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Understanding the Molecular Basis for Differences in Responses of Fish Estrogen Receptor Subtypes to Environmental Estrogens

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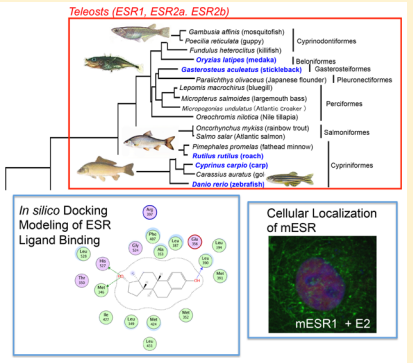
Supporting Information

ABSTRACT: Exposure to endocrine disrupting chemicals (EDCs) can elicit adverse effects on development, sexual differentiation, and reproduction in fish. Teleost species exhibit at least three subtypes of estrogen receptor (ESR), ESR1, ESR2a, and ESR2b; thus, estrogenic signaling pathways are complex. We applied in vitro reporter gene assays for ESRs in five fish species to investigate the ESR subtype-specificity for better understanding the signaling pathway of estrogenic EDCs. Responses to bisphenol A, 4-nonylphenol, and *o,p′*-DDT varied among ESR subtypes, and the response pattern of ESRs was basically common among the different fish species. Using a computational in silico docking model and through assays quantifying transactivation of the LBD (using GAL-LBD fusion proteins and chimera proteins for the ESR2s), we found that the LBD of the different ESR subtypes generally plays a key role in conferring responsiveness of the ESR subtypes to EDCs. These results also indicate that responses of ESR2s to EDCs cannot necessarily be predicted from the LBD sequence alone, and an additional region is required for full transactivation of these receptors. Our data thus provide advancing understanding on receptor functioning for both basic and applied research.



INTRODUCTION

Steroid hormones play fundamental roles in regulating reproductive activities in vertebrates. Estrogens in particular regulate ovarian development, differentiation and maintenance, and oogenesis, as well as stimulating the hepatic synthesis of vitellogenin (VTG) and choriogenin, which are vital for oogenesis in fish.[1,2] These effects are principally mediated through estrogen receptors (ESRs) which belong to the nuclear hormone receptor superfamily. After binding of a ligand to the ligand-binding domain (LBD) of ESR, this complex binds as homo dimer to estrogen response elements (ERE) in the promoter regions of estrogen responsive target genes and regulates their transcription.

Two ESR subtypes (ESR1 and ESR2) have been cloned from amniotes. Despite similar in vitro ERE-binding capacities and comparable affinities for 17β-estradiol (E2),[3,4] ESR1 and ESR2 mediate distinct profiles of gene expression.[5,6] Therefore, ESR1 and ESR2 have been considered to have specific roles and mediate responses to estrogens differently in mammals. In mice, ESR1 is essential for the development and function of reproductive organs, while ESR2 activity has more of a role associated with nonreproductive organs, although its presence (and activity) in the granulosa cells is required for fertility.[7,8] In most teleost species, three subtypes of ESR, namely ESR1 (formerly named ERα), ESR2a (formerly named ERβ2 except for medaka (*Oryzias latipes*) where it is known as ERβ1), and ESR2b (formerly named ERβ1, except for medaka where it is known as ERβ2) have been identified as being encoded by different genes, where ESR2a and ESR2b appear closely related, reflecting a gene duplication event.[9−11] In this paper, we have adopted the ESR nomenclature for classifying different fish ESR subtypes to avoid confusion in comparisons across the study species (see the Supporting Information (SI) for more details on

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the nomenclature that has been adopted to describe ESRs in different fish species). The presence of three subtypes of ESR further complicates understanding of ESR subtype-specific roles. In medaka, the level of VTG induction by several EDCs is positively correlated with expression of ESR2 subtypes.[12] Recent studies using gene knockdown in goldfish primary hepatocytes and zebrafish embryos have demonstrated that ESR2a and/or ESR2b are required for estrogen-mediated upregulation of ESR1 as well as VTG expression.[13,14] These results suggest fundamental contributions of ESR2 subtypes in fish reproduction.

There is a global concern about the presence of EDCs in the environment and their health effects in both humans and wildlife. To date, particular emphasis has been on effects of estrogenic EDCs, and more than 200 chemicals have been identified with estrogenic activity, including pharmaceuticals and industrial and agricultural compounds such as alkylphenols, pesticides, plasticizers, and bisphenols. Most EDCs readily enter the aquatic, and as a consequence, fish are especially at risk of exposure. Effects of some EDCs on fish are proven, and they include delayed onset of sexual maturation, reduced gonadal growth, gonadal deformations, inhibition of spermatogenesis, reduced sperm counts, lowered egg production, skewed sex ratios, and increased prevalence of intersex.[15−19] However, the involvement of different ESR subtypes in mediating the adverse effects of these estrogenic EDCs is so far poorly studied.

Risk of endocrine disruption on fish cannot necessarily be predicted for all species by simply examining receptor activation for a few model fish species[20−24] and comparative analyses are essential for understanding both mechanisms and differences in responsiveness to EDCs. In vitro reporter gene assays have been developed and applied successfully as screening methods to evaluate chemicals with estrogenic effects for a variety of model fish species.[20,21,25] These assays are now being applied to help inform on the similarities and differences between model and sentinel fish species in ESR activation for the different ESR subtypes.[21,25]

Here, we used custom developed in vitro ESR reporter gene assays for five fish species to analyze the ligand-, species-, and subtype-specificity for EDCs. The species adopted were medaka and zebrafish (*Danio rerio*) as laboratory "model" species, three spined stickleback (*Gasterosteus aculeatus*), used for both laboratory and wild population studies, and carp (*Cyprinus carpio*) and roach (*Rutilus rutilus*) that have been used widely for environmental monitoring of EDCs impacts. The test compounds included bisphenol A (BPA), 4-nonylphenol (NP), and dichlorodiphenyltrichloroethane (*o,p′*-DDT), all widely recognized as estrogenic EDCs. We found that each ESR subtype showed distinct responses to EDCs, and these responses for the different ESR subtypes were comparable among five fish species. Given that the ligand sensitivity of ESR1 has been attributed previously to the LBD,[20] we applied in silico docking model analysis and revealed a strong relationship between simulated ligand-LBD interaction potential and ESR activation in the transactivation assay. However, there were some exceptions to this, in particular for ESR2s, where we show no concurrence between simulated ligand−LBD interaction potential and ESR activation in the transactivation assay. Applying the use of GAL4-ESR-LBD fusion proteins and chimera proteins between ESR2a and ESR2b, we show that an additional region to the LBD was required for full transactivation of ESR2b by EDCs. Our findings show that the activity of EDCs acting through ESR2s cannot be necessarily predicted from the LBD sequence alone. The data presented further highlight the utility of transactivation assays for understanding ESR function and for informing on possible risk associated with EDCs acting via fish ESRs.

MATERIALS AND METHODS

Chemical Reagents and Construction of ESRs. E2 was purchased from Sigma-Aldrich (St. Louis, MO), and NP (purity >97.0%), BPA (purity >99.0%), and *o,p′*-DDT (purity >99.5%) were from Kanto-Kagaku (Tokyo, Japan). All compounds tested in the reporter gene assay were dissolved in dimethyl sulfoxide (DMSO, Nacalai, Kyoto, Japan), and the concentration of DMSO in the culture medium did not exceed 0.1%. The expression plasmids used in this study is described in the SI.

Transactivation Assay and Data Analysis. To examine the ligand-sensitivities with the ESRs, transactivation assays using pGL3−4xERE were performed in HEK293 cells (DS Pharma Biomedical, Osaka, Japan) at 37 °C as previously reported.[20] Detailed methods for these assays are provided also in the SI. All transfections were performed at least three times, and results are presented as mean ± SEM from three separate experiments each consisting of three technical replicates per concentration tested. To average the background luciferase activity, data were normalized for responses of the different ESRs to the individual chemical (where zero and one hundred percent were defined as the lowest and the highest response against E2, respectively, for each data set) and dose−response data were analyzed by fitting three parametric nonlinear regression (slope = 1) curves onto the normalized data, and EC_{50} values were calculated from these curves using GraphPad Prism ver.5 (GraphPad Software, San Diego, CA). The statistical analysis was performed with two-factor factorial ANOVA and significance was set at $p = 0.05$.

Construction of GAL4-ESR-LBD and Chimera ESR Proteins. The transactivation assays were performed as described above, with the exception that the GAL4-responsive pG5 vector (Promega) was used as a reporter. E2 was added to the medium at a concentration of 10^{-8} M which induces a maximal response, whereas EDCs were added at 10^{-5} M. This is the maximum concentration to avoid cytotoxity as adopted in previous studies.[20,26] Results are presented as mean ± SEM from three separate experiments each consisting of three technical replicates per concentration tested. Data were normalized for responses of the different ESRs to the individual chemical, where zero and one hundred percent were defined as the vehicle control and the response against E2 (10^{-8} M), respectively, for each data set. The statistical analysis between the receptors was performed with two-way ANOVA with Bonferroni post test and significance was set at $p = 0.05$.

Cellular Localization of mESR Subtypes. The full-coding regions of mESR1, mESR2a, and mESR2b were amplified by PCR and subcloned into the pDsRed-Monomer-N1 vector (Takara). COS-7 (DS Pharma Biomedical) and HEK293 cells were transiently transfected with 200 ng of DsRed-tagged ESR using Fugene HD transfection reagent. After 4 h of incubation, E2 was added to the medium at 10^{-8} M. After 20 h, cells were washed with PBS and fixed in 4% paraformaldehyde. Fluorescence images were taken using a Nikon Confocal Microscope A1Rsi (Nikon, Tokyo, Japan) or inverted microscope CKX41 with DP72 (Olympus, Tokyo, Japan).

Computational Model and Docking Simulation for mESR Subtypes. The homology modeling of the ESR-LBD and the *in silico* analysis of the interaction potential between ligands and the ESR-LBD were performed using the programs of Molecular Operating Environment (MOE) (Chemical Comput-

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Table 1. Gene Transcriptional Activities for E2, BPA, NP, and *o,p′*-DDT Mediated by Each ESR Subtype from Five Fish Species[a]

		E2	BPA	NP	DDT
Medaka ESR1	EC_{50}(M)	1.31×10^{-10}	7.88×10^{-7}	6.20×10^{-7}	9.63×10^{-7}
	95% CI (M)	$(0.92\text{-}1.9)\times10^{-10}$	$(0.5\text{-}1.2)\times10^{-6}$	$(0.37\text{-}1.0)\times10^{-6}$	$(0.69\text{-}1.4)\times10^{-6}$
	RP (%)	100	0.017	0.021	0.014
Medaka ESR2a	EC_{50}(M)	3.25×10^{-11}	6.11×10^{-7}	N.D.	6.14×10^{-7}
	95% CI (M)	$(1.9\text{-}5.7)\times10^{-11}$	$(4.8\text{-}7.7)\times10^{-7}$		$(3.8\text{-}9.9)\times10^{-7}$
	RP (%)	100	0.0053		0.0053
Medaka ESR2b	EC_{50}(M)	8.16×10^{-11}	N.D.	8.64×10^{-7}	N.D.
	95% CI (M)	$(0.63\text{-}1.1)\times10^{-10}$		$(0.63\text{-}1.2)\times10^{-6}$	
	RP (%)	100		0.0094	

		E2	BPA	NP	DDT
Stickleback ESR1	EC_{50}(M)	2.94×10^{-10}	2.95×10^{-7}	9.63×10^{-8}	2.95×10^{-7}
	95% CI (M)	$(1.5\text{-}5.8)\times10^{-10}$	$(0.06\text{-}1.5)\times10^{-6}$	$(0.42\text{-}2.2)\times10^{-7}$	$(0.08\text{-}1.1)\times10^{-6}$
	RP (%)	100	0.10	0.31	0.10
Stickleback ESR2a	EC_{50}(M)	8.44×10^{-11}	1.74×10^{-6}	4.78×10^{-7}	5.33×10^{-7}
	95% CI (M)	$(0.51\text{-}1.4)\times10^{-10}$	$(0.83\text{-}3.7)\times10^{-6}$	$(0.16\text{-}1.4)\times10^{-6}$	$(2.9\text{-}9.9)\times10^{-7}$
	RP (%)	100	0.0049	0.018	0.016
Stickleback ESR2b	EC_{50}(M)	6.30×10^{-11}	N.D.	N.D.	N.D.
	95% CI (M)	$(0.18\text{-}2.3)\times10^{-10}$			
	RP (%)	100			

		E2	BPA	NP	DDT
Zebrafish ESR1	EC_{50}(M)	1.36×10^{-10}	7.88×10^{-7}	6.20×10^{-7}	N.D.
	95% CI (M)	$(0.79\text{-}2.3)\times10^{-10}$	$(0.53\text{-}1.2)\times10^{-6}$	$(0.37\text{-}1.0)\times10^{-6}$	
	RP (%)	100	0.017	0.022	
Zebrafish ESR2a	EC_{50}(M)	2.80×10^{-11}	3.86×10^{-7}	N.D.	1.73×10^{-7}
	95% CI (M)	$(1.7\text{-}4.6)\times10^{-11}$	$(2.3\text{-}6.5)\times10^{-7}$		$(0.62\text{-}4.8)\times10^{-7}$
	RP (%)	100	0.0073		0.016
Zebrafish ESR2b	EC_{50}(M)	2.64×10^{-12}	1.82×10^{-6}	2.82×10^{-7}	1.00×10^{-6}
	95% CI (M)	$(1.1\text{-}6.5)\times10^{-12}$	$(1.4\text{-}2.4)\times10^{-6}$	$(0.92\text{-}8.6)\times10^{-7}$	$(0.52\text{-}1.9)\times10^{-6}$
	RP (%)	100	0.00016	0.00094	0.00027

		E2	BPA	NP	DDT
Carp ESR1	EC_{50}(M)	4.18×10^{-10}	N.D.	N.D.	N.D.
	95% CI (M)	$(2.4\text{-}7.2)\times10^{-10}$			
	RP (%)	100			
Carp ESR2a	EC_{50}(M)	8.01×10^{-11}	1.07×10^{-6}	8.96×10^{-7}	1.36×10^{-6}
	95% CI (M)	$(0.53\text{-}1.2)\times10^{-10}$	$(0.62\text{-}1.8)\times10^{-6}$	$(0.30\text{-}2.7)\times10^{-6}$	$(0.47\text{-}3.9)\times10^{-6}$
	RP (%)	100	0.0075	0.0089	0.0059
Carp ESR2b	EC_{50}(M)	1.57×10^{-10}	N.D.	N.D.	N.D.
	95% CI (M)	$(0.90\text{-}2.7)\times10^{-10}$			
	RP (%)	100			

		E2	BPA	NP	DDT
Roach ESR1	EC_{50}(M)	1.32×10^{-10}	N.D.	4.24×10^{-7}	N.D.
	95% CI (M)	$(0.71\text{-}2.4)\times10^{-10}$		$(2.0\text{-}9.0)\times10^{-7}$	
	RP (%)	100		0.031	
Roach ESR2a	EC_{50}(M)	6.06×10^{-11}	N.D.	4.63×10^{-7}	9.36×10^{-7}
	95% CI (M)	$(0.33\text{-}1.1)\times10^{-10}$		$(0.02\text{-}8.8)\times10^{-6}$	$(0.42\text{-}2.1)\times10^{-6}$
	RP (%)	100		0.013	0.0065
Roach ESR2b	EC_{50}(M)	2.33×10^{-10}	N.D.	N.D.	N.D.
	95% CI (M)	$(1.0\text{-}5.4)\times10^{-10}$			
	RP (%)	100			

[a]95% CI; 95% confidence intervals of EC50. RP, relative potency; (EC50 E2/EC_{50} chemical X) × 100. N.D.; not-determinable because of weak estrogenicity.

ing Group, Montreal, Canada). To build homology models of mESR-LBDs, the crystal structures of human (h)ESR1-LBD, hESR2, and the interaction between hESR1-LBD and BPA were obtained from the Protein Data Bank (PDB: entries 1A52, 2FSZ, and 3UU7, respectively).[27−29] The most stable ligand-binding modes with the ESR-LBD were determined based on the lowest U-total value and each docking simulation was evaluated with a U-dock score (kcal/mol). Detailed methods are available in the SI.

RESULTS

Cloning and Phylogeny of Fish ESR Subtypes. We collected information on all ESR subtypes from medaka, three-spined stickleback, zebrafish, common carp, and roach from Genbank and Ensembl databases. Although most teleosts are predicted to have three ESRs (ESR1, ESR2a, and ESR2b), information for only one ESR2 subtype was available in the database for both stickleback (ESR2a) and roach (ESR2b). To isolate stickleback ESR2b and roach ESR2a, partial DNA fragments were amplified from the liver of each fish species by RT-PCR using degenerate oligonucleotides.[22] DNA fragments similar to ESR were obtained from both stickleback and roach cDNAs, and using the RACE technique, full length ESR2 cDNAs, including the ATG start site and TGA/TAA terminal signal, were cloned. Based on the phylogenic analysis (see below), these isolated ESRs were identified as stickleback ESR2b

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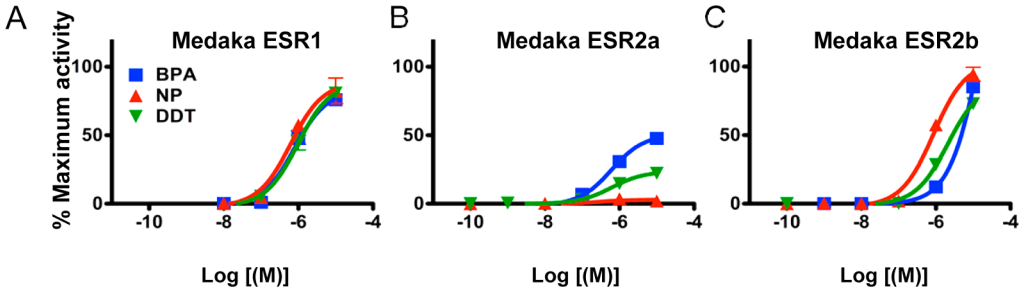

Figure 1. Concentration−response profiles of medaka (m)ESRs activated by EDCs. Reporter activities of mESR1 (A), mESR2a (B), and mESR2b (C) by bisphenol A (BPA), 4-nonylphenol (NP), and *o,p′*-DDT (DDT) were tested. Dose−response curves fitted on data normalized between 0 and 100%, where zero and one hundred were defined as the smallest and the largest values of E2 response, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

and roach ESR2a. The stickleback ESR2b gene (LC006094) comprises 1956 nucleotides and encodes a protein of 651 amino acids; the roach ESR2a gene (LC006093) comprises 1653 nucleotides and encodes a protein of 550 amino acids.

Comparison of the amino acid sequences of the three ESR subtypes across the five fish species showed that all sequences could be subdivided into 5 domains as defined by Krust et al.[30] (SI, Figure S1). The putative DNA-binding domain (DBD; C domain) and LBD (E domain) showed high similarities across the species analyzed (ESR1: DBD, 96−97%; LBD, 80−90%; ESR2a: DBD, 97%, LBD, 87−90% and ESR2b: DBD, 97−100%, LBD, 78−97%, all compared to the respective medaka sequence). The A/B-, D-, and F-domains had lower homologies (ESR1: A-domain, 32−58%; D-domain, 32−49%; F-domain, 7−24%; ESR2a: A-domain, 43−62%; D-domain, 36−52%, F-domain, 16−32% and ESR2b: A-domain, 38−66%; D-domain, 40−62%, F-domain, 7−15%, all compared to the respective medaka sequence). On exclusion of the hypervariable A/B and F domains, the neighbor-joining phylogenetic tree for ESRs resulted in the predicted two separate clades ESR1 and ESR2 (SI, Figure S2). In addition, the teleost ESR2 clade subdivides as a result of duplication of the ESR2 gene.

Estradiol-Induced Transcriptional Activity of Fish ESR Subtypes. Differences in translational activities of ESR1s mediated by the natural estrogen, E2, were small among species with only a 3.2-fold difference in ESR1 sensitivity between the most (medaka, $EC_{50} = 1.31 \times 10^{-10}$ M) and the least (carp, $EC_{50} = 4.18 \times 10^{-10}$ M) sensitive species (Figure S3A, in the SI, and Table 1). Similarly, the differences in ESR2a in response to E2 were also small with a 3.0-fold difference between the most (zebrafish, $EC_{50} = 2.80 \times 10^{-11}$ M) and the least (stickleback, $EC_{50} = 8.44 \times 10^{-11}$ M) sensitive species (Figure S3B, in the SI, and Table 1). In all species, ESR2a was more sensitive to E2 compared with ESR1. The EC_{50} of ESR2b showed an 88-fold difference in sensitivity between the most (zebrafish, $EC_{50} = 2.64 \times 10^{-12}$ M) and the least (roach, $EC_{50} = 2.33 \times 10^{-10}$ M) sensitive species (Figure S3C, in the SI, and Table 1). Overall, with the exception of zebrafish, the EC_{50} values for ESR2b for E2 were between the EC_{50}s for ESR1 and ESR2a. These results indicate that ESR subtype specificity and species differences are minimum in terms of the responses to E2.

Transactivation of Fish ESRs Exposed to EDCs. The dose−response curves and calculated EC_{50} values indicated species- and ESR subtype-differences in the sensitivity of ESRs to BPA, NP, and *o,p′*-DDT (Figure 1 and Table 1). Compared with E2, they all exhibited relatively weak estrogenic activities in inducing transactivation of all ESR subtypes examined.

BPA, NP and *o,p′*-DDT transactivated mESR1 and reached the maximum level of E2-induced transactivation at 10^{-5} M (Figure 1A). However, they were found to be very weak estrogens for mESR2a; even at 10^{-5} M, BPA induced half of the maximum level of E2-induced transactivation (Figure 1B). In contrast, these chemicals activated mESR2b, and the order of responsiveness to the different chemicals (at 10^{-6} M) was NP< *o,p′*-DDT < BPA (Figure 1C).

Although the pattern of transactivation of ESR1 in other fish species varied widely with the different chemicals, for all five species higher maximum activities EC_{50} values were generally observed for ESR1 compared with ESR2a (Figure S4, in the SI, and Table 1). When comparing maximum responses of ESR2a and ESR2b, the EDCs tested were more effective in activating ESR2b compared with ESR2a (SI: stickleback, Figure S4E,F; zebrafish, Figure S4H,I; and carp, Figure S4K,L). Both forms of the roach ESR2 exhibited lower transactivation abilities in response to EDCs (SI, Figure S4N,O). Taken together, these results showed that each ESR subtype responded differently to EDCs, but that the comparative responses for any one ESR subtype seems to be conserved among the fish species.

Cellular Localization of mESR Subtypes. We next investigated the possible reasons for the differential sensitivities among ESR subtypes using medaka as a model. Ligand-dependent nuclear translocation has been suggested to be a possible explanation for variable differences in transactivity between two medaka androgen receptor subtypes.[31] In contrast, experiments on cellular localization of mESRs showed a persistent nuclear localization of all three subtypes independent of the presence or absence of E2 under the culture conditions we adopted both in COS7 and HEK293 cells (Figures 2 and S5), suggesting that the nuclear localization step was not the cause of the differential sensitivities among subtypes.

In Silico Analysis of Ligand-Binding to mESRs. We have previously reported that ligand sensitivities of ESR1 can be attributed, at least in part, to the LBD.[20] The DBDs of all three mESR subtypes showed high similarities (Figure S6). These findings, together with the finding above of a persistent nuclear localization of all three subtypes independent of the presence or absence of E2, suggested that ligand-binding as the most likely step responsible for subtype selectivity rather than DNA-binding or nuclear translocation. To gain further insight into the ligand sensitivity among ESR subtypes relating to the LBD, we applied in silico analysis of all three mESR-LBD, which is based on a crystal structure of hESR-LBD as a template.[27−29] The homology models of mESR-LBD have close resemblance to hESRs-LBD, with low root-mean square deviation (RMSD) values (0.562 Å for ESR1, 0.757 Å for ESR2a, and 0.820 for ESR2b).

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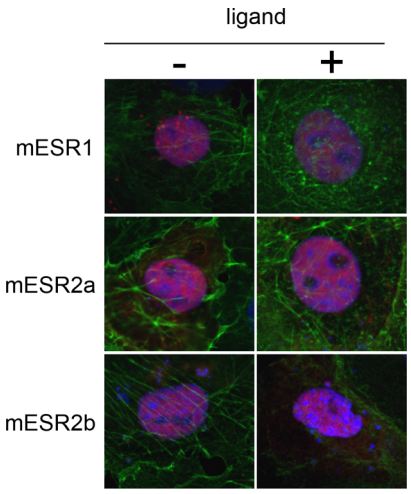

Figure 2. Cellular localization of medaka (m)ESR subtypes in COS7 cells. All mESR subtypes are localized in the nucleus in the presence or absence of E2, suggesting that nuclear localization step does not participate in the differential sensitivities among subtypes. The chromatin DNA and actin were stained with DAPI (blue) and Phalloidin (green), respectively.

Amino acid residues forming the ligand-binding pocket of the mESR1 for E2 were conserved with hESR1[28,32] (Figure 3A). For example, the hydroxyl group at position C3 of E2 was hydrogen-bonded to the Leu390 of the mESR1, whereas the hydroxyl group at position C17 interacted with His527 (Figure 3A). In addition, Glu356, which is known to form a hydrogen-bond with E2 in the hESR1[28,32] was located at a position close to C3, suggesting a possible electrostatic interaction between them. The interaction energy (U-dock) between mESR1 and E2 was −58.06 kcal/mol. Docking simulations between mESR1 and EDCs were performed, assuming that EDCs occupy the ligand-binding pocket with the lowest binding energy. The in silico analysis revealed that at least one side of residues (Glu356) interact with the hydrogen-bonded-, hydroxyl-, or chloride-group between ESR1 and EDCs (Figure 3B−D). In contrast, His527 in mESR1 did not interact with the hydrogen bonds with EDCs, suggesting a lower binding affinity. Given that both sides of the hydroxyl group participated in the hydrogen bond with Glu356 and Met424, the interaction energy between BPA and mESR1 was −48.03 kcal/mol, which was lower (suggesting a more stable binding) compared to NP (−39.58 kcal/mol) or *o,p′*-DDT (−33.17 kcal/mol) (Figure 3C,D).

Amino acid residues of the ESR2s interacting with E2 were conserved between human and medaka.[29] In particular, Glu316 (mESR2a) and Glu355 (mESR2b) formed hydrogen bonds with the ligand (Figure 3E,I), and His487 (mESR2a) and His526 (mESR2b) were located in close proximity to the hydroxyl group of C17 of E2. The interaction energies of mESR2a and mESR2b with E2 were estimated to be −53.87 and −55.95 kcal/mol, respectively, showing a similar binding potential compared to mESR1. For mESR2a, modeling suggests that BPA forms hydrogen bonds with Glu377 and Lys298 (Figure 3F), both not participating in interactions with E2 (Figure 3B). NP formed a hydrogen bond with Glu276, whereas *o,p′*-DDT did not form this bond (Figure 3G,H). Accordingly, the interaction energy of *o,p′*-DDT was the highest among the EDCs used in this experiment (−49.29, −47.37, and −36.88 kcal/mol for BPA, NP and *o,p′*-DDT, respectively). For mESR2b, a hydrogen bond was observed between Glu355 and the hydroxyl substitution of the BPA and NP (Figure 3J,K), whereas no strong interaction was detected between *o,p′*-DDT and mESR2b (U-dock estimated as −30.85 kcal/mol; Figure 3L).

Molecular Mechanisms of Differential Response among ESR Subtypes. The predicted interaction energies between ESRs and EDCs showed similar values among the subtypes, but this was not consistent with the transactivation assay, in which ESR2a showed lower transactivation abilities in response to EDCs than those of other ESRs. We then evaluated the above in silico simulation results through a series of empirical studies applying the GAL4 system. In this system, upon ligand-binding to the GAL4-ESR-LBD fusion protein, this complex interacts with the GAL4 binding site and activates the reporter transcription (Figure 4A). The process is completely depending on the LBD and thus enables us to empirically evaluate the interaction between ESR-LBD and the ligands.[33]

E2 similarly transactivated all GAL4-mESR-LBD fusion proteins. For ESR1, the EDCs tested induced transactivation of GAL4-mESR1-LBD with the order in responsiveness of BPA > NP > *o,p′*-DDT (Figure 4A), consistent with the predicted interaction energy in silico (Figure 3B−D). On the other hand, NP activated GAL4-mESR2b-LBD weakly but did not activate GAL4-mESR2a-LBD. BPA and *o,p′*-DDT failed to activate either of the GAL4-mESR2-LBD fusion proteins (Figure 4A). This suggests that mESR2-LBD can interact with the EDCs but requires additional domain for the EDC-driven transactivation in the GAL4 system. We therefore constructed chimera proteins in which LBDs were exchanged between mESR2a and mESR2b. All mESR variants, including the two chimera mESRs, showed ERE-driven reporter activity in response to E2. On the other hand, chimera mESR2s showed that the response to EDCs was much more comparable to the mESRs which the LBD originated from (Figure 4B). These results indicated that (1) differences in the transactivation among ESR subtypes largely depend on the LBD; (2) a region additional to the LBD is required for full transactivation of ESR2b by EDCs; and (3) such a region can be substituted by counterparts of mESR2 subtypes.

DISCUSSION

Various EDCs have been reported to affect sex determining processes and alter reproductive output, particularly in fish and amphibian species. For fish, EDC exposures can be continuous and in some cases they occur at high levels. Thus, fish species have been used extensively as models for research into the effects of EDCs and as sentinels for endocrine disruption in wildlife populations. In assessments of the effects of chemicals, generally few studies have taken into consideration "species diversity" and/or the "genetic diversity". A whole genome duplication event occurred coincident with the teleost radiation[9−11,34] resulting in duplicated paralogous genes and subsequently induced neo-functionalization (i.e., acquiring a new function), subfunction-alization (the original gene function is split and distributed to different paralogues), or inactivation/gene loss.[35] As a consequence in teleosts, estrogen signaling is mediated through at least three ESR subtypes and each subtype will likely show differential responses to ligands. This complicates the understanding of estrogen signaling pathways and the deleterious effects of the EDCs on those pathways to affect physiological function.

Species Differences of ESR Subtypes in Response to EDCs. All ESR subtypes responded to the endogenous estrogen, E2, in a similar manner, except for zebrafish ESR2b that showed a high sensitivity compared with the other fish ESR2bs studied.

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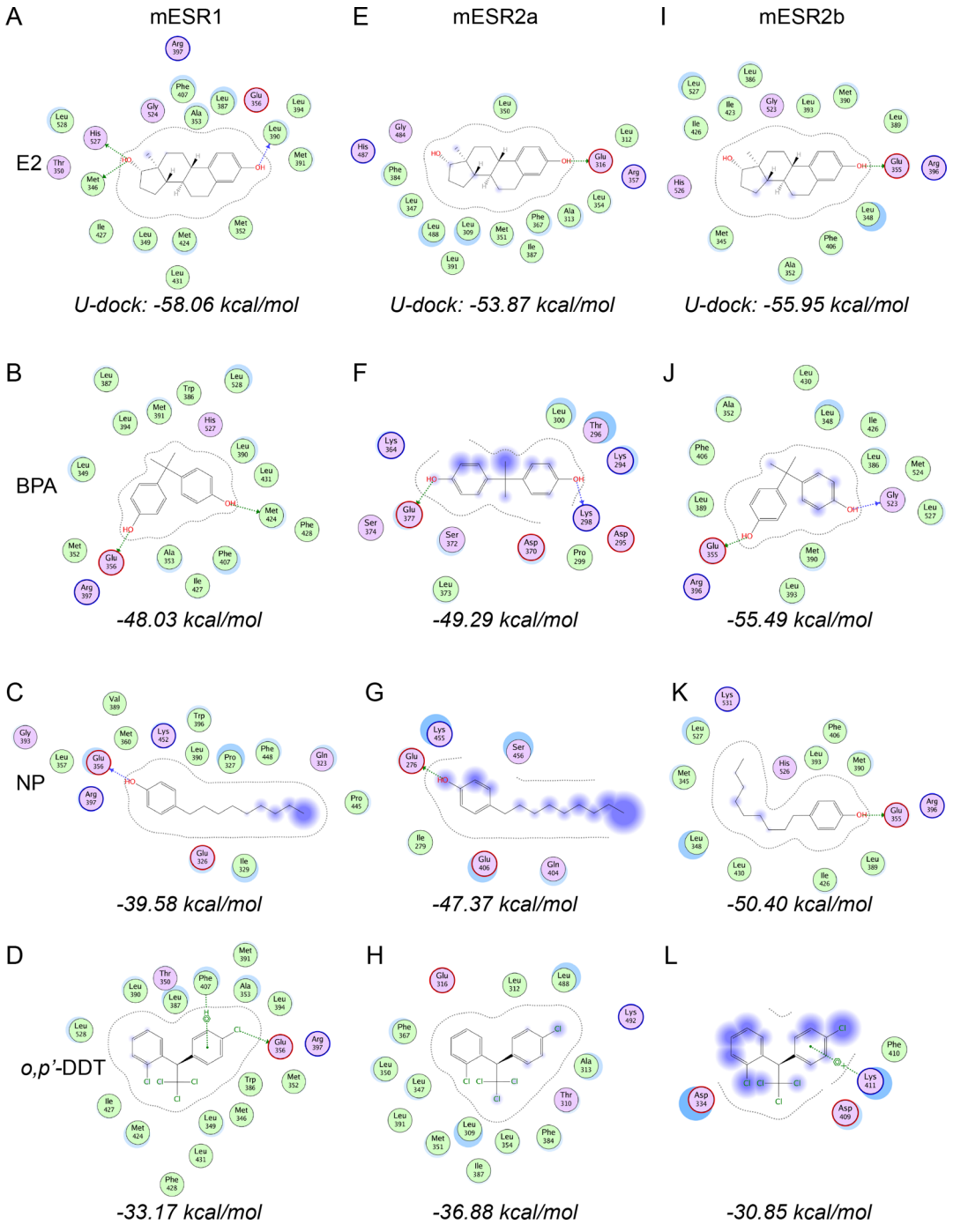

Figure 3. Predicted interaction of amino acid residues of medaka (m)ESRs and the ligands. Amino acid residues of mESR1 (A–D), mESR2a (E–H), and mESR2b (I–K) predicted to interact with E2 (A, E, I), BPA (B, F, and J), NP (C, G, and K), and *o,p′*-DDT (D, H, and L) are depicted. Polar (pink) and hydrophobic (green) amino acid residues interacting with ligands are indicated. Green dotted arrows indicate hydrogen bond. Purple circles indicate exposed region of the ligand.

This likely reflects functional conservation in the associated physiological processes during evolution. In contrast, there were clear differences in the responses to EDCs. ESR2a exhibited the weakest reporter activity based on the maximum response. ESR1 and ESR2b both responded to BPA, NP, and *o,p′*-DDT, but the sensitivity (EC_{50}) of ESR2b was generally lower compared with the responses for ESR1. Our data thus show that these functional responses to estrogenic EDCs for each ESR have persisted during evolution in different teleost species. We also found, however, that there are differences in the responsiveness of each ESR between fish species. For example, carp and roach ESRs (cyprinid fish) were relatively less sensitive to EDCs which might, at least in part, bear testimony to their ecological niche and tolerance to chemical contamination.

The gene network involved in ESR regulation and the interrelationships between the different ESRs has not been elucidated fully. However, a gene knockdown experiment in goldfish primary hepatocytes has revealed that ESR2a and/or ESR2b are required for estrogen-mediated induction of ESR1.[14,36] Furthermore, morpholino-oligonucleotide knock-

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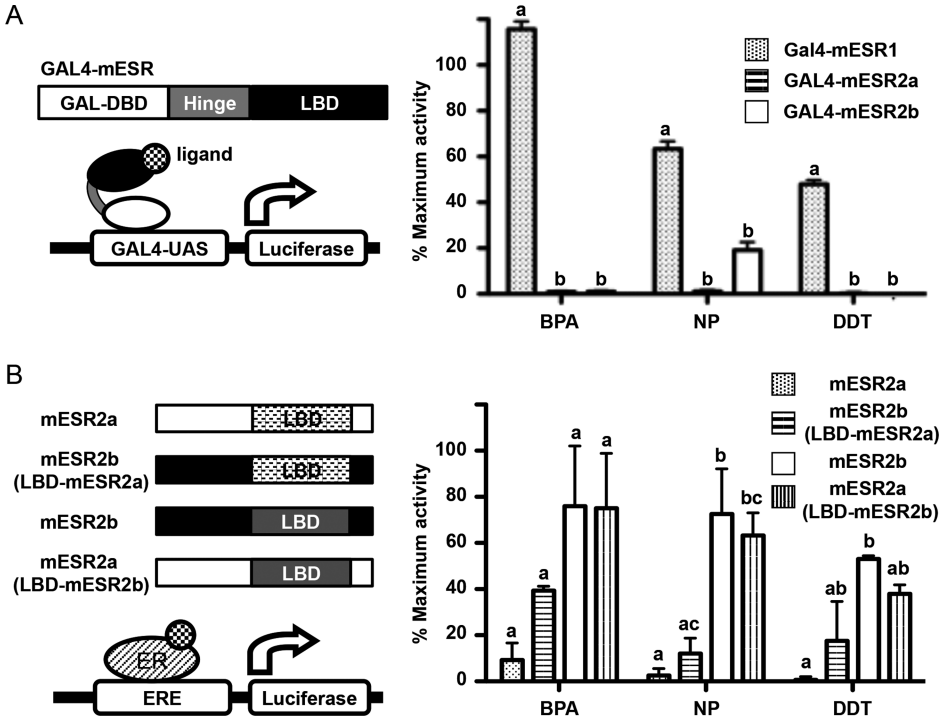

Figure 4. Ligand-binding domain (LBD)-dependent transactivation of medaka (m)ESR subtypes in response to EDCs. The contribution of LBDs of mESRs to differential responses to EDCs was investigated using the GAL4 system (A) and conventional assays with wild type and LBD-chimera mESRs (B). For ligands, 10^{-5} M of EDCs were added to the medium. Data normalized between 0 and 100%, where zero and one hundred were defined as the values for vehicle control and the E2 (10^{-8} M) in each data set, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested. * indicates a statistical difference between the subtypes ($p < 0.05$).

down experiments in zebrafish embryos revealed interactive expression of ESRs.[13] Although morpholino knockdown experiments are now known to cause nonspecific and/or off-target effects in some cases and this can potentially lead to misinterpretation of gene network analyses,[37] it is not surprising that coordinated regulation processes of ESRs are required for the induction of estrogen target genes such as VTG. This illustrates that even though ESR1 may act a primary signaling pathway for responding to EDCs (and thus acts as a sensitive biomarker for exposure estrogenic EDCs) responses of the ESR2s to EDCs are also crucial for assessing the effects of these chemicals in exposed animals.

Identifying the Source of Variation in the Responses of ESR to Estrogenic EDCs. We previously reported that the species differences in activation of fish ESR1 by EDCs depends on the LBD.[20] The LBD confers the ligand-binding and here various cofactor interactions can affect the responses to estrogens.[38] The LBD, therefore, most likely contributes to subtype variation in response to EDCs.

To evaluate the mode of ligand-binding to ESRs, we applied a 3D structure-based computational method. The docking model analysis revealed that the overall architecture of the three mESR-LBDs is similar and amino acid residues interacting with E2 are highly conserved. As a consequence, the interaction energies (U_dock) between E2 and the different mESR subtypes are comparable. This is consistent with the similar responses of all three mESRs to E2 in our transactivation assays. The hydroxyl group at position C3 of E2 forms a hydrogen-bond with Leu390 of mESR1, Glu316 of mESR2a and Glu355 of mESR2b, whereas the hydroxyl group at position C17 interacts with His527 of mESR1 and possibly with His487 of mESR2a and His526 of mESR2b. The electrostatic interactions between these amino acid residues and the hydroxyl groups at positions C3 and C17 are important for the maximum transactivation of mESRs. Because electrostatic interactions, such as hydrogen bonds, are much stronger than van der Waals forces, an electrostatic interaction network is likely to be the main element explaining the different preferences of EDCs to the ESR subtypes. In addition, lack of interactions between the histidine residues and the ligand induce a substantial reorientation of the imidazole ring of the histidine, possibly destabilizing the interaction between the ligand-binding pocket and the ligand.[27,39] Nonetheless, two hydroxyl groups of BPA form a hydrogen bond with mESR1 whereas there was only one for NP, but no hydroxyl group in *o,p′*-DDT, resulting in the difference of an order of interaction energy between mESR1 and EDCs as follows: BPA ≪ NP < *o,p′*-DDT. Importantly, this order is consistent with our empirical GAL4-mESR1-LBD assay, showing the robustness of our simulation.

Likewise, the two phenol groups of BPA possibly form hydrogen bonds with Glu377/Lys298 of mESR2a and Glu355/Gly523 of mESR2b. The interaction energy between mESR2s and BPA was estimated to be lower than that between mESR1 and BPA. ESR2a is generally predicted to interact stably with EDCs (lower interaction energy), although this is not consistent with a weak or even lack of mESR2a transactivation by EDCs.

To further investigate the contribution of LBD and to add empirical observation, we performed reporter gene assays using the GAL4 system, which can eliminate potential dependence of the ESR on the N-terminus and DNA-binding domain of the protein.[33] However, we did not observe any transactivation of GAL4-mESR2a-LBD or GAL4-mESR2b by the EDCs. This discrepancy between the GAL4 system and the intact receptor transactivation assay suggested that additional features are involved in EDC-induced activation of mESR2b. By constructing

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and applying chimera mESRs in which the LBD of mESR2a was replaced by the corresponding region of mESR2b and vice versa, we confirmed that such a region can be replaced by counterparts of mESR2 subtypes. Taken together, our results suggest that, although the LBD plays a major role for the differences seen in the transactivation among mESR subtypes, this does not account fully for the differences seen between the mESR2s. The N-terminus within AF-1 is indispensable for BPA-induced transactivation of hESRs.[27] These results indicate that, in addition to the LBD, highly structural modifications caused by ligand-binding and probably involving in AF-1 within the N-terminal domain are necessary for transactivation responses, at least for the mESR2b. It remains to be established whether or not EDCs actually bind to ESR2a, and therefore, the mode of binding between them, if any, needs to be further addressed to establish this, which could be established though in vitro binding assays and/or crystal structural analysis.

The DBD is highly conserved among ESR subtypes and is thus likely to share largely overlapping properties of DNA-binding. However, ligand−receptor interactions may result in significant modulation within the C-terminal extension of the core DBD,[40] and this could affect DNA-binding of the ESRs. It has been also shown that ESR can activate signaling pathways and modulate gene expression independently of direct binding to DNA.[41,42] It could be the case therefore that some EDCs may have potent effects by modulating ER via noncanonical pathways in vivo.

In this study, we show that the ESR reporter gene assay system can be usefully applied in the analysis of ligand-induced ESR transactivation across different fish species to identify potentially sensitive species, to help understand the roles of different ESR subtypes in estrogen signaling and to identify functionally important interactions that confer species and ESR subtype specificity for EDCs. These molecular analyses derived from the transactivation assays, together with the in silco analyses help in our understanding on the functional divergence of the ESR subtypes in fish and for informing on possible risk associated with exposure to EDCs in fish. Interactions of EDCs with the ESRs and the interactions between ESRs, however, may vary depending on the stage of reproductive development, metabolism, season, and abiotic factors, such as water temperatures and ultimately in vivo studies using life stages of interest are essential in the final risk analysis for estrogenic EDCs.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: Nomenclature for ESRs in the fish species in this study. Figure S1: Domain structures of each ESR subtype. Figure S2: Evolutionary relationships among fish ESRs. Figure S3: Concentration−response profiles of fish ESRs activated by E2. Figure S4: Concentration−response profiles of all ESR subtypes from five fish species activated by EDCs. Figure S5: Cellular localization of medaka ESR subtypes in HEK293 cells. Figure S6: Domain structures and sequence comparison of mESR subtypes. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b00704.

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Notes

The authors declare no competing financial interest.

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