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DEVELOPMENT OF A MOLECULAR BIOMARKER FOR DETECTING INTERSEX AFTER EXPOSURE OF MALE MEDAKA FISH TO SYNTHETIC ESTROGEN

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Abstract—Although intersex of fish species has been widely reported in aquatic environments, there is no appropriate biomarker of the effects to assess the occurrence of intersex. In the present study, mRNA expression profiles of 14 genes, which are either involved in ovary development and maintenance or have relatively high mRNA transcription levels in ovarian gene expression profiles in fish species, were comprehensively evaluated in male Japanese medaka (*Oryzias latipes*) exposed to 17α-ethynylestradiol (EE2) to investigate their utility as indicators of the severity index of intersex. Of these 14 genes, mRNA expression of a novel gene, termed ovary structure protein 1 (OSP1) in the present study, showed female-like expression pattern with the highest transcription levels, and displayed the most significantly positive correlation with the severity index of intersex ($r^2 = 0.8215$, $p < 0.0001$). The full-length mRNA sequence of OSP1 is 802 bp, with an open reading frame (ORF) of 642 bp that encodes a 214-amino acid peptide. Reverse transcription polymerase chain reaction and in situ hybridization revealed that OSP1 was expressed exclusively in the ovaries, specifically in the oogonia and primary oocytes in ovaries and in testis with intersex. These results suggest that the analysis of mRNA expression of OSP1 can be used to indicate intersex in male medaka. Environ. Toxicol. Chem. 2012;31:1765–1773. © 2012 SETAC

Keywords—Biomarker of effect Intersex Ovary structure protein 1 Ovary development Endocrine-disrupting chemicals

INTRODUCTION

Environmental estrogenic substances such as natural and synthetic estrogens, some pesticides, and other manmade chemicals can cause gonadal growth retardation, testicular degeneration, and intersex (primary oocytes within testicular tissue) at relatively low concentrations [1–3]. In particular, intersex of fish has been widely used as direct evidence of the feminization of wild fish from freshwater and marine environments worldwide [4–6]. Incidences of intersex as great as 100% have been reported for the male roach (*Rutilus rutilus*) inhabiting lakes and rivers of the United Kingdom [4]. Severe intersex in male fish is related to impaired reproductive success by affecting sperm quality, fertilization success, and the ability to produce viable offspring [7,8]. Therefore, there are important ecotoxicological implications for developing biomarkers of effect that allow for quantitative evaluation of intersex in fish species.

Vitellogenin (VTG), the precursor protein for egg yolk, has been extensively used as a sensitive biomarker for assessing the estrogenic activities of chemicals in aquatic environments [9,10]. Because of its associations with fish feminization, several studies have attempted to establish relationships between VTG levels and the severity index of intersex in males of a variety of fish species to determine whether VTG is a reliable indicator of the occurrence of intersex. However, poor relationships were observed in subsequent laboratory experiments and field studies [11–13]. Vitellogenin is a large serum phospholipoglycoprotein produced only in the liver of mature females and is transferred by the bloodstream to the ovary, where it is taken up and modified by developing eggs [14]. The production of VTG is directly regulated by the circulating concentrations of 17β-estradiol (E2), but not by the gonadal status of the organism [15], although it could be affected by the stage of gonadal development. Consequently, VTG is not suitable to indicate the occurrence of intersex, even though the production of VTG is sensitive to the short-term effects of the external environmental estrogens. Because an appropriate biomarker of effect should be specific, sensitive, and directly related to the histopathological damage [16], in principle, genes associated with ovarian development or maintenance should be useful as biomarkers of intersex because similar oocyte structures were thought to be present in the testis of male fish with intersex. Some ovarian-specific genes such as FIGα (factor in the germline α), 42Sp50 (major protein component of a 42S nucleoprotein particle), and zona pellucida (ZP) protein have been detected at the early stages of sexual differentiation and development in several fish species and have been used as biomarkers for indicating oocyte differentiation status in female fish [17–19]. In addition, analysis of gene expression profiles in gonads of several species of fish has been used as an effective method to screen for genes involved in controlling ovarian development or maintenance—which may serve as biomarkers of intersex [20–23].

In the present study, 14 candidate genes known to be involved in ovarian development and maintenance [17–19], or that have relatively high mRNA transcription levels in studies of ovarian gene expression profiles in different fish species [20–23], were investigated as potential candidates to develop as molecular biomarkers of effect for indicating intersex occurrence. Female-like expression patterns and mRNA abundances were determined to be two basic criteria for initial screening, considering that an appropriate biomarker of effect should be specific and sensitive. In addition, the period of sexual differentiation of male fish is the most sensitive developmental stage for intersex induction [24,25]; therefore it is important to screen the biomarker genes just after the completion of sexual

All Supplemental Data may be found in the online version of this article.

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differentiation of medaka fish (~21 d post hatching [dph]). In the present study, gene expression analysis was conducted at 21 dph of medaka fish (after the completion of sexual differentiation) and at 90 dph (the period of sexual maturity) to identify relatively stable biomarkers that are expressed throughout the whole gonadal developmental process. The transcriptional profiles of these 14 candidate genes in male Japanese medaka exposed to 17α-ethinylestradiol (EE2) were measured by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR), and the relationships between their transcript levels and the severity index of intersex were then investigated. For comparison, the mRNA expression of 45 genes, well known to be involved in sexual differentiation (10 steroid hormone receptors, 9 steroidogenesis enzymes, and 26 ovary/testis differentiation-related regulatory proteins) were also measured. A novel gene, selected from the ovarian gene expression profiles of medaka fish (*Oryzias latipes*) [21], termed OSP1, was proposed as the most effective biomarker of effect due to its high level of mRNA transcription and its strong association with intersex. The tissue distribution and cellular localization of OSP1 was then investigated by RT-PCR and in situ hybridization (ISH) to further understand its functions during ovary development. This novel biomarker can provide a direct warning of intersex occurrence in male medaka exposed to estrogenic endocrine disruptors. The approach used in the present study should be applicable to developing intersex biomarkers in other fish species.

MATERIALS AND METHODS

Test chemicals

The 17α-ethynylestradiol was purchased from Fluka Chemie, and dimethyl sulfoxide (DMSO) was purchased from Sigma.

Experimental fish

Japanese medaka, orange red strain, were cultured in flow-through tanks under conditions that facilitated breeding (16:8-h light/dark cycle) and were fed live brine shrimp (*Artemia nauplii*) twice daily. Water used in the experiment was filtered through activated carbon and had a hardness of 8.1 ± 0.1 mg/L $CaCO_3$, pH 7.7 ± 0.2, dissolved oxygen 7.8 ± 0.3 mg/L and was maintained at $25 \pm 1^{\circ}$C.

Exposure design

For initial screening of the candidate biomarker genes, the larvae were exposed to a single concentration of EE2 or a vehicle control (0.005% DMSO) on the first day after hatching (two tanks per treatment). The EE2 concentrations in the water were measured by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS), and the actual concentration for the EE2 exposure group was 74.8 ± 10.2 ng/L. This concentration used in the initial screening was designed to induce serious intersex in male medaka fish. The exposure was performed in 3-L tanks filled with 2 L of carbon-filtered water; half of the water in each tank was replaced daily with fresh carbon-filtered water. At 14 dph, larvae were transferred into 12-L tanks that were provided with with a continuous supply of water containing EE2 or the vehicle control. A flow-through system with a twofold volume of water flowing through every 24 h was used. Gonads of six male fish and six female fish in each treatment group were sampled after the completion of sexual differentiation (21 dph) and at the period of sexual maturity (90 dph) for qRT-PCR analysis of gene expression. Reverse-transcription PCR and gelose gel electrophoresis were used to determine genotypic sex of medaka larvae using gene-specific primers against the sex determination gene DMY. The sequences of the primers were as follows: DMY-F: 5′-GGCCGGGTCCCCGGG-TG-3′; and DMY-R: 5′-CTGGTACTGCTGGTAGTTGTG-3′ [26].

To further investigate the relationships between gene transcription levels and the severity index of intersex, medaka larvae collected on the first day after hatching were exposed to a series of EE2 concentrations. The EE2 concentrations in the water were measured by UPLC-ESI-MS/MS, and the actual concentrations for EE2 exposure groups were 5.6 ± 0.3, 16.1 ± 2.7, 29.5 ± 2.9, 40.6 ± 4.3, 51.4 ± 5.6 ng/L, respectively (two tanks per treatment). These EE2 concentrations were chosen to induce different severity indices of intersex based on previous reports in fish species [27,28]. The exposure continued for 90 d. On the last day of exposure, six male fish were sampled from each treatment group. The testis from each fish was gently divided into two equal parts, one of which was stored in liquid nitrogen until required for analysis of gene expression and the other in 10% formaldehyde solution for histological determination of the severity index of intersex.

Gene expression analysis

For the analysis of gene expression, medaka larvae or gonads from adult fish were snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from the samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To prevent genomic DNA contamination, the total RNA was digested by DNase I (TaKaRa Biotechnology) and purified. The total RNA recovered from DNase I digestion was measured at 260 and 280 nm using a spectrophotometer. The 260-nm reading was used to estimate the concentration of total RNA. The 260:280 nm ratios, as well as a 1% agarose–formaldehyde gel stained with ethidium bromide, were used to verify the RNA quality in each sample. The RNA concentrations were adjusted to 100 ng/μl for first-strand cDNA synthesis.

First-strand cDNA synthesis and qRT-PCR were performed according to the methods described previously [15]. Primers for quantification of mRNA abundance of each gene were designed using Premier Primer 5.0 (Premier Biosoft International) (Table 1). To minimize the problems associated with DNA contamination, the primers were designed to span at least one intron of the genomic sequence whenever possible. Ribosomal protein L7 (RPL-7), which has been demonstrated to be an appropriate endogenous control for gene-expression profiling in studies of endocrine-disrupting chemicals [15], was used as the internal control. Relative expression was evaluated by the comparative cycle threshold method described by Applied Biosystems.

Development of the severity index of intersex

The classification of the severity index of intersex based on histopathological observation of gonads is considered to be approximate and discontinuous and therefore does not accurately reflect the intersex status in cases of subtle differences [29]. Therefore, to comprehensively evaluate the occurrence of intersex, the classification of severity index of intersex described in previous papers [4,30,31] was modified in accordance with Equation 1, which incorporated the scores for developmental stages and numbers of oocytes within individual fields of view (Supplemental Data, Fig. S1). Because of the positive

Table 1. Primers pairs used for quantitative real-time reverse transcription–polymerase chain reaction

Gene	Accession no.	Sequences (first row, forward primers; second row, reverse primers)	Amplicon size (bp)
RPL-7	DQ118296	5′-CGCCAGATCTTCAACGGTGTAT-3′ 5′-AGGCTCAGCAATCCTCAGCAT-3′	72
FIGα	AF128805	5′-TGCAGCAGAGGATGGAGACAT-3′ 5′-TTAATCCCTCGAAGCTTGATCG-3′	116
ELF-4E	AF128806	5′-GGTTCTGGTTGGAGACGCTTT-3′ 5′-TCCTTTGGCTCGGATGTTG-3′	98
Qd	AF128816	5′-TGGCAAAGAAGGTGCTGATC-3′ 5′-ATGTCCTCAGCAGTGGCAGTT-3′	163
42Sp43	AF128814	5′-CCATATTGCACGCAAAGCC-3′ 5′-CACGCCACAGGTAAATGAGACT-3′	139
42Sp50	AF128815	5′-ACGCTGGGGGTTAAGCAAAT-3′ 5′-TAGCCGTAGAGGTTTGTTGATTGT-3′	128
ZPA	AF128807	5′-TTCCTGGTGACTCCAATGACG-3′ 5′-TCGCAACAGGTTCAGGAGTGTA-3′	69
ZPB	AF128808	5′-ATACTCCCCAGGAGACGGTGTA-3′ 5′-CGATGACAGCGTTGTTCACAG-3′	88
ZPC	AF128809	5′-ATGGCAGTGAATGCAGAGCTG-3′ 5′-AACCGGCTGTCAGTCCAAACT-3′	74
Foxl2	AB252055	5′-CCTCACGCTGTCTGGCATCT-3′ 5′-CCAGCCTTTCTTGTTCTTCTCAT-3′	73
Vasa	AB063484	5′-AGGACTCCATCTTCTCCCACTACA-3′ 5′-GGCCTCCTCAAAGGTCATGAT-3′	116
Sycp1	AB207974	5′-TGTCAGCAGAAGATAGCAGAC-3′ 5′-CTTCTTTCTCTTTTCAAGACCC-3′	112
Fstl	NP	5′-GTGGACCAGACAAACAACGCA-3′ 5′-GCTACACTGGATGTCCTCGCA-3′	213
Tra2	AB079121	5′-AGTACGGCCCACTGAGCGAA-3′ 5′-GAACCACGACGAGAACCACCA-3′	250
OSP1	NP[a]	5′-CCCAAGGAAGTGTCCCAAAT-3′ 5′-ATAACCACCATACTGTCCAGAAGG-3′	116

[a] NP is the nucleotide sequence that was identified in our lab and not published.

relationship between oocyte developmental stage and its related diameter [31], each field of view received a score for the average diameter of oocytes to refer to their developmental stages.

$$\mathrm{TSI} = N \times \frac{\sum(\mathrm{Dn})}{n} \quad (1)$$

where TSI is the severity index of intersex; Dn is the diameter of each individual oocyte; n is the number of oocytes used for calculating their related diameter; and N is the number of oocytes within the same individual fields of view. In addition, the field of view was set as 100×100 μm, and the average TSI for three separate sections in one slide was calculated.

The OSP1 cDNA clone and sequence analysis

The full-length cDNA of OSP1 was determined by 3′-/5′-RACE (rapid amplification of cDNA ends) using the 3′-/5′-Full RACE Core Set (Takara) according to the manufacturer's protocol. Gene-specific primers (Table 2) for 3′-/5′-RACE were designed based on the partial mRNA sequence identified in the NCBI (accession number: AF128822). The genomic DNA sequence and chromosomal location of OSP1 were determined by using the BLAST algorithm at the Medaka Genome Project Working Draft (http://www.ensembl.org/Oryzias_latipes/Info/Index). Gene organization (exon/intron boundaries) was examined by comparison with the full-length cDNA, and confirmed by the recognized consensus sequence GT/AG in the introns adjoining the splicing junctions (G-T at the 5′ and A-G at the 3′ terminus of each intron). The deduced amino acid sequence of medaka OSP1 was obtained from GenBank using the ClustalX program, and adjusted manually using BioEdit 7.0.1. Based on the alignment, the protein sequence similarities were calculated using the identity matrix in BioEdit.

In situ hybridization

Section ISH was performed as described previously [32] with slight modifications. In brief, fresh-frozen samples were embedded in Tissue-Tek OCT compound (Sakura Finetek USA), cut in 6-μm sections, and mounted on silanated slides. Sections were digested with proteinase K (1 μg/ml), and then fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS). Slides were hybridized with 80 μl of hybridization buffer without probes for 2 h at 37°C to reduce nonspecific binding of probes, and then hybridized at 37°C for 18 h in 80 μl of hybridization buffer containing 1 μg/ml digoxigenin (DIG)-labeled RNA probe. The posthybridization washes were as follows: twice at 37°C for 15 min in $2 \times$ SSC; twice at 37°C for 15 min in $1 \times$ SSC; 30 min in NTE buffer containing 20 μg/ml RNase A (Sigma) at 37°C and 20 min three times in $0.1 \times$ standard saline citrate at 37°C. Sections were incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 2 h at 37°C. After washing twice with a washing buffer containing 0.1 M Tris-HCl and 0.15 M NaC1(pH 7.5), sections were incubated in 200 μl of detection buffer containing nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP) for 16 h at room temperature. Finally, the color reaction was stopped by washing the sections for 15 min in 10 mm Tris-HCl and 1 mm ethylenediamine tetraacetic acid (EDTA) (pH 8.1).

Whole-mount ISH was performed as described previously [33]. In brief, the medaka gonads were fixed in 4% paraformaldehyde/PBS for 4 h and then transferred to 30% sucrose/

Table 2. Primers used for gene cloning of ovary structure protein 1 (3′-RACE and 5′-RACE) and in situ hybridization (ISH)

Primers		Sequences
Primers for 3′-RACE	Forward primer 1	5′–AAGAGCCTTCTGGACAGTATGGTGG-3′
	Forward primer 2	5′–CTATCCTGAACAAGAGCCAGCCAAG-3′
	Adaptor primer	5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTT-3′
	Nested primer	5′-CGCTACGTAACGGCATGACAGTG-3′
Primers for 5′-RACE	Forward primer 1	5′–TGGGGTGTAGCGGAACTGACTGAA-3′
	Forward primer 2	5′–CTGCCATATTTGGGACACTTCCTTG-3′
	Outer primer	5′–CATGGCTACATGCTGACAGCCTA-3′
	Inner primer	5′–CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3′
Primers for ISH	Forward primer	5′- AGCAGAATGAGGGTGTTGTGGATTTGTC–3′
	Reverse primer	5′- GGCTTGGCTGGCTCTTGTTCAGGATA–3′

PBS overnight at 4°C. After fixation, the gonads were treated with proteinase K (50 μg/ml) for 20 min at 37°C. Hybridization with the sense or antisense probes at a final concentration of 1 ng/ml was performed at 42°C overnight. Detection and color reaction were performed with a 1,000-fold dilution of alkaline phosphatase-conjugated anti-DIG antibody and with fresh color reaction buffer containing NBT and BCIP.

In addition, the negative control consisted of sections that were hybridized with equal amounts of sense probe under the same ISH conditions as described above. With each set of ISH experiments, a section with no probe was also added to control nonspecific binding. The sense and antisense DIG-labeled RNA probes were obtained by cloning a partial sequence of the OSP1 cDNA (278 bp) into the pGEM-T Easy vector (Promega) and transcribing it in vitro using an RNA labeling kit (Roche) in accordance with the manufacturer's protocol. Primer pairs are listed in Table 2.

Chemical analysis

The actual concentrations for EE2 exposure groups were determined by using solid phase extraction concentration combined with UPLC-ESI-MS/MS. In brief, 500-ml water samples were collected from each exposure tank, spiked with 10 ng of EE2-d4 (Wako Pure Chemical) as internal standard, and extracted through hydrophilic lipophilic balanced (HLB) cartridges (6 ml, 500 mg; Waters) at a flow rate of 5 to 10 ml/min. The cartridge was preconditioned with 6 ml of ethyl acetate (Fisher Chemical), 6 ml of acetonitrile (Fisher Chemical), and 12 ml of distilled water. Then the cartridges were rinsed with 10 ml of distilled water and dried under nitrogen flow. Target analytes were subsequently eluted with 15 ml of ethyl acetate. The eluates were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 ml of methanol (Fisher Chemical) for UPLC-ESI-MS/MS analysis. The analytical methods for UPLC-ESI-MS/MS are provided in the Supplemental Data (Supplemental Data, Table S1). The method detection limit of EE2 for a 500-ml water sample was 0.2 ng/L, and the actual concentrations were found to be below the detection limit for all the vehicle control aquaria.

Statistical analysis

Statistical analyses were conducted using SPSS 11.5 (SPSS). All data are expressed as mean ± SD. The normality of each dataset was assessed by the Kolomogrov–Smirnov one-sample test, and homogeneity of variance was determined by a Levene's test. Differences among each exposure group were analyzed by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered to be significant.

RESULTS

Gene expression profiles

The transcription levels of 14 candidate genes (FIGα, Elf-4e, 42Sp43, 42Sp50, ZPA, ZPB, ZPC, Qd, Fst1, Foxl2, Sycp1, Tra2a, Vasa, and OSP1) in gonads of medaka exposed to 74.8 ng/L EE2 were measured by qRT-PCR. Among those genes selected for analysis, eight (FIGα, Elf-4e, 42Sp43, 42Sp50, ZPA, ZPB, ZPC and Qd) were chosen because they are well known to be involved in ovary development or maintenance [17–20], and the remaining six (Fst1, Foxl2, Sycp1, Tra2a, Vasa, and OSP1) were selected based on their high mRNA transcription levels determined in previous studies of ovarian gene expression profiles of fish species [21–23]. Of the 14 genes, the transcription levels of 13 (FIGα, Elf-4e, 42Sp43, 42Sp50, ZPA, ZPB, ZPC, Qd, Fst1, Foxl2, Sycp1, Tra2a, and OSP1) and nine (FIGα, 42Sp43, 42Sp50, ZPA, ZPB, ZPC, Qd, Tra2a, and OSP1) genes, at 21 and 90 dph, respectively, had female-like expression patterns in EE2-exposed male medaka fish with transcription levels comparable to those of female medaka fish (Fig. 1). At the 21-dph stage, the increase in mRNA

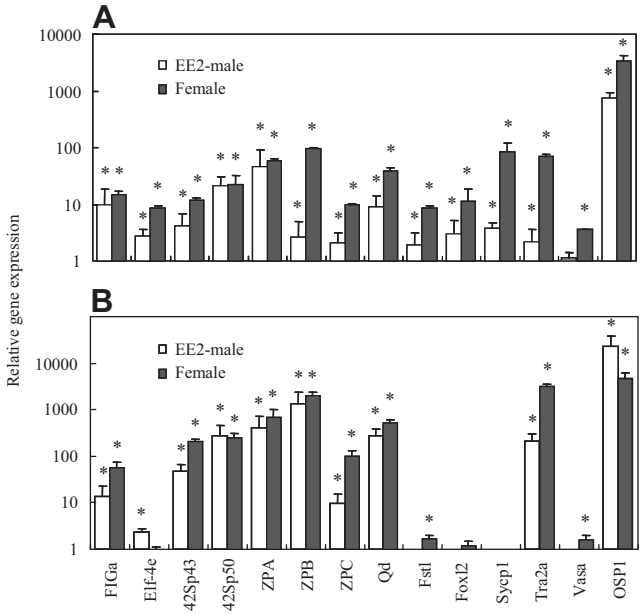

Fig. 1. Relative transcription levels of 14 candidate genes in gonads of male medaka fish after exposure to 74.8 ng/L 17α-ethynylestradiol (EE2). (**A**) Exposure from 0 to 21 dph. (**B**) Exposure from 0 to 90 dph. Fold-change values were calculated as the ratio between the gene expression levels of males in the EE2 exposure group and the mean expression levels of the control males. Ribosomal protein L7 was used as the internal control, and data are presented as means ± standard deviation ($n = 6$). $^{*}p < 0.05$ compared with the control males.

levels of OSP1 in male fish were the greatest (747-fold), followed by ZPA (47-fold), 42Sp50 (21-fold), FIGa (10-fold), and Qd (ninefold). This was similar to the gene expression changes at the 90-dph stage, at which time the fold change in mRNA levels of OSP1 were also the greatest (22,501-fold), followed by ZPB (1,139-fold), ZPA (412-fold), 42Sp50 (280-fold), and Qd (279-fold).

Of the 45 sexual differentiation-related genes, including 10 steroid hormone receptors, 9 steroidogenesis enzymes, and 26 ovary/testis differentiation-related regulation proteins, only a subset of genes (DMC1, WNT4, CXCR4, CYP19A, 17β-HSD1, and Era) had female-like expression patterns in EE2-exposed male medaka fish with transcription levels comparable to those of female medaka fish. The expression profiles of all genes are shown in the Supplemental Data (Supplemental Data, Table S2). Compared with the mRNA abundance of the 14 genes related to ovary development, the changes in mRNA abundance of all female-like genes were relatively low, for example, DMC1 (1.2-fold), WNT4 (1.6-fold), CXCR4 (-1.5-fold), CYP19A (-2.3-fold), 17β-HSD1 (1.3-fold), and ERα (5.8-fold) at the 21-dph stage and DMC1(1.5-fold), WNT4 (1.1-fold), CXCR4 (3.6-fold), CYP19A (6.5-fold), 17β-HSD1 (11.4-fold), and ERα (-13.9-fold) at the 90-dph stage. Of these genes, the mRNA abundance of ERα and 17β-HSD1 was greatest at the 21- and 90-dph stages, respectively (5.8-fold increase of ERα at the 21-dph stage and 11.4-fold increase of 17β-HSD1 at the 90-dph stage). However, the mRNA abundance of these genes was not greater at both sampling times (13.9-fold downregulation for ERα at the 90-dph stage and 1.3-fold upregulation for 17β-HSD1 at the 21-dph stage).

Relationship between gene expression and the severity index of intersex

Six candidate genes (FIGα, 42Sp50, ZPA, ZPB, Qd, and OSP1) were selected based on their female-like expression patterns and relatively high mRNA abundance, as described above, to investigate the relationships between their transcription levels and the severity index of intersex. Significant positive relationships were observed between the mRNA abundance of 42Sp50, Qd, ZPA, ZPB, and OSP1 as well as the severity of intersex (Fig. 2). The transcription levels of FIGα were not significantly positively correlated with the severity of intersex ($r^2 = 0.1028$, $p = 0.054$), although FIGα also had female-like expression patterns and relatively high mRNA abundance.

The association between OSP1 gene expression in each testis sample and the corresponding EE2 exposure concentration was further investigated. Although a dose-dependent induction of OSP1 transcription levels was observed for exposure groups, there were some testis samples with induced OSP1 gene expressions at relatively low EE2 exposure concentrations (5.6 and 16.1 ng/L EE2) in which the occurrence of intersex could not be detected by the optical microscope system we used (Fig. 3).

Clone and sequence analysis of medaka OSP1

To explore the biological functions of OSP1 and verify its potential role as a suitable biomarker for indicating the occurrence of intersex, gene clone and sequence analysis were conducted. The full-length mRNA sequence was 802 bp, including an 86-bp 5′ untranslated region (UTR) and a 74-bp 3′ UTR (including the poly A tail) (Fig. 4). The open reading frame of 642 bp (including the stop codon) encodes a polypeptide of 214 amino acids, of which the first 16 residues were predicted to comprise a signal peptide. The mature peptide of OSP1 was predicted to have 2.0 negative charges at pH 7.0, and has a molecular weight of 2.3 kDa and an isoelectric point of 5.65. The OSP1 gene was located in region 21,754,079 to

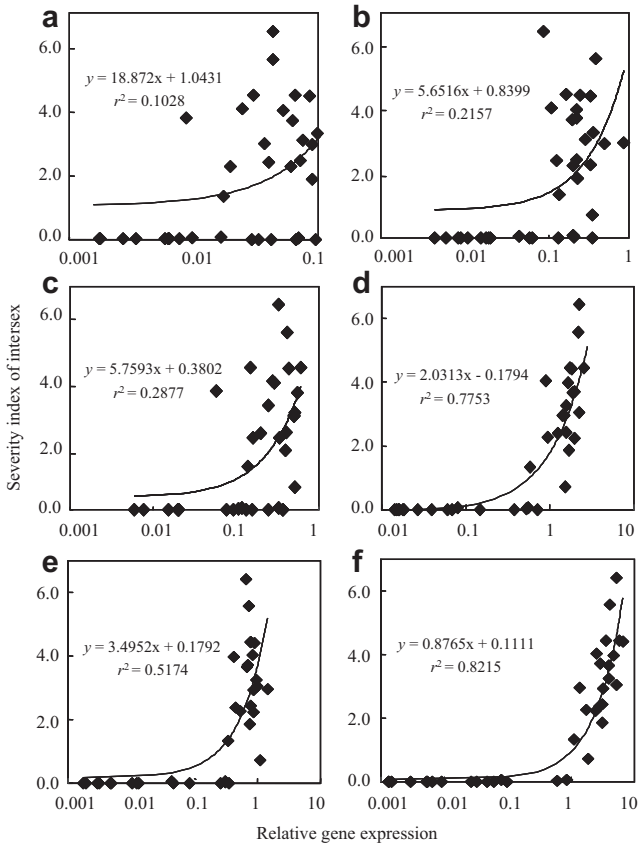

Fig. 2. Relationships between the gene transcription levels and the severity index of intersex. Ribosomal protein L7 was used as the internal control to calculate the relative mRNA expression. (**a**) FIGα ($p = 0.054$); (**b**) 42sp50 ($p = 0.004$); (**c**) Qd ($p = 0.001$); (**d**) ZPA ($p = 0.000$); (**e**) ZPB ($p = 0.000$); (**f**) OSP1 ($p = 0.000$).

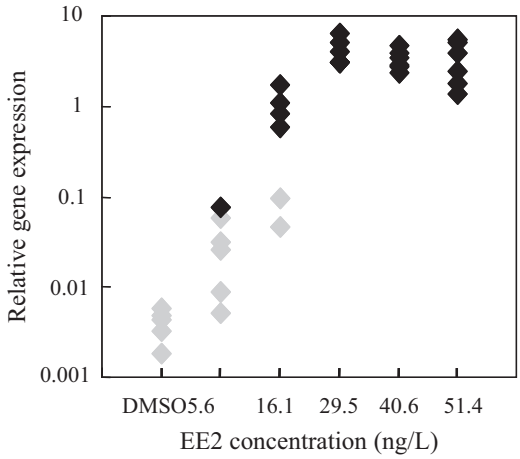

Fig. 3. Relative transcription levels of ovary structure protein 1 in gonads of males exposed to 17α-ethynylestradiol (EE2). Ribosomal protein L7 was used as the internal control to calculate the relative mRNA expression. Samples with intersex seen by the histopathological observation are marked as ◆ and samples without intersex are marked as ◆. DMSO = dimethyl sulfoxide.

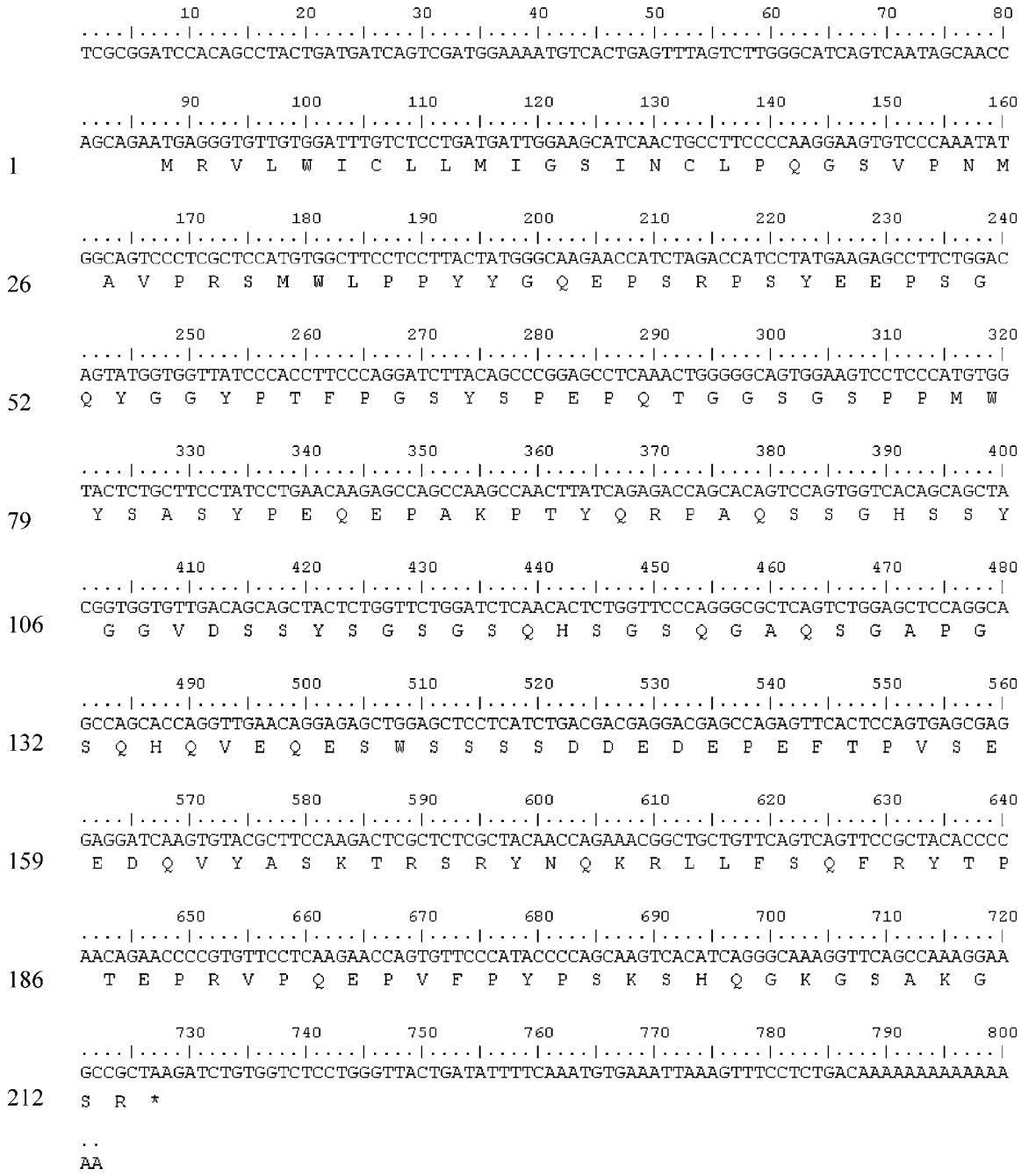

Fig. 4. Nucleotide and deduced amino acid sequence of ovary structure protein 1 cDNA isolated from gonads of Japanese medaka (orange red strain, *Oryzias latipes*). The numbers above the sequence indicate the nucleotide positions, and the values on the left correspond to the amino acid residues.

21,755,173 of chromosome 6 and consisted of five exons and four introns. The exon–intron boundaries of the OSP1 gene have conserved the classical "gt-ag" intron splice motifs. Additional analysis of the promotor region of the OSP1 gene revealed the presence of putative GATA-1, GATA-2, SOX-5, and SRY binding sites scattered throughout the sequence (data not shown).

A search for homologous sequences of OSP1 using the Ensembl database (http://www.ensembl.org/Multi/blastview) identified six additional copies of OSP cDNAs, termed OSP2 to OSP7, in the medaka genome. Products of these seven genes were related to each other, and comparisons of the deduced peptide sequence of each cDNA revealed that approximately 126 amino acid residues (∼59.2%) are identical among these proteins (Supplemental Data, Fig. S2). In ovaries from female medaka, the mRNA abundance of the OSPs has similar transcription levels at both the 21- and 90-dph stages (∼2,800–3,400-fold changes and 2,400–7,500-fold changes, respectively). However, in gonads from male medaka exposed to 74.8 ng/L of EE2, mRNA expression of OSP1 was 636-fold upregulated at the 21-dph stage and 16,876-fold upregulated at the 90-dph stage compared with the males from the DMSO control, whose levels were much higher than for other OSPs (∼7–477-fold upregulated at 21 dph and 1,637–11,476-fold upregulated at 90 dph) (Supplemental Data, Fig. S3). Furthermore, in male medaka fish sampled at 21 dph, the mRNA abundances for all OSPs exposed to EE2 were significantly less compared with the females in the DMSO control group. However, at 90 dph, the mRNA abundances of most OSPs in males exposed to EE2 were greater (although not significantly

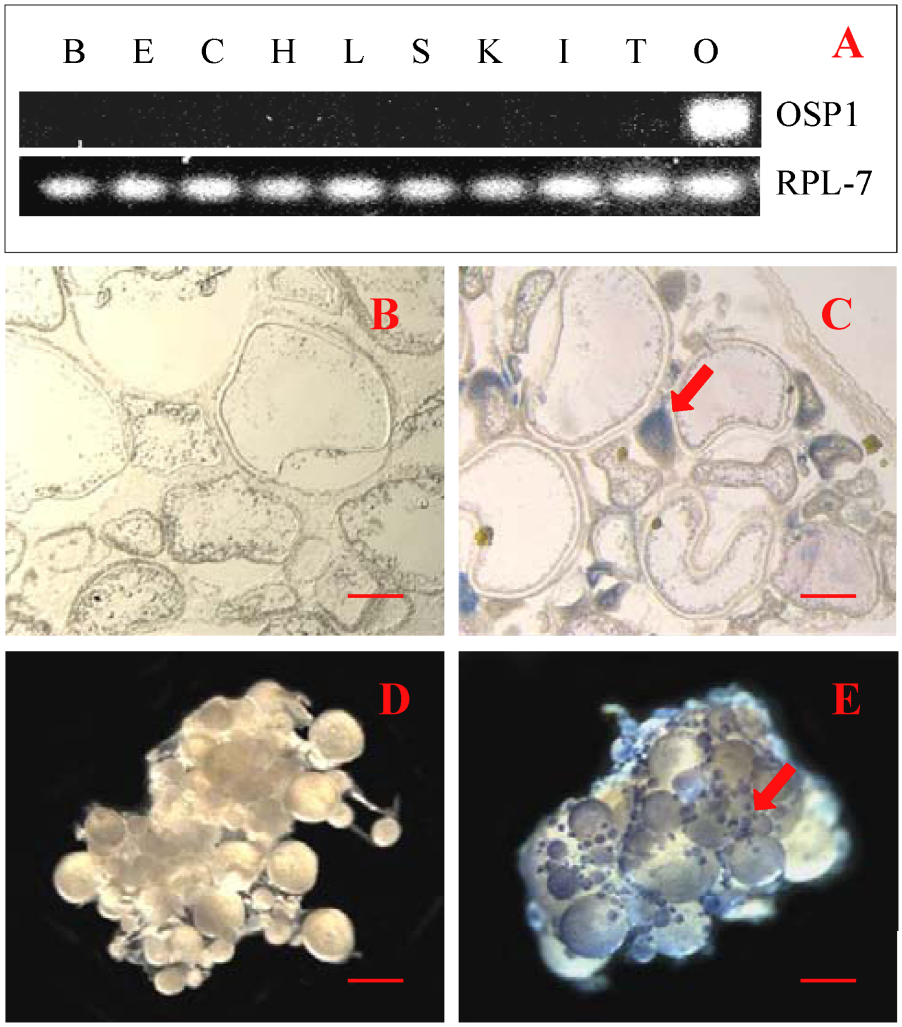

Fig. 5. Tissue distribution and cellular localization of ovary structure protein 1 (OSP1) gene expression. (**A**) Tissue distribution of OSP1 expression determined by reverse transcription–polymerase chain reaction in brain (B), eye (E), cheek (C), heart (H), liver (L), spleen (S), muscle (M), intestine (I), testis (T), and ovary (O). (**B–E**) Section in situ hybridization (ISH) and whole-mount ISH of OSP1 in gonads of adult female medaka fish. The arrows show OSP1 localization. (**B**) and (**D**) are the negative controls. Scale bar = 50 μm in (**B**), (**C**); 400 μm in (**D**), (**E**). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

different) than those in females in the DMSO control group, showing a large upregulation from 21 to 90 dph.

Tissue distribution and cellular localization of OSP1

Tissue distribution of OSP1 gene expression was determined in brain, eye, gill, heart, liver, spleen, muscle, intestine, testis, and ovary of adult medaka by RT-PCR and agarose gel electrophoresis. It was found that OSP1 transcripts were expressed exclusively in the ovaries (Fig. 5A). This was further verified by the results of ISH. Both section and whole-mount ISH using the antisense RNA probes revealed that the OSP1 mRNA was more abundant and homogeneous in the oogonia and early oocytes and that the hybridization signal was weak in the later previtellogenic and vitellogenic oocytes (Fig. 5B–E). Ovary structure protein 1 gene transcripts in male medaka fish with intersex were further measured by section ISH. Antisense DIG hybridization signals were specifically detected in the primary oocytes in the testis (Fig. 6).

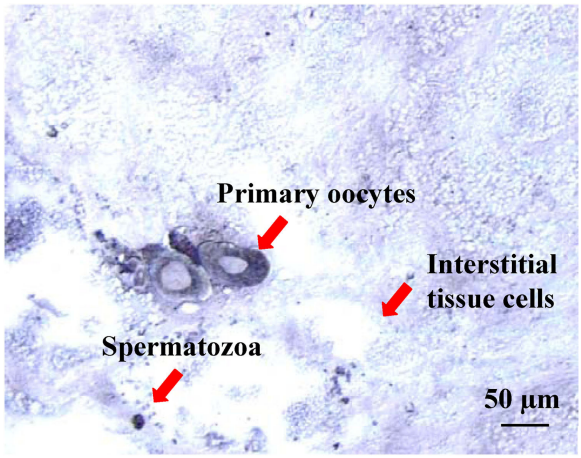

Fig. 6. Localization of ovary structure protein 1 gene expression by in situ hybridization in male fish with intersex. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

DISCUSSION

Intersex of fish species has been widely reported throughout the world and has been used as direct evidence of the

feminization of wild fish from freshwater and marine environments [4–6]. Although intersex of fish has been related to impairment of reproductive success by affecting sperm quality, fertilization success, and the ability to produce viable offspring [7,8], little concern has been expressed regarding the development of appropriate molecular effect biomarkers for detecting the occurrence of intersex in fish species.

Of the 14 candidate genes either involved in ovary development and maintenance, or with relatively high mRNA transcription levels in ovarian gene expression profiles in fish species, OSP1 showed a female-like expression pattern with the highest transcription levels in males at both 21 and 90 dph (747-fold and 22,501-fold, respectively), followed by ZP proteins and 42Sp50 (Fig. 1). It should be noted that OSP1 was a novel gene selected from datasets of ovarian gene expression studies using an oligonucleotide microarray, called NO.TC20436 [21]. Because only a partial expressed sequence tag of OSP1 was identified, its basic functions are still unclear. Compared with other candidate genes, the transcription level of OSP1 in the gonad of male medaka fish exposed to EE2 was the highest, similar to its transcription level in normal female medaka fish (Fig. 1). This finding suggested that similar oocyte structures would be present in the testis of male fish with intersex and that OSP1 would be a potential biomarker gene for detecting the occurrence of intersex. Furthermore, considering that some sex differentiation-related genes (like CYP19A, SOX9, and DMRT1) have also been considered as biomarkers for indicating sex differentiation and the development process in previous studies [11,34,35], the gene expression profiles of the 45 candidate genes involved in sexual differentiation were also investigated (Supplemental Data, Table S2). Among these genes, only DMC1, WNT4, CXCR4, CYP19A, 17β-HSD1, and ERα showed female-like expression patterns in EE2-exposed male medaka fish with transcription levels comparable to those of control females. These results are consistent with gene expression during the estrogen-induced feminization process in other fish species such as rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) [23,36]. Because the changes in transcription levels of all these genes with female-like expression patterns were relatively low, they are unlikely to be useful as a biomarker of intersex in male fish exposed to estrogenic substances.

Of the six genes selected (FIGα, 42Sp50, ZPA, ZPB, Qd, and OSP1) with female-like expression patterns and relatively high mRNA abundance (as described above), significant positive relationships with the severity index of intersex in male medaka were observed for the mRNA abundance of 42Sp50, Qd, ZPA, ZPB, and OSP1 (Fig. 2). It should be noted that the mRNA abundance of FIGα, which has been used as a biomarker of sexual differentiation and development in female medaka [17], was not significantly positively correlated with the severity of intersex. This could be due to the low expression levels of this gene. Of the genes with significantly positive relationships, the r^2 and p values for OSP1 were 0.8215 and 0.000, respectively, which was higher than those of others, suggesting that the mRNA abundance of OSP1 is the most correlated to the histopathological changes. Thus, OSP1 was demonstrated to be the most suitable biomarker of effect because of its relatively highest transcription levels compared with other genes together.

In addition, an association between OSP1 gene expression in each testis sample and its corresponding EE2 exposure concentration was found (Fig. 3). It was observed that some testis samples induced OSP1 gene expressions at relatively low EE2 exposure concentrations (5.6 and 16.1 ng/L EE2), but intersex could not be detected in these testis samples using light microscopy. This phenomenon was perhaps due to the low detection limit of the optical microscope system. The diameters of the observable oocytes as seen by an optical microscope system are usually greater than 20 μm [6,30]; therefore, it is difficult to distinguish the smaller ones from the spermatozoids and other somatic cells, because the diameters of the oogonias and early primary oocytes are approximately 5 to 10 μm in medaka fish [37]. These results suggest that OSP1 gene expression is a more sensitive tool for indicating the occurrence of intersex compared with traditional histopathological observation.

Ovary structure protein 1 transcripts were expressed exclusively in the ovaries, and they were more abundant and homogeneous in the oogonia and early oocytes, with a weak signal in the later previtellogenic and vitellogenic oocytes (Fig. 5B–E). This gene expression pattern was similar to well-known genes such as ZPC, 42Sp50, and FIGα [17–19], indicating its crucial role in early ovarian development process. This phenomenon was further confirmed by the results obtained by analyzing the OSP1 promoter region, where the presence of putative GATA-1, GATA-2, SOX-5, and SRY binding sites were observed to be scattered throughout the sequence, suggesting that expression of OSP1 would be controlled by a complex gene regulatory network. Because the transcription factors GATA-1, GATA-2, SOX-5, and SRY regulate sexual differentiation and development [38–41], it is likely that OSP1 is involved in ovarian development, as expected. Ovary structure protein 1 gene transcripts in male medaka fish with intersex were also specifically detected in the primary oocytes in the testis (Fig. 6), further confirming it was a sensitive and specific biomarker of effect that can be used to indicate the occurrence of intersex of male fish.

In conclusion, a specific and sensitive molecular biomarker of effect for determining the severity index of intersex in medaka was developed. It can be expected that this marker will provide a powerful tool for direct warning of intersex occurrence in male medaka under exposure to estrogenic endocrine disruptors. Further investigation is required to determine the exact functions of the OSP1 gene and its potential application to wild fish species.

SUPPLEMENTARY DATA

Table S1. Parameters for analysis of 17α-ethynylestradiol by ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry.

Table S2. Relative transcription levels of 45 target genes involved in sexual differentiation.

Fig. S1. Severity index of intersex of medaka fish exposed to different concentrations of EE2.

Fig. S2. Alignment of the deduced peptide sequences of CDS regions of OSPs.

Fig. S3. Relative transcription levels of OSPs in gonad of medaka fish after exposure to EE2. (197 KB DOC).

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