3049–3058.
41. Mayer WE, O'Huigin C, Tichy H, Terzic J, Saraga-Babic M. 2002. Identification of two Ikaros-like transcription factors in lamprey. *Scand J Immunol* 55:162–170.
42. O'Brien JS. 1962. The role of the folate coenzymes in cellular division. A review. *Cancer Res* 22:267–281.
43. Miller WR, Larionov A, Renshaw L, Anderson TJ, White S, Hampton G, Walker JR, Ho S, Krause A, Evans DB, Dixon JM. 2007. Aromatase inhibitors—Gene discovery. *J Steroid Biochem Mol Biol* 106:130–142.
44. Pellegrini E, Menuet A, Lethimonier C, Adrio F, Gueguen MM, Tascon C, Anglade I, Pakdel F, Kah O. 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish. *Gen Comp Endocrinol* 142:60–66.
45. Forlano PM, Schlinger BA, Bass AH. 2006. Brain aromatase: New lessons from non-mammalian model systems. *Front Neuroendocrinol* 27:247–274.
46. Garcia-Ovejero D, Azcoitia I, Doncarlos LL, Melcangi RC, Garcia-Segura LM. 2005. Glia-neuron crosstalk in the neuroprotective mechanisms of sex steroid hormones. *Brain Res Brain Res Rev* 48:273–286.
47. Sierra A, Lavaque E, Perez-Martin M, Azcoitia I, Hales DB, Garcia-Segura LM. 2003. Steroidogenic acute regulatory protein in the rat brain: Cellular distribution, developmental regulation and overexpression after injury. *Eur J Neurosci* 18:1458–1467.
48. Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, Kah O. 2005. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *J Comp Neurol* 485:304–320.
49. Fraser HM. 2006. Regulation of the ovarian follicular vasculature. *Reprod Biol Endocrinol* 4:18.
50. Gruemmer R, Klein-Hitpass L, Neulen J. 2005. Regulation of gene expression in endothelial cells: The role of human follicular fluid. *J Mol Endocrinol* 34:37–46.
51. Miyabayashi K, Shimizu T, Kawauchi C, Sasada H, Sato E. 2005. Changes of mRNA expression of vascular endothelial growth factor, angiopoietins and their receptors during the periovulatory period in eCG/hCG-treated immature female rats. *J Exp Zool A Comp Exp Biol* 303:590–597.
52. Selman K, Wallace RA, Sarka A, Xiaoping Q. 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *J Morphol* 218:203–224.
53. Pavelock K, Braas K, Ouafik L, Osol G, May V. 2001. Differential expression and regulation of the vascular endothelial growth factor receptors neuropilin-1 and neuropilin-2 in rat uterus. *Endocrinology* 142:613–622.
54. Julio-Pieper M, Lara HE, Bravo JA, Romero C. 2006. Effects of nerve growth factor (NGF) on blood vessels area and expression of the angiogenic factors VEGF and TGFβ1 in the rat ovary. *Reprod Biol Endocrinol* 4:57.
55. Salas C, Julio-Pieper M, Valladares M, Pommer R, Vega M, Mastronardi C, Kerr B, Ojeda SR, Lara HE, Romero C. 2006. Nerve growth factor–dependent activation of trkA receptors in the human ovary results in synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J Clin Endocr Metab* 91:2396–2403.
56. Hirota Y, Osuga Y, Nose E, Koga K, Yoshino O, Hirata T, Yano T, Tsutsumi O, Sakuma S, Muramatsu T, Taketani Y. 2007. The presence of midkine and its possible implication in human ovarian follicles. *Am J Reprod Immunol* 58:367–373.
57. Sundfeldt K, Piontkewitz Y, Billig H, Hedin L. 2000. E-cadherin–catenin complex in the rat ovary: Cell-specific expression during folliculogenesis and luteal formation. *J Reprod Fertil* 118:375–385.
58. Harandian F, Farookhi R. 1998. Contact-dependent cell interactions determine hormone responsiveness and desensitization in rat granulosa cells. *Endocrinology* 139:1700–1707.
59. Halbleib JM, Nelson WJ. 2006. Cadherins in development: Cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* 20: 3199–3214.
60. Egawa M, Yoshioka S, Higuchi T, Sato Y, Tatsumi K, Fujiwara H, Fujii S. 2003. Ephrin B1 is expressed on human luteinizing granulosa cells in corpora lutea of the early luteal phase: The possible involvement of the B class Eph-ephrin system during corpus luteum formation. *J Clin Endocr Metab* 88:4384–4392.
61. Lindner V, Booth C, Prudovsky I, Small D, Maciag T, Liaw L. 2001. Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell–cell interaction. *Am J Pathol* 159:875–883.
62. Johnson J, Espinoza T, McGaughey RW, Rawls A, Wilson-Rawls J. 2001. Notch pathway genes are expressed in mammalian ovarian follicles. *Mech Dev* 109:355–361.
63. Irving-Rodgers HF, Rodgers RJ. 2006. Extracellular matrix of the developing ovarian follicle. *Semin Reprod Med* 24:195–203.
64. Huet C, Pisselet C, Mandon-Pepin B, Monget P, Monniaux D. 2001. Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: Relationships between cell shape and function. *J Endocrinol* 169:347–360.
65. Wayne CM, Fan HY, Cheng X, Richards JS. 2007. Follicle-stimulating hormone induces multiple signaling cascades: Evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. *Mol Endocrinol* 21:1940–1957.
66. Pangas SA, Jorgez CJ, Matzuk MM. 2004. Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. *J Biol Chem* 279:32281–32286.
67. Knight PG, Glister C. 2006. TGF-β superfamily members and ovarian follicle development. *Reproduction* 132:191–206.
68. Pierre A, Pisselet C, Monget P, Monniaux D, Fabre S. 2005. Testing the antagonistic effect of follistatin on BMP family members in ovine granulosa cells. *Reprod Nutr Dev* 45:419–425.
69. Tse SW, Broderick JA, Wei ML, Luo MH, Smith D, McCaffery P, Stamm S, Adreadis A. 2005. Identification, expression analysis, genomic organization and cellular location of a novel protein with a RhoGEF domain. *Gene* 359:63–72.
70. Daggett DF, Boyd CA, Gautier P, Bryson-Richardson RJ, Thisse C, Thisse B, Amacher SL, Currie PD. 2004. Developmentally restricted actin-regulatory molecules control morphogenetic cell movements in the zebrafish gastrula. *Curr Biol* 14:1632–1638.
71. Teubner A, Sobek-Klocke I, Hinssen H, Eichenlaub-Ritter U. 1994. Distribution of gelsolin in mouse ovary. *Cell Tissue Res* 276:535–544.
72. Kamangar BB, Gabillard JC, Bobe J. 2006. Insulin-like growth factor-binding protein (IGFBP)-1, -2, -3, -4, -5, and -6 and IGFBP-related protein 1 during rainbow trout postvitellogenesis and oocyte maturation: Molecular characterization, expression profiles, and hormonal regulation. *Endocrinology* 147:2399–2410.
73. Brannstrom M, Mayrhofer G, Robertson SA. 1993. Localization of leukocyte subsets in the rat ovary during the periovulatory period. *Biol Reprod* 48:277–286.
74. von Schalburg KR, Rise ML, Brown GD, Davidson WS, Koop BF. 2005. A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. *Biol Reprod* 72:687–699.
75. Ohnishi J, Ohnishi E, Shibuya H, Takahashi T. 2005. Functions for proteinases in the ovulatory process. *Biochim Biophys Acta* 1751:95–109.
76. Hasan S, Hosseini G, Princivalle M, Dong JC, Birsan D, Cagide C, de Agostini AI. 2002. Coordinate expression of anticoagulant heparan sulfate proteoglycans and serine protease inhibitors in the rat ovary: A potent system of proteolysis control. *Biol Reprod* 66:144–158.
77. Smith MF, Ricke WA, Bakke LJ, Dow MP, Smith GW. 2002. Ovarian tissue remodeling: Role of matrix metalloproteinases and their inhibitors. *Mol Cell Endocrinol* 191:45–56.
78. Zhou C, Wu J, Borillo J, Torres L, McMahon J, Bao Y, Lou YH. 2005. Transient expression of CC chemokine TECK in the ovary during ovulation: Its potential role in ovulation. *Am J Reprod Immunol* 53:238–248.
79. Committee on Toxicity Testing and Assessment of Environmental Agents NRC. 2007. *Toxicity Testing in the 21st Century: A Vision and a Strategy.* National Academies Press, Washington, DC.