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Transcriptomic responses in Japanese medaka (*Oryzias latipes*) exposed to 17β-estradiol

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ABSTRACT

The effects of 17β-estradiol (E2) were evaluated using the medaka DNA microarray representing 36,398 genes. We first evaluated chronic effects on medaka exposed to E2 at different concentrations for 60 days posthatch. At ≥ 30 ng/L of E2 severe reproductive impairments such as sex reversal were observed. Larval medaka, *Oryzias latipes*, (within 24 hrs posthatch) were then exposed to E2 at various concentrations (3, 30, 100 ng/L) for up to 7 days. Microarray analyses of the E2-exposed larvae revealed that exposure to E2 up-regulated and down-regulated 339 and 105 genes, respectively. The up-regulated genes included ones involved in the p53 signaling pathway, apoptosis, and growth and development, in addition to well-known biomarkers such as vitellogenin and choriogenins. Down-regulated genes included heat shock proteins and estrogen receptors. Most of the up-regulated genes encoding the p53 signaling pathway, apoptosis, and growth and development exhibited a dose-dependent increase in gene expression, whereas the down-regulated genes in the heat shock protein category showed a dose-dependent decrease in gene expression. Time course experiments suggested that the E2 treatment attenuated the time-dependent changes in gene expressions of these genes. Among the genes related to oocyte maturation, estrogen-regulated genes such as choriogenins and vitellogenins were dramatically induced in response to E2 exposure, whereas other steroid-regulated genes such as zona pellucida-domain proteins did not change in gene expression by the E2 treatment. Results suggest that transcriptomic studies on larval medaka help elucidate the effects caused by endocrine disruptors on various biological pathways in vertebrate development.

Keywords: Affinity chromatography; 17β-estradiol; medaka; DNA microarray; Endocrine disruption; Feminization.

1. Introduction

There is a weight of evidence that humans and wildlife are susceptible to endocrine disruption by various natural and synthetic chemicals introduced into the environment [1]. Endocrine disrupting chemicals (EDCs) have the potential to constitute a threat to the reproductive health of organisms, increase the risk of tumor development, and may cause other adverse effects on differentiation, growth, and development [1]. During the past decade, there have been numerous attempts to elucidate the mechanism of endocrine disruption in vertebrates, particularly in fish. Teleost fish, such as Japanese medaka (*Oryzias laptipes*) [2-5], zebrafish (*Danio rerio*) [6, 7], and fathead minnow (*Pimephales promelas*) [8], have been models for mechanistic studies on endocrine disruption. Studies have revealed that many EDCs alter endocrine function by interacting hormone receptors, such as estrogen receptors (ERs), androgen receptors (ARs) and thyroid hormone receptors [9]. Recent studies have shown that reproduction in vertebrates can be disrupted by modulating the hypothalamic-pituitary-gonadal (HPG) axis, by either directly interacting with sex hormone receptors, or by compensatory actions [10]. Various molecular biomarkers have been suggested for screening and testing EDCs, including the yolk protein precursor vitellogenin (VTG) [11], nuclear hormone receptors (e.g., ER and AR) [12, 13], steroidogenic enzymes (e.g., aromatase) [14], and gonadotropins [15]. It is, however, difficult to interpret adverse effects on complicated biological processes based on changes in expression of a few genes. There is clearly a demand for more comprehensive and integrated approaches to better understand the physiological status of an organism affected by EDCs.

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Microarray technology provides the mRNA expression profile of virtually all known genes simultaneously to gain a more comprehensive assessment of the chemical impacts on organisms [16]. A transcriptomic approach has the potential not only to allow an insight into mechanisms of toxicity, but also to provide informative data on novel toxic modes of action. Consequently, transcriptomic studies help link unique gene expression profiles elicited by toxicant exposures and biological pathways. For example, gene expression profiling of EDC-exposed fish may aid in the elucidation of effects caused by the EDCs on various biological pathways besides the HPG axis, including metabolism, biosynthesis, secretion, energy production and cell growth, resulting in a more comprehensive understanding of endocrine disruption.

In this work, we investigated the effects of 17β-estradiol (E2) exposure on larval medaka using a medaka microarray representing 36,398 genes. Transcriptional responses of genes were examined to elucidate the effects of the exogenous E2 exposure on various biological pathways in developmental stages of medaka. We found that genes involved in the p53 signaling pathway, apoptosis, growth and development were up-regulated by the exogenous E2, in addition to well-known estrogen-dependent biomarkers such as VTGs and choriogenins (CHGs). By contrast, genes such as heat shock proteins (HSP) and ERs were down-regulated by E2 treatment. Time course experiments suggest that E2 treatment appeared to attenuate the time-dependent changes of some gene expressions in developmental stages. Results indicate that transcriptomic approaches can be used for integrated evaluation of endocrine disruption of developmental stages.

2. Materials and methods

2.1 *Test chemicals*

17β-Estradiol (E2) was obtained from Sigma Chemical Industries, Ltd. (St. Louis, MO, USA) and dimethyl sulfoxide was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). An E2 stock solution was prepared by dissolving E2 in dimethyl sulfoxide.

2.2 *Test organism*

Japanese medaka (*Oryzias laptipes*, orange-red strain) were originally obtained from the National Institute for Environmental Studies (Tsukuba, Japan), and have been maintained at the Japan Pulp & Paper Research Institute, Inc. The brood stock was maintained at 24 ± 1ºC in UV-disinfected, dechlorinated, carbon-filtered tap water with a 16 h light-8h dark photoperiod. The fish were fed *Artemia nauplii* (<24 h after hatching) twice a day.

2.3 *Exposure design*

Exposures of E2 were carried out by a continuous flow-through system, designed to maintain constant concentrations of E2 throughout the exposure experiments. Briefly, the E2 stock solution was delivered to a mixing glass chamber by a mini-chemical pump unit (Oriental Mortar Co., Ltd., Tokyo, Japan) and diluted with carbon-filtered tap water continuously delivered at a flow rate of 100 mL/min. The E2 test solution overflowed the mixing chamber to enter each test glass aquaria containing 23L of the test solution.

For chronic experiments, 25 larval medaka (within 12 hours posthatch) were exposed to 0 (control), 1, 3, 10, 30, and 100 ng/L of E2 (dimethyl sulfoxide with a final concentration of 1:2,500 v/v water) until 60 days posthatch. At the end of the exposures, phenotypic males and females were selected and separated (as determined by secondary sexual characteristics of the fins) for further reproduction tests.

For microarray experiments, larvae were exposed to E2 for 1, 2 and 7 days by the continuous flow-through system as for the chronic exposures except in 1L test chambers. E2 concentrations used were 0 (control), 3, 30, and 100 ng/L for 2 and 7 days of exposure and 0 and 100 ng/L for 1 day of exposure (dimethyl sulfoxide with a final concentration of 1:2,500 v/v water). Each E2 treatment had two replicates with 90 larvae for each chamber. At day 1, 2 and 7 of the exposures, triplicate samples (30 larvae/sample) from each chamber respectively were collected, flash-frozen in liquid nitrogen, and stored in liquid nitrogen until RNA extraction.

All experiments were conducted with a 16 h light-8 h dark photoperiod and at 24 ± 1ºC, except for reproduction tests, when the water temperature was raised to 27 ± 1ºC to stimulate spawning. The fish were fed *Artemia nauplii* (<24 h after hatching) twice a day.

2.4 *Biological assessment*

1) Reproduction tests

At the end of the chronic exposures, sexual counterparts (2 months posthatch) for the E2-exposed fish were selected from the brood stock. Each mating pair of the E2-exposed fish and a sexual counterpart was put into a reproduction test chamber (1L) with circulated carbon-filtered tap water without E2. Fecundity (spawned eggs/pair/d) and fertility (fertilized eggs/total spawned eggs) were examined daily for each mating pair during 7 consecutive days and calculated for each E2 treatment group. At the end of the reproduction tests, the E2-exposed fish were separated from their sexual counterparts for gonad histology and genotypic sex determination.

2) Gonad histology

The E2-exposed fish were sacrificed, and their body lengths and weights were measured. Gonads were then sampled and weighed for the gonadosomatic index (GSI) calculation. The gonads were immersed in Bouin's fixative (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) overnight. The gonads were then dehydrated and embedded in paraffin wax using a Shandon Excelsior ES (Thermo Fisher Scientific Co., Waltham, MA, USA). Serial longitudinal sections (5 μm of thickness) were prepared using a microtome. The sections were stained with hematoxylin and eosin, mounted with Mount-Quick (Daido Sangyo Co., Ltd., Kobe, Japan), and examined under a light microscope.

3) Genotypic sex determination

To identify sex reversal by the chronic exposures of E2, genotypic sex, XX or XY, was determined by detecting the presence of two major sex determination/differentiation genes (DMY/DMRT1bY and DMRT1) [17-19]. For this, we collected a piece of the caudal fin of all individuals for each treatment and extracted their total DNA using the DNeasy ® Tissue and Blood Kit (Qiagen Inc., CA, USA). A PCR analysis was then performed to detect both DMY and DMRT1 using a primer set, PG17.5 and PG17.6 (the nucleotide sequences: CGGGTGCCCAAGTGCTCCCGCTG and GATCGTCCCT CCACAGAGAAGAGA, respectively) according to Matsuda et al. [19]. The PCR products were analyzed electrophoretically with DNA 7500 Nano LabChip Kit using Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA).

2.5 *Microarray experiment*

1) Total RNA extraction and cRNA preparation

Each frozen sample was ground with a mortar and pestle in liquid nitrogen. Total RNA was isolated from the homogeneous powder using the Qiagen RNeasy Lipid Tissue Midi Kit (Qiagen) following procedures recommended by the manufacture. The quantity and purity of the total RNA were examined photometrically by 260nm/280nm and 260nm/230nm ratios using a NanoDrop ND1000 spectrophotometer (Nano Drop Technologies, DE, USA) and electrophoretically with RNA 6000 Nano LabChip Kit using Agilent Bioanalyzer 2100 (Agilent Technologies). Only RNA samples with RNA Integrity Number (RIN) values above 9.0 were used for further cRNA preparations.

cRNA was prepared from the total RNA using the Quick Amp Labeling Kit (Agilent Technologies) following procedures recommended by the manufacturer. Briefly, 500 ng of the total RNA was reverse transcribed to cDNA followed by synthesis of cRNA incorporated with cyanine 3 (Cy3)-labeled nucleotide. cRNA was then purified using RNeasy mini columns (Qiagen). The quality of the cRNA samples was verified by total yield of the cRNA and the incorporation rates of Cy3 calculated based on the spectrophotometric measurement using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2) Microarray analysis

A medaka DNA microarray containing 36,398 genes was designed by the Chemical Evaluation and Research Institute (CERI) (Tokyo, Japan). The custom medaka DNA microarray with a 4 x 44K format was developed by Agilent Technologies based on the design. cRNA was fragmented to an average size of 100 bp. Hybridization was performed with single cRNA derived from one biosource (one-color hybridization). Hybridization, washing, and scanning were carried out following standard procedures (Agilent Technologies). The data used in further analyses consisted of local background-corrected median intensities that were greater than 2.6 standard deviations above the local mean background. The median of the selected raw data was calculated for each array. Normalization to the median was then applied to the raw intensities across the arrays. Differentially expressed genes were selected by a t-test with a p-value<0.05 for at least one treatment group at day 1, 2 or 7, and based upon a change greater than two-fold in comparison with the control group. Selected genes were categorized based on a biological process using the Gene Ontology (GO) (http://www.geneontology.org/) and UniProtKB (http://www.uniprot.org/uniprot/). Dose-response and time-dependent relationships in gene expression levels of the categorized genes were analyzed using the cumulative chi-squared method [20]. All microarray data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) platform (http://www.ncbi.nlm.nih.gov/geo/) under platform number: GSE17633.

3. Results

3.1 *Chronic effects of E2 on medaka*

We first evaluated chronic effects on medaka exposed to E2 at 1, 3, 10, 30, and 100 ng/L for 60 days posthatch. Table 1 summarizes the results of the chronic experiments. From the observation of external sexual characteristics at 60 d posthatch, all fish in the 100 ng/L treatment group were phenotypically female (Table 1). PCR analyses of the caudal fins were then conducted to determine genotypic sex of all individuals in each E2 treatment using PG17.5 and PG17.6 primers (see "Materials and Methods"). With these primers, only one PCR product (DMRT1 fragment) was detected for the XX (female) genotype, whereas two PCR products (DMY and DMRT1 fragments) were generated for the XY (male) genotype. Of the phenotypic females, two (out of 13 individuals) and eight (out of 20 individuals) were determined as genotypic males in 30 ng/L and 100 ng/L treatment groups, respectively (Table 1). Sex reversal rates, (no. of sex-reversed males)/(no. of total genotypic males), were calculated to be 22.2% for the 30 ng/L treatment group and 100% for the 100 ng/L treatment group. No sex reversal was detected at or less than 10 ng/L of E2 treatment.

Gonad histology showed that there was no adverse effect in E2-treated males at or less than 3 ng/L or in all female individuals, whereas intersex gonads (testis-ova: oocytes in the testes) (Fig. 1) were present in one male of the 10 ng/L group and three males of the 30 ng/L group, respectively (Table 1).

The mean fecundities and fertilities of the E2-exposed males paired with unexposed females decreased significantly (ANOVA followed by a pair-wise t-test; Table 1) at 10 and 30 ng/L of E2. The mean fertilities of the E2-exposed females paired with unexposed males were significantly reduced in all E2-treatment groups except in the 30 ng/L group, whereas the mean fecundities of the E2-exposed females decreased significantly only in the 100 ng/L group (Table 1). No statistically significant difference was observed in GSIs of the E2-exposed females for all E2 treatment groups, whereas those of

Table 1. Chronic effects on medaka exposed to various concentrations of E2 for 60 days posthatch.

E2 concn. (ng/L)[1]	n	Phenotype sex			Gonadal histology (No. of fish)			GSI (%)[2]		Fertility (%)[2]		Number of eggs [2] (Number of eggs/pair/day)	
		male	female	female									
		Genotype sex			Testis	Ovary	Testis-ova	Male	Female	Male	Female	Male	Female
		XY	XX	XY									
0	21	10	10	0	10	10	0	0.019±0.0066	0.12±0.012	96.8±1.7	96.6±1.2	37.5±8.6	27.7±8.6
1	22	10	12	0	10	12	0	0.011±0.0035**[3]	0.11±0.029	82.7±11.6	91.9±4.9*	33.7±11.6	35.8±10.5
3	21	8	13	0	8	13	0	0.015±0.0045	0.12±0.031	77.5±26.7	89.8±5.1**	35.1±9.7	31.7±9.4
10	25	13	12	0	13	12	1	0.012±0.0036*	0.11±0.02	63.4±34.5*	78.4±19.4*	26.2±9.6*	25.7±4.5
30	20	7	11	2	7	13	3	0.013±0.0031*	0.12±0.043	55.7±32.1*	93.3±8.1	25.1±9.8*	20.3±10.4
100	20	0	12	8	0	20	ND[4]	ND	0.13±0.056	ND	69.8±26.4**	ND	17.5±13.2*

[1] Nominal concentrations.

[2] Data expressed as mean ± standard deviation.

[3] Significantly different compared with controls (*p<0.05, **p<0.01)

[4] ND: not detemined because all genotypic males were sex-reversed to phenotypic females.

the E2-exposed males decreased significantly in all E2 treatment groups except in the 3 ng/L group, although there appeared to be no dose-dependent effect (Table 1). Based upon the chronic experiments, 3, 30 and 100 ng/L of E2 were used in the exposure experiments for the DNA microarray analysis as low, medium, and high doses, respectively.

3.2 *Reproducibility of gene expression profiles in larval medaka*

For DNA microarray analysis, we employed larval medaka instead of mature fish, because critical steps in sex determination occur during the larval stage [21]. Because adult fish have normally been used for DNA microarray analyses [22-26], we first evaluated the validity of using larval medaka for DNA microarray experiments. The mean yields of total RNA from each sample (containing 30 larvae) were 46.5 μg, 42.6 μg and 58.1 μg for 1, 2 and 7 days of exposure, respectively. The 260 nm/280 nm ratios and RIN values of the total RNA samples used for microarray analyses ranged from 1.98 to 2.13 and from 9.7 to 10.0, respectively, indicating a high quality of the total RNA samples from larval medaka. We next examined reproducibility of gene expression in larval medaka. Table 2 shows correlation coefficients of gene expression patterns among controls for 1, 2 and 7 days posthatch. The correlation coefficient values ranged from 0.88 to 1.00 for control samples (an average value: 0.95), indicating that variances between mRNA expression profiles of larval medaka were small. These results demonstrate that highly reproducible gene expression data can be obtained from larval medaka under the experimental conditions used in this study.

3.3 *Gene expression profiles in response to E2 exposures*

1) Overview of functional categories of genes regulated by E2

Transcriptional changes were examined for the E2-exposed larvae using the medaka microarray. Differentially expressed genes were selected for each treatment based on the t-test and magnitude of change compared with the control group. Based

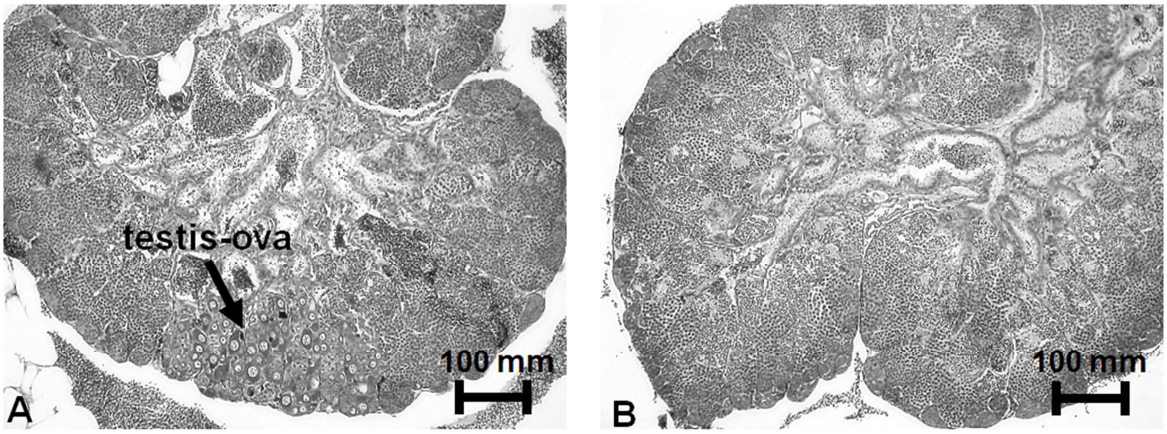

Figure 1. Effects of 17β-estradiol (E2) treatment on the testis in medaka. (A) Testis-ova observed in male medaka exposed to 100 ng/L E2 for 2 months posthatch. (B) Normal testis in control male medaka.

Table 2. List of correlation coefficients among control samples.

		1day[1]			2day[2]			7day[3]		
	No.	1	2	3	1	2	3	1	2	3
1day	1	1	0,97	0,98	0,96	0,97	0,98	0,92	0,87	0,88
	2		1	1,00	0,98	0,99	0,99	0,93	0,92	0,92
	3			1	0,98	0,99	0,99	0,93	0,92	0,93
2day	1				1	0,98	0,97	0,92	0,94	0,95
	2					1	0,99	0,93	0,92	0,93
	3						1	0,93	0,91	0,91
7day	1							1	0,91	0,92
	2								1	1,00
	3									1

[1] Control samples at day 1
[2] Control samples at day 2
[3] Control samples at day 7

on these criteria, 339 and 105 genes were determined as up- and down-regulated genes by the E2 treatment, respectively (up- or down-regulated in at least one exposure group) (Supplementary Table 1). Fig. 2 shows the numbers of up- and down-regulated transcripts for 1, 2 and 7 days exposure at different E2 concentrations. The numbers of up-regulated transcripts in the E2-exposed larvae dramatically increased at day 7, suggesting that the E2 treatments induced various physiological changes at and after day 7.

The up- and down-regulated genes were then categorized into functional groups (Fig. 3). Approximately 60% of both the up- and down-regulated genes were unknown for functions. Approximately 1/4 of the up- or down-regulated genes with known functions were in the "metabolism" category. The other 20% were assigned to "regulation of biological process" or "stress response", respectively.

2) Genes related to sexual differentiation and development

Table 3 summarizes expression profiles of representative genes related to sexual differentiation and development.

In the "hypothalamic-pituitary-gonadal (HPG) axis" category a few genes, such as brain aromatase, were significantly induced and exhibited a dose-dependent increase with E2 treatment at day 7. Expressions of estrogen receptor (ER)-related genes and androgen receptor (AR) α showed a dose-response decrease with E2 treatment. The remaining genes in the HPG category did not exhibit a dose-response relationship with E2 exposure. Time-dependent changes (1 to 7 days) in gene expression were observed for approximately 50% of the genes in this category such as 17-β hydroxysteroid dehydrogenase type 3 and nuclear receptor coactivator 7. Exposure to 100ng/L of E2 appeared to attenuate the time-dependent changes in several genes, particularly 17-β hydroxysteroid dehydrogenase type 3, activin A receptor type II, and nuclear receptor coactivator 7.

In the "oogenesis" category, vitellogenins (VTGs), choriogenins (CHGs) and L-SF precursors (L-SFs) were strongly induced with 30 ng/L and 100 ng/L of E2. The expression levels of these genes increased sharply with the E2 treatments in a dose-response and time-dependent manner. Time-dependent increases in gene expressions were observed for a factor in the germ line α (FIGα) and zona pellucida (ZP)-domain proteins, although these genes did not exhibit dose-dependent changes with E2 treatment.

Protamine in "spermatogenesis" did not show either a dose-response or time-dependent change when treated with E2.

3) Other genes with known functions

Table 4 summarizes expression profiles of the up- or down-regulated genes with known functions besides the genes related to sexual differentiation and development.

In the "regulation of biological process" category we found the up-regulated genes mainly belonged to three groups; "p53 signaling", "apoptosis", and "development and growth". Most of these genes exhibited a dose-dependent increase in gene expressions, although the increase was not as dramatic as VTGs or CHGs (Table 3). Approximately 70% of the genes in the "regulation of biological process" and "metabolism" categories showed a time-dependent decrease in gene expression for controls, suggesting that these genes may play important roles during the early larval stage. The E2 treatments appeared to compensate for the decrease in expression of these genes. Consequently, these genes had similar expression levels until day 7.

Many genes encoding heat shock proteins (HSPs) were down-regulated by the E2 treatments, showing a dose-response decrease in expression levels. Most of these genes exhibited a time-dependent increase in gene expression for controls. The E2 treatments, in this case, appeared to attenuate the time-dependent induction of the HSPs.

4. Discussion

It is well known that exogenous E2 adversely affects sexual development and reproduction of wildlife including fish [1]. Various mechanistic studies have been conducted on the

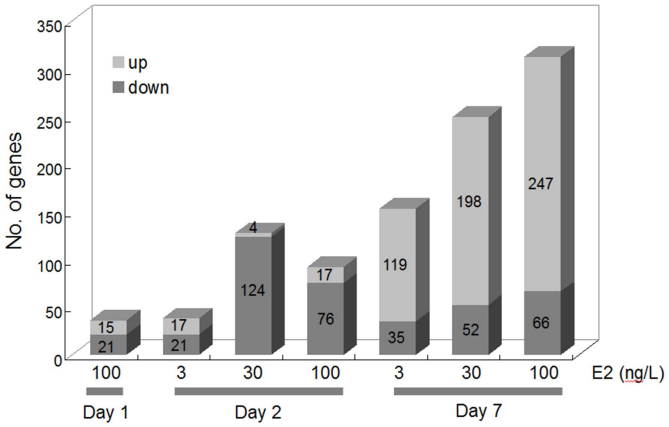

Figure 2. Numbers of up- and down-regulated genes in larval medaka exposed to 17β-estradiol.

endocrine disrupting effects of E2 mainly using fish species [2, 23, 27, 28]. Most of the studies, however, focused on elucidating the effects on the HPG axis by measuring the expression levels of several biomarker genes [11, 27-30]. A more integrated approach such as toxicogenomics is needed for a better understanding of endocrine disrupting phenomena. In this paper, we conducted a comprehensive analysis of the physiological effects of E2 on medaka using the medaka DNA microarray.

Larval medaka, instead of adult fish, were used for both chronic experiments and the transcriptomic analysis in this study, although adult fish have normally been used in various studies on endocrine disruptors [2, 4, 5, 24-26, 31]. Reasons why adult fish have been used are as follows: 1) the morphological sex is easily distinguished by observing secondary sexual characters [32], so that exposure experiments can be done separately for females and males; 2) internal organs can easily be separated compared with larvae, so that assays for biomarkers such as hepatic VTGs [11] can easily be conducted. One of the disadvantages of using adult fish is difficulty in evaluating effects of chemicals on development and sexual differentiation. Because organisms at early developmental stages are susceptible to endocrine disruptors [33], it is crucial to detect transcriptomic changes in embryonic, larval or juvenile stages to understand the mechanisms of endocrine disruptions. In fact, several key genes are expressed in late embryonic and early larval stages: a factor in the germ line α (Figα) at 1 day after hatching; aromatase at 4-10 days after hatching; DMY at embryonic stage 36 [34-36]. Thus, we decided to expose larval medaka to E2 immediately after hatching for chronic and microarray experiments.

We first conducted chronic experiments to determine E2 concentrations for microarray experiments. The full life cycle test by Seki et al. [33] showed that E2 caused reproductive impairment and feminization to medaka even at as low as 10 ng/L. Similar results were obtained in our study, although our experimental design was slightly different. In the work by Seki et al. [33], mating pairs were selected from the same E2-treatment group and exposed to E2 at the same concentrations for 30 days. The reproductive impairments might be caused during sex differentiation, gonad development, reproductive behavior or fertilization. With this experimental design, it might be difficult to clarify which sex is susceptible to E2 and at which developmental stages reproductive impairments were caused. Thus, in our study, the E2-exposed fish were paired with a non-exposed counterpart. Reproduction tests were then conducted without E2 exposure. Significantly lower fertility and egg production in the E2-exposed male (Table 1) suggest that E2 exposure might affect testicular development and reproductive behavior of male medaka. In female medaka, oogenesis, not oocyte development, could be adversely affected by E2 exposure, suggested by significantly lower fertility but no effect on GSIs (Table 1). Results also implied that a major part of adverse effects on both males and females were caused during development, not during reproduction tests.

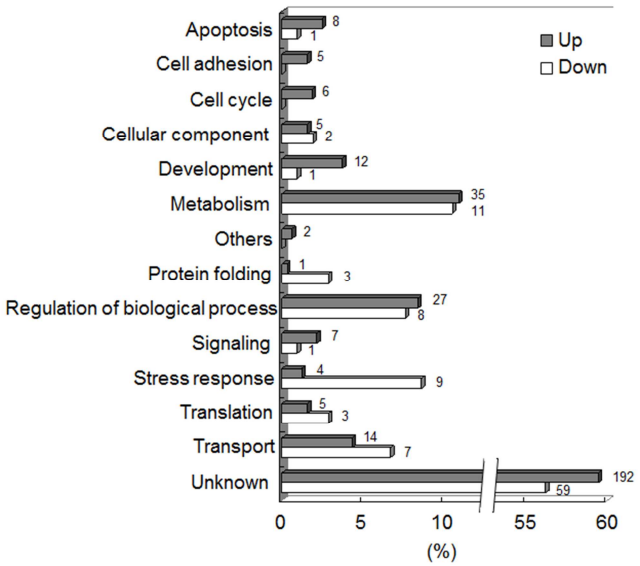

Figure 3. Functional categories of the up- and down-regulated genes in larval medaka exposed to 17β-estradiol based on the Gene Ontology (http://www.geneontology.org/).

Another different experimental design used in this study was genotype sex determination using PCR. With the primers used, only one PCR product (DMRT1 fragment) was detected for the XX (female) genotype, whereas two PCR products (DMY and DMRT1 fragments) were generated for the XY (male) genotype [19]. The sex of medaka is normally determined by observing secondary sexual characters (anal fins). Sex ratios have been compared by statistical analyses in many endocrine disruption studies [2-4, 18, 31]. Sex reversal, however, can only be confirmed by genotypic sex determination. Our results indicate that sex reversal occurred at ≥ 30ng/L of E2, higher than the E2 concentrations causing reproductive impairment (Table 1).

Changes in gene expression in larval medaka exposed to E2 were investigated using the medaka DNA microarray containing 36,398 features. Difficulty in sampling individual tissues from larvae led us to choose whole-body sample preparations. We first examined the variance in mRNA expression levels between pairs of control samples (30 larvae/sample) at day 1, 2 and 7. The average correlation coefficient among control samples was 0.95 (Table 2), indicating that mRNA expression profiles obtained by microarray analysis of medaka larvae are highly reproducible. The reproducibility makes larval medaka an ideal test animal for evaluating effects of chemicals, because of the small size of larvae compared with adult fish: for exposure, only a small space is required, and variances among samples should be smaller than for adults because one sample contains 30 larvae. Differentially expressed genes were then determined in the E2-exposed larvae for 1, 2, and 7 days. For 1 and 2 days of exposure, only 15 and 17 genes, respectively, were determined as induced genes even in the 100 ng/L E2 treatment groups (Fig. 2), suggesting that larvae must be exposed for at least 7 days for comprehensive analysis of the responses of genes to various chemicals.

Table 3. Expression profiles of genes related to sexual differentiation and development in larval medaka exposed to 17b-estradiol (E2).

Probe ID	Dose-response at day 7[1]				Time-dependence[2]						Description
	E2 (ng/L)			Dose-dependent increase (I) or decrease (D)[3]	Control		Time-dependent increase (I) or decrease (D)[3]	E2 100ng/L		Time-dependent increase (I) or decrease (D)[3]	
	3	30	100		2 day	7 day		2 day	7day		
"Hypothalamic-pituitary-gonadal axis"											
NP418483	1.27*	1,14	1.30*	I	1,26	0.76*	D	0,96	0,88	-	Activin A receptor type II
NP835138	1,03	1,08	1,45	-	1,16	0,82	-	0,91	0,97	-	Activin/inhibin b A chain protein
TC43331	0,90	0,63	0,78	-	1,00	1,54	-	0,86	1,05	-	Gonadotropin-releasing hormone receptor 1
TC43341	1,19	1,20	1,38	-	1,02	0.62*	D	0,96	0,75	-	Gonadotropin-releasing hormone receptor 2
TC43335	0,58	0,65	0,80	-	0,94	1,02	-	0,87	0,72	-	Gonadotropin-releasing hormone receptor 3
AU171640	1,22	1,14	1,38	-	1.16*	0,80	-	0,95	0,84	-	Neuropeptide Y
TC44068	1,06	0,93	0,98	-	0,96	1,03	-	0.85*	0,85	-	11b-hydroxysteroid dehydrogenase type 3
BJ733965	1,24	1,36	1,29	-	0,95	0.66*	D	0,94	0,98	-	17b-hydroxysteroid dehydrogenase type 3
TC44446	1,25	1,31	1,42	-	1,13	1,03	-	1,08	1,15	-	20b-hydroxysteroid dehydrogenase
AM149489	1,22	1,33	1,48	-	1,07	1,03	-	1,15	1,29	-	homolog to 20b-hydroxysteroid dehydrogenase
TC59351	1,02	1,06	1,25	-	0,88	0,95	-	1,14	0,91	-	3b-hydroxysteroid dehydrogenase
NP828099	1.39*	2.17*	4.41*	I	1,94	0,76	-	1,10	0,98	-	Brain aromatase (Cytochrome P450 19A2)
TC43315	1,15	1,23	1,07	-	0,96	1.61*	I	1,00	1.49*	I	Cytochrome P450 19A1
NP855454	1,18	1,06	1,24	-	1,04	0.54*	D	1,08	0,57	D	Cytochrome P450 11b
NP418747	1,02	1,35	2,57	I	1,19	1,14	-	1,02	2,40	-	Estrogen receptor
NP423661	2,24	1,41	1,56	-	1,23	1,52	-	0,77	1,17	-	Estrogen receptor b
AU169990	0,54	0.26*	0,92	-	0,91	1,03	-	0,99	1,23	-	Estrogen-related receptor a
TC43574	0,56	0.32*	0.23*	D	0,94	3.85*	I	1,02	0,82	-	Nuclear receptor coactivator 7 (140 kDa estrogen receptor-associated protein)
TC56094	0,50	0.21*	0.11*	D	0,81	4.07*	I	1,08	0,67	-	Nuclear receptor coactivator 7 (140 kDa estrogen receptor-associated protein)
NP863975	0.47*	0.57*	0.27*	D	0.62*	5.10*	I	0,89	2,18	-	Androgen receptor a
TC56245	0,99	1,03	0,95	-	0,94	0,96	-	0,83	1,04	-	Androgen receptor b
"Oogenesis"											
TC53300	4,77	144.51*	439.96*	I	66.21*	13,75	I	4.72*	91.52*	I	Choriogenin H
TC57172	2,03	152.66*	1383.36*	I	4,19	4,03	-	7.86*	449.77*	I	Choriogenin Hminor
TC55876	1,83	81.07*	741.72*	I	2,93	2,13	-	6.81*	394.28*	I	Choriogenin Hminor
TC54002	1,56	116.85*	1075.94*	I	5,48	3,35	-	7.70*	475.67*	I	Choriogenin Hminor
BJ915456	0,49	12,10	94.38*	I	3,01	4,24	-	2,64	204.90*	I	Choriogenin Hminor
TC59626	2,01	2.20*	2.24*	I	1,33	0.71*	D	1,19	1,44	-	homolog to Choriogenin Hminor

TC52879	1,04	1,45	2.14*	I	1,02	1,10	-	1,17	2.04*	I	homolog to Choriogenin Hminor
TC61048	1,25	5,66	107.15*	I	0,91	0.52*	D	1,26	81.63*	I	homolog to Choriogenin Hminor
TC54239	1,07	120.94*	708.48*	I	3,34	3,48	-	11.17*	477.58*	I	homolog to Choriogenin L
BJ923281	3,32	50,55	3670.37*	I	0,24	0,71	-	1,65	5502.26*	I	homolog to Choriogenin L
TC52912	1,59	20,68	70.60*	I	1,32	1,33	-	2.99*	52.21*	I	similar to Choriogenin H
TC57609	1,48	2.03*	2.35*	I	0,97	0,92	-	1,07	2.27*	I	Vitellogenin
TC52966	0,87	194,70	3007.41*	I	1,00	1,77	-	3,36	4582.71*	I	homolog to Vitellogenin 1
TC53352	1,04	1,54	24.58*	I	0,95	0.36*	-	0,90	10,90	-	homolog to Vitellogenin 1
TC58422	1,23	85,45	2427.07*	I	1,12	1,25	-	1,58	2409.29*	I	homolog to Vitellogenin 1
TC59208	0.58*	79,01	2734.28*	I	2,01	1,62	-	1,71	2279.88*	I	homolog to Vitellogenin 1
TC56202	2.09*	266,37	5208.38*	I	2.68*	2.41*	I	2,16	3619.01*	I	homolog to Vitellogenin 1
TC59433	2,12	98.65*	724.88*	I	3,85	3,27	-	8.42*	426.26*	I	homolog to Vitellogenin 1
TC59419	2,79	143.59*	606.12*	I	8,35	2.68*	-	5.01*	90.75*	I	homolog to Vitellogenin II
TC60262	2.85*	2.95*	4.26*	I	0,92	0.30*	D	0,92	1,25	-	L-SF precursor
TC53637	3,91	115.57*	306.04*	I	29,97	20,49	-	5.09*	90.89*	I	L-SF precursor
TC56827	1,94	184.37*	1667.81*	I	1,72	1,42	-	10.24*	660.27*	I	L-SF precursor
BJ918864	0,72	130.91*	1076.61*	I	1,64	1,76	-	10.63*	629.76*	I	L-SF precursor
TC59032	0,99	227.01*	1479.28*	I	1,48	2,46	-	13.65*	743.48*	I	homolog to L-SF precursor
BJ908450	1,21	192.71*	1507.28*	I	2,65	2,72	-	10.87*	630.97*	I	homolog to L-SF precursor
TC60472	1,04	109.27*	877.79*	I	2,34	2,81	-	9.66*	545.81*	I	homolog to L-SF precursor
BJ911644	2.04*	2.37*	2.45*	I	1,21	0,91	-	2,63	2.94*	I	homolog to L-SF precursor
TC45512	1,39	2,06	1,07	-	1,39	10.34*	I	1,11	9.57*	I	FIGa
TC43292	1,69	2,18	0,97	-	0,64	119.23*	I	1,90	212.80*	I	ZPAX
TC43334	1,84	2,59	0,93	-	0,99	28.57*	I	0,86	24.37*	I	ZPB domain containing protein
TC43355	1,53	2,24	0,94	-	2,72	283.52*	I	1,75	217.86*	I	ZPC domain containing protein 2
TC43477	1,68	2,65	1,10	-	1,27	46.15*	I	2,20	79.43*	I	ZPC domain containing protein 2
AM145452	2,55	3,57	1,74	-	1,24	2,97	-	0,69	2,19	I	ZPC domain containing protein 3
TC43321	1,46	2,03	0,99	-	2.80*	54.02*	I	4,93	48.22*	I	ZPC domain containing protein 4
BJ893265	2,97	3,70	3,15	-	1,06	1,06	-	1,27	4.15*	I	ZPC domain containing protein 5
TC43318	1,90	2,62	0,97	-	0,53	29.96*	I	1,76	65.95*	I	ZPC5
"Spermatogenesis"											
NP418658	0,80	0,92	1,51	-	1,53	1,40	-	0,14	0,34	-	Protamine

[1]Gene expressions at day 7 expressed as the fold change compared with controls at day 7.

[2]Time-dependent changes in gene expressions expressed as the fold change compared with control or the E2 100ng/L group at day 1.

[3]Dose- or time-dependent changes in gene expressions were evaluated using the cumulative chi-squared analysis. "I" and "D" indicate significant dose- or time-dependent increase and decrease ($p<0.05$), respectively. "-" indicates no significant dose- or time-dependent change.

*Significantly different ($p<0.05$) compared with controls at day 7 (dose-response) and with control or the E2 100ng/L group at day 1 (time-dependence), respectively.

Table 4. Expression profiles of genes with known functions in larval medaka exposed to 17b-estradiol (E2).

Probe ID	Dose-response at day 7[1]				Time-dependence[2]						Description
	E2 (ng/L)			Dose-dependent increase (I) or decrease (D)[3]	Control		Time-dependent increase (I) or decrease (D)[3]	E2 100ng/L		Time-dependent increase (I) or decrease (D)[3]	
	3	30	100		2 day	7 day		2 day	7day		
"Regulation of biological process"											
(a) p53 signaling											
TC43995	3.29*	4.55*	4.02*	I	0,77	0.24*	D	0,79	0,98	-	weakly similar to ankyrin repeat domain-containing protein 9
TC44618	3.86*	4.58*	4.14*	I	0,75	0.22*	D	0,88	0,96	-	weakly similar to ankyrin repeat domain-containing protein 9
TC45259	1.76*	1.76*	2.19*	I	1,38	0,67	D	1,15	1,13	-	BHLH protein DEC1b
TC46609	1,82	1,64	2.39*	I	1,32	0.48*	D	1.14*	1,00	-	similar to CBP/p300-interacting transactivator
TC46691	2.58*	2.08*	2.24*	I	1.72*	1,13	-	1,18	1,27	-	BHLH protein DEC1a
TC48131	1,65	2.03*	2,08	-	1,05	0.56*	D	1,09	1,18	-	similar to CCAAT/enhancer binding protein delta2
TC60219	1,18	1,37	4,48	I	1,06	0,78	-	0,89	4,15	I	weakly similar to apoptosis-stimulating of p53 protein 2
TC61529	1,72	2,16	1,40	-	0,98	1,12	-	1,15	1,47	-	similar to hypoxia-inducible factor 1 a
(b) apoptosis											
AM149239	2.18*	2.08*	1.94*	I	1,12	0,82	-	1,08	1.50*	I	similar to peroxisome proliferator-activated receptor g
AU178411	1,55	1.71*	2.09*	I	1,16	0,76	D	1,05	1,05	-	weakly similar to intestinal Muc 2-like protein
TC44023	1.79*	1.77*	2.18*	I	1,01	0.48*	D	0,98	0,99	-	homolog to ubiquitin
TC47349	1.64*	1.69*	2.03*	I	1,06	0.54*	D	0,97	0,99	-	homolog to ubiquitin
TC51915	2.15*	2.16*	2.23*	I	1,16	0.48*	D	1,06	0,98	-	similar to interferon regulatory factor 2 binding protein 2 isoform B
TC52486	1.82*	1.80*	2.15*	I	0,96	0.44*	D	0,95	0,95	-	homolog to ubiquitin
TC55411	2,20	2.28*	2.56*	I	1,21	0.57*	D	1,19	1,36	-	similar to caspase-9
TC55763	2.06*	2.33*	2.60*	I	1,26	0.65*	D	1,09	1,37	-	homolog of ring finger protein 36 isoform b
TC58172	2.13*	2.41*	2.52*	I	1,22	0.60*	D	1,22	1,39	-	weakly similar to caspase-9 precursor
(c) growth and development											
AM138341	1,56	1.88*	2.17*	I	1,01	0.37*	D	1,02	0,85	-	similar to HMG-box transcription factor 1
AU170803	2,92	5,07	2.55*	I	0,75	0,70	-	1,93	1,53	-	similar to zinc finger RNA-binding protein
AU178201	2.06*	1.90*	1.89*	I	0,92	0.53*	D	0,89	0,96	-	similar to connective tissue growth factor precursor
BJ011762	1,59	1,63	2.05*	I	1,11	0.30*	D	0,97	0.51*	D	similar to keratin

BJ025587	1,32	1,97	2,70	-	0,99	0,97	-	1,06	2,61	-	TUDOR
BJ708117	7,36	3.60*	7.30*	I	1,89	0,90	-	0,69	2.50*	I	similar to growth-arrest-specific protein 1 precursor (GAS-1)
BJ736582	1.56*	1.54*	2.04*	I	1,15	0.4*	D	1,09	1,05	-	similar to kinetochore protein Spc24
BJ880895	2,02	1,93	1,95	I	1,04	0,79	-	0,80	1.57*	I	similar to growth arrest and DNA- damage-inducible protein GADD153
NP1445793	2.57*	1,97	1,69	-	1.64*	1.97*	I	1,09	1,52	-	BHLH protein DEC2
TC43357	1.94*	2.08*	2.18*	I	1,61	1,09	-	1,14	1.76*	I	HoxC10a
TC45582	1,96	2.17*	2.62*	I	1,18	0.53*	-	1,25	1,79	-	homolog to Growth arrest and DNA-damage-inducible protein GADD45 g
TC45587	2,68	3.14*	9.05*	I	1,77	0.30*	D	1,53	2,09	-	similar to insulin-like growth factor binding protein-1 (IGFBP1)
TC48028	1,21	1,37	2.35*	-	0,91	0.38*	D	0,68	1,18	-	similar to protein Jumonji
TC51745	2.24*	2.55*	2.74*	I	0,98	0.40*	D	1,14	1,11	-	similar to cyclin-G2
TC52709	1.71*	1.90*	2.03*	I	1,23	0.66*	D	1,00	1,18	-	similar to development and differentiation enhancing factor-like 1
TC53134	2.13*	1.97*	2.01*	I	0,88	0.45*	D	0,93	0,90	-	similar to connective tissue growth factor precursor
TC53171	2.58*	3.31*	2.56*	I	1,04	0.72*	D	1,20	1.77*	I	similar to krueppel-like factor 11
TC57898	1.70*	1.77*	2.02*	I	1,35	0,94	-	0,82	1.81*	I	similar to growth arrest and DNA- damage-inducible protein GADD153
"Metabolism"											
cytochrome P450											
TC58366	1,29	1.56*	2.47*	I	1,04	0.27*	D	0,99	0,63	D	cytochrome P450 monooxygenase CYP2K1
TC58597	1,36	1.68*	2,15		1,01	0.31*	D	0,98	0,63	-	similar to cytochrome P450 2K5
TC43435	1,04	1.22*	1.13*	I	1,45	2.39*	I	1,15	1.82*	I	Cytochrome P450 3A
"Stress response" and "Metabolic process"											
heat shock protein											
AU169726	0,87	0,31	0,23		1,53	1,50	-	0,89	0.59*	D	stress-70 protein
TC52094	0,78	0.69*	0.40*	D	1,21	2.76*	I	1,27	1,07	-	similar to 47 kDa heat shock protein
TC53141	0,62	0,59	0.48*		1,17	1.90*	I	1.25*	0.75*	D	homolog to novel protein similar to heat shock protein 90-a
TC60176	0,74	0.54*	0.37*	D	0,88	1.91*	I	0,89	0,78	-	homolog to heat shock 70 kDa protein 5
TC61286	0,62	0,53	0.41*		1,05	2.10*	I	0,97	0,75	-	homolog to heat shock protein 90 a
TC62024	0,61	0.46*	0.46*	D	0,69	1.52*	I	0,89	1,10	-	heat shock cognate 71 kDa protein
TC49227	0,57	0.41*	0.47*	D	0,91	2.43*	I	0,92	1,14	-	homolog to 10 kDa heat shock protein mitochondrial

[1]Gene expressions at day 7 expressed as the fold change compared with controls at day 7.

[2]Time-dependent changes in gene expressions expressed as the fold change compared with control or the E2 100ng/L group at day 1.

[3]Dose- or time-dependent changes in gene expressions were evaluated using the cumulative chi-squared analysis. "I" and "D" indicate significant dose- or time-dependent increase and decrease ($p<0.05$), respectively. "-" indicates no significant dose- or time-dependent change.

*Significantly different ($p<0.05$) compared with controls at day 7 (dose-response) and with control or the E2 100ng/L group at day 1 (time-dependence), respectively.

Dose-response and time-dependent microarray analyses were then carried out to evaluate the E2 effects on larval development. Table 3 shows the expression profiles of the genes involved in sexual differentiation and development. The endocrine control of reproduction in medaka involves the hypothalamic-pituitary-gonadal axis [37]. The gonadotropin-releasing hormone (GnRH) is synthesized in the preoptic/hypothalamic area of the brain and stimulates secretion of gonadotropins [38]. In teleost fish such as medaka, gonadotropins have two forms: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [38]. FSH and LH are primarily involved in E2 biosynthesis and oocyte maturation, respectively. E2 plays critical roles in oocyte growth and is synthesized via the steroidogenesis pathway [39]. The steroidogenesis pathway includes various enzymes, such as 17β-hydroxysteroid dehydrogenase (HSD), 3β-HSD, 20β-HSD and aromatase. E2 is finally converted from testosterone, catalyzed by cytochrome P450 aromatase [39]. Medaka has two distinct genes for aromatase: 19A1 in the ovarian follicular layers and 19A2 in the brain, which play important roles in reproduction and neurogenic activity, respectively [40]. In this study, only a few genes, particularly brain aromatase 19A2, exhibited a significant dose-dependent induction by the E2 treatments (Table 3). The remaining genes in the HPG axis did not show either dose- or time-dependent responses to E2 exposure (Table 3). These results suggest that the estrogen treatments may affect neurogenic development in the brain, but appear not to modulate E2 biosynthesis, in line with the work on adult medaka by Zhang et al. [29].

As shown in previous studies [11, 27, 28,41], genes related to oogenesis, such as VTGs, CHGs and L-SFs, were strongly induced even at larval stages exposed to E2 (Table 3). VTGs and CHGs are precursor proteins of egg yolk and the egg envelope, respectively, that are synthesized in the liver [27, 28, 41]. VTGs, CHGs and L-SFs have been shown to be good biomarkers for environmental estrogens in fish, due to their sensitivities and specificities to E2 [11, 27, 28, 41]. Some of these genes were induced more than one thousand times relative to controls at 100 ng/L of E2 (Table 3), confirming that the VTGs and CHGs are the most sensitive biomarkers for estrogens. The induction of these genes exhibited both a dose- and time-dependent relationship and appeared to correlate with gonadal abnormalities and sex reversal rates. More studies, however, have to be done to elucidate the relationship between feminization phenomena and induction of the oogenesis-related genes.

Other genes related to the egg envelope, called the zona pellucida (ZP)-domain are specifically expressed in the oocyte [42]. Significant increases in gene expression of the ZP genes were observed in controls at day 7 (Table 3), indicating that gene expression during early oogenesis can be evaluated at day 7 posthatch. Although egg envelope formation seems to involve both CHG and ZP gene expressions [42], the E2 treatments did not cause any induction of the ZP genes (Table 3). In mammals, a factor in the germ line α (FIGα) plays a key role in the oocyte-specific expression of ZP genes [43]. In this study, FIGα did not exhibit dose-response gene expression, but showed a time-dependent increase in gene expression (Table 3). These results imply that FIGα may be involved in the expression of ZP genes, although further studies are necessary to clarify the interaction between FIGα and ZP genes in medaka.

Protamine is expressed at the initial stage of spermiogenesis in medaka and involved in the maintenance of the condensing state in sperm nuclei [44]. The gene expression of protamine did not show either dose- or time-dependent change with E2 treatments (Table 3). E2 exposure did not cause significant changes in gene expression of FIGα, ZPs or protamine even in mature fish [22], suggesting that E2 may not be involved in gene expression of these genes.

Interestingly, some of the ER-related genes were down-regulated by the E2 treatments (Table 3). It is known that ERs play important roles in defeminization of the male brain and, therefore, sexual behavior. Exposure of E2 during development (gestation and shortly after birth) has been suggested to suppress ER expressions in mammal brains through feedback inhibitions of its ligand, E2 [45]. ER expression levels increased when male rats were deprived of E2 after birth, whereas E2 supplements caused subsequent declines in ER expressions [46, 47]. These studies suggest that the expression levels of neural ERs might be influenced by endogeneous E2 levels during development. It was also reported that ERβ in the brain of goldfish was down-regulated by E2 exposure [48]. Taken together with our results, it is implied that excess administration of E2 may cause the repression of ER-related genes to maintain E2 homeostasis in larval medaka. Some genes encoding HSPs were also down-regulated, and exhibited a dose-response relationship to E2 (Table 4). ERs exist as monomers in the cytoplasm without their ligands, and form a multi-protein complex with HSP90 and HSP70 in the presence of E2 [49, 50]. In our study, the expressions of both HSPs and ER-related genes increased in a time-dependent manner for controls, which appeared to be attenuated by the E2 treatments (Table 3 and 4). It is, however, speculative to state that ERs and HSPs are co-regulated. More work needs to be done to understand the mechanism of HSP suppression by E2 exposure.

Besides the sexual development related gene expressions, many genes encoding p53 signaling, apoptosis, and growth and development were significantly induced even at as low as 3 ng/L of E2 (Table 4). The expression levels of these genes declined significantly later than day 2 for controls. The E2 treatments maintained these expression levels even at day 7 (Table 4). Some genes encoding p53 signaling, apoptosis, and development and growth are known to be involved in both development and stress response [51-55]. Genes related to the p53 signaling play important roles in cell cycle arrest [53, 54]. The p53 signaling pathway is required in embryonic development and DNA repair [53, 54]. Apoptosis is the process of programmed cell death that may occur in multicellular processes, including mammalian embryonic development [51, 52]. Apoptosis is also triggered when cells are damaged be-

yond repair (such as DNA damage) [51, 52]. GADD45, GADD153, and GAS1 related to DNA damage and cell cycle arrest are also known as genes associated with the regulation of growth during organogenesis in the rat fetus [55]. On the other hand, some of the cytochrome P450 enzymes convert E2 to 4-OH-E2, which undergoes metabolic redox cycling between hydroquinone and quinone, formed via semiquinone intermediates [56, 57]. The semiquinone is a free radical that can react with molecular oxygen to form oxygen reactive species, resulting in cell and DNA damage. Our results imply that the contribution of cytochrome P450-mediated oxidative stress to p53 signaling and apoptosis might be rather small, because only a few cytochrome P450s were significantly induced at high concentrations of E2. The p53 signaling and apoptosis at 3 and 30 ng/L of E2 could be caused by the stimulation of cell growth and development. Estrogens have been used as promoting agents for growth of cattle and sheep and also used for the estrogen therapy in Turner's syndrome [58]. In our experiments, significant increases were observed in body lengths and weights of the E2 30 ng/L group compared with controls (data not shown), suggesting that the larval growth might be promoted at low concentrations of E2.

In this study, we investigated physiological effects of medaka exposed to E2 using the combination of chronic experiments and transcriptomic analyses. Larval medaka instead of adult fish were used in this study to evaluate the effects of E2 on sexual differentiation and development. Larvae were fairly sensitive to E2 in gene expression, and the gene expression profiles were highly reproducible. Using medaka DNA microarray, the transcriptomic approach was conducted to determine the physiological effects of E2. Results suggest that E2 may affect not only the steroid receptor-mediated pathway, but also other physiological activities, such as apoptosis, cell growth and development. Because the gene expression profiles of the E2-exposed larval medaka were similar to those of mammals, larval medaka can be an excellent vertebrate model for a mechanistic study of endocrine disruption.

5. Supplementary material

Supplementary material regarding this manuscript is available online in the web page of JIOMICS.

http://www.jiomics.com/index.php/jio/rt/suppFiles/29/0

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