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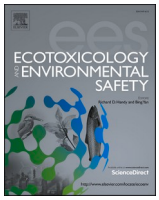

Identification of receptors for eight endocrine disrupting chemicals and their underlying mechanisms using zebrafish as a model organism

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ABSTRACT

Herein, eight common endocrine disrupting chemicals (EDCs) were exposed to zebrafish (*Danio rerio*) to investigate the relationship between different EDCs and their activated estrogen receptors. Under acute exposure, we identified five major malformation types whose incidence and deformity modes differed among EDCs. Luciferase analysis divided the EDC receptors into four categories: (i) triclosan (TCS), 17ß-estradiol (E2) and estriol (E3) mainly activated GPER expression; (ii) bisphenol A (BPA), *p*-(tert-octyl) phenol (POP), 17α-ethynylestradiol (EE2), E2 and E3 activated ERβ expression; (iii) E2 and E3 acted on both GPER and ERβ; and (iv) estrone (E1) and 9,9-bis(4-hydroxyphenyl)fluorene (BHPF) had little effect on the two receptors. *In vivo* immunofluorescence experiments on 96-hpf larvae provided evidence that TCS and POP acted on GPER and ERβ, respectively, while E2 acted on the two receptors simultaneously. Luciferase activities in the promoter regions of *gper* (−986 to −488) and *erβ* (−1998 to −1496) were higher than those in other regions, identifying these key regions as targets for transcription activity. TCS promoted GPER expression by acting on the JUND transcription factor, while POP promoted ERβ expression by activating the Foxl1 transcription factor. In contrast, E2 mainly regulated transcription of GPER and ERβ by Arid3a. These findings provide compelling evidence that different EDCs possess varying estrogen receptors, leading to differential regulatory pathways and abnormality symptoms. These results offer an experimental strategy and fundamental information to assess the molecular mechanisms of EDC-induced estrogen effects.

1. Introduction

Endocrine disrupting chemicals (EDCs) are compounds that interfere with the endocrine system of organisms. EDCs modulate hormone receptors and affect the production, storage and uptake of hormones, or the action of a hormone within a specific target tissue or organ (Lee et al., 2018). There are numerous known or suspected EDCs present in aquatic environments, with sewage treatment plant (STP) effluent being a major environmental source (Xu et al., 2015; Mansouri et al., 2020). Domestic STP effluents may contain mixtures of EDCs, such as natural (17β-estradiol, estrone and estriol) and synthetic estrogens (17α-ethinylestradiol, alkylphenol ethoxylates and bisphenol A) (Gorelick et al., 2014). Collectively, those EDCs that interact with estrogen receptors are known as environmental estrogens or xenoestrogens (Kerdivel et al., 2013), and their occurrence in STP effluent is strongly correlated with adverse physiological effects on fish (Sakalli et al., 2018).

In a toxicological study of EDCs, our previous research observed that different estrogen species produced varying effects on zebrafish (*Danio rerio*), possibly resulting from differential target and effector organs (Wang et al., 2020). EDCs affect a wide range of physiological processes in reproductive and non-reproductive organs/tissues in zebrafish by means of interaction between estrogen and biomembrane receptors (Saraswat et al., 2016). Zebrafish (*Danio rerio*) is a model organism that can be used for human cardiovascular development and function studies due to conserved estrogen signaling (Huttner et al., 2013). In general, estrogens bind with two classes of receptors: nuclear hormone receptors

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(ERα and ERβ) that are ligand-dependent transcription factors, and the G protein-coupled estrogen receptor (GPER, also known as GPR30), an integral membrane protein. Lin et al. (2017) reported that TCS acted on GPER, but not conventional receptors (ERα and ERβ). Additionally, high throughput RNA-seq identified differentially expressed genes induced by TCS or BPA exposure varied greatly because of their different estrogenic target molecules (Sun et al., 2020). For example, BPA inhibited lipid oxidation, while TCS mainly disrupted lipid synthesis and transport at the transcription level in larval zebrafish.

Estrogens can act on organisms by binding to nuclear receptors (ERα and ERβ), which are ligand-dependent transcription factors that directly regulate gene expression. There was general consensus among researchers with this viewpoint before GPR30 was first characterized as an estrogen membrane receptor a decade ago (Jeyakumar et al., 2011). Thomas et al. (2010) identified the important functions of this novel receptor (now known as GPER) as an intermediary in estrogen actions in mammalian neuroendocrine, cardiovascular, immune, pancreatic, musculoskeletal and renal systems. GPER has been detected in mammalian reproductive tissues such as ovary, oocytes, breast, oviduct, uterus, testis and prostate (Krejčířová et al., 2018). However, there are conflicting results regarding the role of GPER in reproduction, particularly in uterine function (Micevych et al., 2017). There is emerging evidence that GPER mediates several reproductive functions in mammals, including E2 stimulation of primordial follicle formation, uterine proliferation, endometrial cell growth, enhancement of the uterine contractile response to oxytocin in females, and proliferative and apoptotic pathways during spermatogenesis in males (Nishie et al., 2017). Given their importance to human and organism health, it is crucial to identify the specific receptors for estrogen pollutants to mitigate their health risks, and highlight their toxicity effects and molecular action mechanisms.

Herein, we used zebrafish (*Danio rerio*) as a model organism, which has several unique advantages in toxicology research compared to rodents, such as high-resolution imaging of the whole body and early developmental processes (similar to humans). Zebrafish are suitable for high throughput toxicological studies due to their high spawning capacity. Zebrafish have been used for decades to assess chemically mediated effects and are generally accepted and recommended for use in evaluating chemical hazards in aquatic environments (Wang et al., 2020; Hamm et al., 2019). In studying gene functions and the toxicity mechanisms of pollutants, they facilitate genetic manipulation by micro-injection *in vivo*. Furthermore, fluorescent labeling enables *in vivo* detection of the distribution and accumulation of pollutants in zebrafish.

This study investigated the toxicity effects of eight EDCs on embryonic-larval zebrafish development: estrone (E1), 17ß-estradiol (E2), 17α-ethynylestradiol (EE2), estriol (E3), triclosan (TCS), *p*-(tert-octyl) phenol (POP), bisphenol A (BPA) and 9,9-bis(4-hydroxyphenyl) fluorene (BHPF). EDCs may trigger a range of pathological characteristics and phenotypic malformations due to their different target molecules and effector organs, though they all have similarly estrogenic effects (Mansouri et al., 2020; Moreman et al., 2017). Therefore, it is possible to reveal toxicity mechanisms according to their different targets or action receptors. Herein, we disclose the relationship between similar or differential malformations and action receptors among the eight EDCs by construction of a firefly and *Ranilla* luciferase reporter system and qRT-PCR of the related transcription factors. At the molecular level, we elucidated whether the eight EDCs activated different estrogen receptors. Our research findings offer new insights on ecological risk assessment of EDCs, and provide practical significance for the prevention, diagnosis and treatment of EDC-induced diseases.

2. Material and methods

2.1. Ethical statement

Our experimental protocols followed the guidelines for ethical use of animals formulated by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University. Accordingly, we performed dissection of larval and adult zebrafish on ice to minimize suffering.

2.2. Chemical reagents

TCS (99.9%, CAS No. 3380-34-5), BPA (99.9%, CAS No. 80-05-7) and BHPF (97.0%, CAS No. 3236-71-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). E3 (98.0%, CAS No. 50-27-1), E2 (98.0%, CAS No. 50-28-2), E1 (99.0%, CAS No. 53-16-7), EE2 (99.0%, CAS No. 57-63-6) and POP (97.0%, CAS No. 140-66-9) were obtained from Aladdin (Shanghai, China). The molecular structures for these eight EDCs are shown in Fig. 1. EDC standard solutions were dissolved in acetone and stored at −20 °C. Cycloleucine (98.0%, CAS No. 52-52-8) and betaine (98.0%, CAS No. 107-43-7) were gratis supplied by Macklin Reagent (Shanghai, China). We acquired acetone from Sinopharm Chemical Reagent (Shanghai, China) and formamide (99.0%, CAS No. 75-12-7) and xylene (98.5%, CAS No. 1330-20-7) from Aladdin (Shanghai, China).

2.3. Zebrafish maintenance and selection criteria for EDC-exposure concentrations

Wild-type (AB strain) zebrafish (*Danio rerio*) were maintained in dechlorinated and filtered water at 28 °C with a 14:10-h, light:dark photoperiod (light on at 8 a.m.). Exposure concentrations for the eight EDCs were based on their respective LC_{50} and EC_{50} values for embryonic and adult zebrafish (Oliveira et al., 2009; Moreman et al., 2017), environmentally relevant concentrations, and our preliminary experimental results. Exposure levels for TCS, BPA and BHPF were set at 200 μg/L, which fell between 33 and 50% of their respective LC_{50} values (261–420 μg/L) (Oliveira et al., 2009; Moreman et al., 2017; Lin et al., 2017; Zhang et al., 2018; Sun et al., 2020). Similarly, an exposure concentration of 125 μg/L was selected for POP based on *ca.* 50% of its LC_{50} value (261 μg/L). In contrast, exposure levels for the four steroid hormones (E1, E2, E3 and EE2), as positive controls for the estrogenic effect, were all chosen to be 10 μg/L according to their 96-hpf EC_{50} values of 2.9–8.9 μg/L (Segner et al., 2003; Van den Belt et al., 2004; Bakos et al., 2019). The control group was treated with 0.0025% acetone (Zhang et al., 2018); no obvious phenotypic malformations were observed for larval and adult zebrafish at this acetone concentration. We used system water (dechlorinated tap water purified by a circulation system) to dilute the stock EDC solutions to appropriate exposure concentrations, and the exposure solution was renewed daily to maintain stable exposure concentrations throughout the experimental period.

2.4. qRT-PCR analysis

Zebrafish larvae were rinsed using phosphate buffer solution (PBS, Solarbio, Beijing, China) in a 1.5-ml RNase-free EP tube. Total RNA was isolated and purified from tissues or whole-mount larvae using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was transcribed to cDNA with a First-Strand cDNA Synthesis Kit (#170–8890, Bio-Rad, Hercules, CA, USA). Primers were synthesized by Sangon Biotechnology (Shanghai, China) using elfα as the endogenous reference for genes (Table S2). The qRT-PCR was performed using an ABI Prism 7500 Sequence Detection System (PerkinElmer, Applied Biosystems, Foster City, CA, USA). The cycle process followed 40 cycles at 95 °C for 5 min, 95 °C for 15 s and 60 °C for 1 min. Quantification adopted the $2^{-\Delta\Delta Ct}$ method as described by Sun et al. (2020). All PCR reactions included three biological replicates and each biological replicate included three technological replicates.

2.5. Site-directed mutagenesis

We aligned multiple nucleotide sequences using the modified Clustal

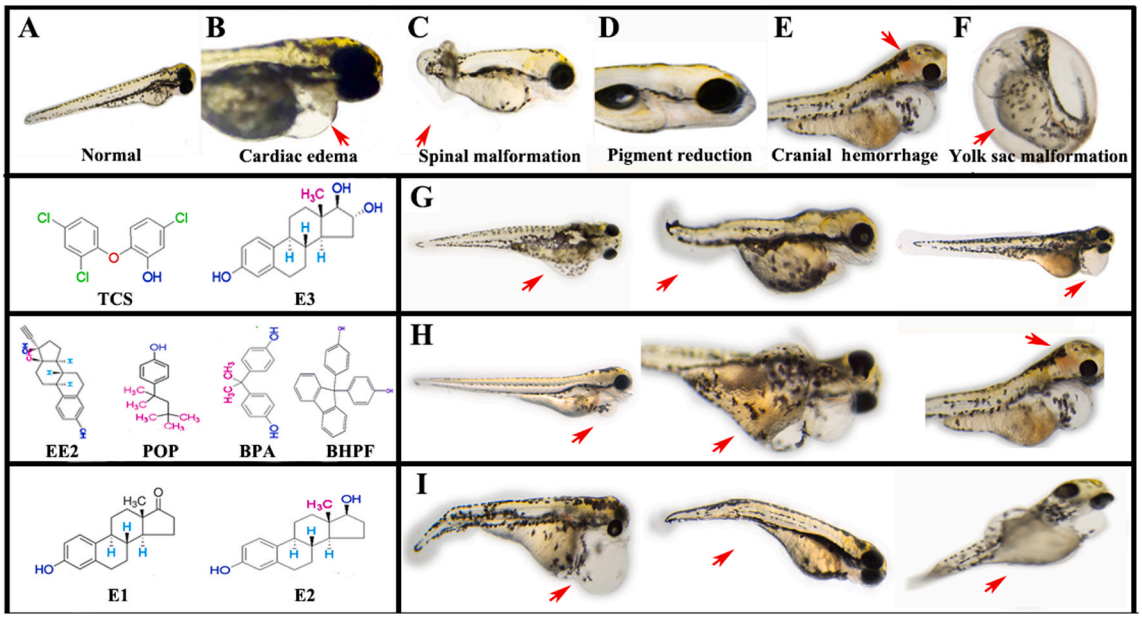

Fig. 1. Chemical structures for eight EDCs and their induced malformations to zebrafish larvae. **Notes:** A, normal; B, cardiac edema; C, spinal malformation; D, pigment reduction; E, cranial hemorrhage and F, yolk sac malformation. G, TCS and E3-induced main phenotype malformations; H, EE2, POP, BPA and BHPF-induced main phenotype malformations; I, E1 and E23-induced main phenotype malformations.

W algorithm of the Vector NTI 8 software package (Invitrogen). Phosphorylation site prediction was performed using GPS 3.0 software (Group-based Prediction System, v3.0; http://gps.biocuckoo.org/). The promoter was cloned into the expression plasmid pcDNA3 and expressed under the control of the cytomegalovirus promoter. Several individual point mutations were introduced with ~40-mer synthetic oligonucleotides using the Quick-Change XL-mutagenesis kit (Stratagene, San Diego, CA, USA). Sequencing of DNA constructs utilized an aliquot (8 μL) of the amplified product that was transferred into the T1 competent cell and resuscitated with a 250 μL coating of LB medium at 37 °C for 16 h. A single colony was selected for amplification overnight, and 1 mL of bacterial solution was sequenced by Sangon Biotechnology (Shanghai, China).

2.6. Construction of estrogen-receptor reporter plasmid and luciferase assay

We extracted zebrafish genomic DNA using the EasyPure Genome DNA Kit (Transgene, Shenzhen, China) according to manufacturer's protocols. PCR operations strictly followed our previous report (Sun et al., 2020). Validated plasmids containing target fragments were gel-purified and ligated to pEASY-Blunt Simple Cloning Vector (Transgene). Several clones were sequenced by BGI (Beijing, China) and searched against the genome database using the ensemble BLAST/BLAT (http://www.ensembl.org/Danio_rerio/Tools/Blast?db=core;redirect=no). Primers used for the promoter activity assay are listed in Table S3.

PCR products containing GPER and ERβ promoters were independently digested with SacI and HindIII or NcoI restriction endonucleases (New England Biolabs, Ipswich, MA, USA), and fragments containing the promoter region were collected with an EasyPure® Quick Gel Extraction Kit (Transgene). Promoter fragments of GPER were inserted into pGL3.0-basic (Promega Bio Sciences, San Luis Obispo, CA, USA) using SacI and NcoI sites. ERβ promoter fragments were inserted into pGL3-basic using SacI and HindIII sites. Plasmids, including various lengths of *GPER* or *ERβ* promoter regions and the pRL-TK vector in a ratio of 2:1, were microinjected (~2 nL) into the one-cell-stage of zebrafish embryos; the pRL-TK vector served as an internal control reporter. After injection, embryos were incubated at 28 °C for 48 h for luciferase assay. Protein extracts of 48-hpf embryos in passive lysis buffer were placed in an ice bath, and aliquots of the extracts (20 μL) utilized for luciferase assays using a Dual Luciferase Reporter Assay System (Promega Bio Sciences) following manufacturer's instructions (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Whole-mount immunofluorescence histochemistry (WIHC)

We performed WIHC on 72-hpf larvae following exposure to EDCs as described above. Primary antibodies (rabbit) for GPER and ERβ were purchased from ABclonal (Woburn, MA, USA). The secondary antibody was Fluor 488-conjugated goat anti-mouse (GeneTex, Irvine, CA, USA). We recorded fluorescent intensity using a fluorescence microscope.

2.8. Statistical analysis

Each EDC-exposure treatment and control group was carried out using three biological replicates and three technological replicates; all data are reported as mean ± standard deviation (n = 3). Each biological replicate included 20 zebrafish (female:male = 1:1) and thus 60 zebrafish (3 × 20) were used in each group for RNA-seq, qRT-PCR, WIHC, luciferase analysis and histopathological observation. We used one-way analysis of variance (ANOVA) to analyze the effects of EDC exposure, followed by Tukey's post-hoc test to independently compare each EDC exposure treatment with the control group. A similar approach was used to explore statistical differences among treatments based on the bioinformatic analysis of sequencing data. All statistical analyses were conducted with SPSS 18.0 (IBM Corp., Armonk, NY, USA) at a $p < 0.05$, $p < 0.01$ or $p < 0.001$ significance level.

3. Results

3.1. Relative toxicity of eight EDCs to zebrafish embryonic development

Following zebrafish exposure to eight EDCs, we observed five major malformation types: cardiac edema (Fig. 1B), spinal malformation (Fig. 1C), pigment reduction (Fig. 1D), cranial hemorrhage (Fig. 1E) and yolk sac deformity (Fig. 1F). The incidence and type of deformity differed among EDC species (Table 1). The main teratogenic effects

observed in TCS and E3 treatments included cardiac edema (9.7–100%) and spinal malformation (~12%) (Fig. 1G). The incidence of spinal malformation was the highest among deformities, except for E2 (10.6%). In contrast, the main teratogenic effects upon exposure to BPA, BHPF, EE2 and POP included cardiac edema and yolk sac deformity (Fig. 1H). In the E1 and E2 treatments, the main deformities were spinal malformation, cardiac edema (100%) and yolk sac deformity. Notably, the highest percentages of malformation were cardiac edema in the EDC-exposure treatments (Table 1), especially for the POP, E1, E2, EE2 and E3 treatments (56–100%). In sharp contrast, no deformities were observed in the control group (Fig. 1A).

3.2. Luciferase assay of *gper* and *erβ* genes after EDC exposure

To assess whether EDCs activated different estrogen receptors, a double luciferase reporter system for *gper* and *erβ* genes was constructed. We employed the *gper* promoter region from −1998 to −1 bp and the *erβ* promoter region from −1969 to −1 bp to successfully construct the receptor reporter system (Fig. S2). The activities of promoter segments were detected at 48 hpi (hours post injection) by transferring *gper* and *erβ* promoter region constructs and pGL 3.0 Basic into zebrafish embryos. Subsequently, we injected embryos with a single EDC for 48 h to observe the fluorescence intensity ratio of firefly to *Ranilla* (Fig. 2A).

Up-regulation of the *gper* gene was observed in zebrafish embryos following TCS, E2 and E3 exposure (Fig. 2B), with luciferase activities significantly increased by 1.1–1.3-fold ($p < 0.05$ for E2; $p < 0.01$ for TCS; $p < 0.001$ for E3) compared to the control group. Similarly, *erβ* expression was significantly increased by 1.2–1.7-fold in the BPA ($p < 0.05$), E2 ($p < 0.01$), EE2 ($p < 0.001$) and POP ($p < 0.05$) treatments relative to the control group (Fig. 2C). In contrast, no significant change in *erβ* expression was observed in the TCS, BHPF, E1 and E3 treatments. Consequently, we posit that the eight EDCs can be divided into three categories: (i) GPER as the estrogen receptor for TCS, E2 and E3; (ii) ERβ as the estrogen receptor for BPA, E2, EE2 and POP; and (iii) no sensitivity of BHPF and E1 to either GPER or ERβ. The lack of sensitivity for BHPF and E1 implies that their estrogen receptors belong to other target molecules, or they have no estrogenic effect. Notably, E2 activated both GPER and ERβ receptors, demonstrating the complexity of its estrogenic effect.

3.3. Promoter activity analysis of *gper* and *erβ* and screening of transcription factors

To understand promoter characteristics of the *gper* and *erβ* genes and their main regulatory sites, we analyzed the promoter regions by construction of a series of reporter plasmids containing different regions of the promoters (Fig. 3A and C). Promoter segment activities were detected at 48 hpi by injecting *gper* and *erβ* region constructs and pGL 3.0 Basic into zebrafish embryos; pRT-TK was co-injected as an internal reference plasmid with the above constructs and the promoter-less plasmid into zebrafish embryos. We chose TCS, E2 and POP as representative EDCs (as described in Section 3.2) for luciferase activity analysis of different promoter regions on GPER and ERβ.

The promoter activities of *gper* in the regions from −1496 to −1 bp and −1998 to −1 bp were approximately 4~5-fold higher compared to that from the promoter-less construct (pGL 3.0 Basic). Promoter activities in the region from −986 to −1 bp contrasted with those from −488 to −1 bp and were significantly higher (~2-fold), suggesting that the region from −986 to −488 bp is an important transcription-factor binding site and key activity-regulatory site (Fig. 3A and B). In comparison, promoter activities of *erβ* in the region from −499 to −1 bp were approximately 1~2-fold higher than those from the promoter-less construct (pGL 3.0 Basic). However, the promoter activities of *erβ* from −945 to −1 bp and −1491 to −1 bp were lower than those from −499 to −1 bp, suggesting a possible negative control element(s) between −1945 and −499 bp. Additionally, a positive control element(s) might be present between −1969 and −1491 bp as promoter activity was elevated in the region from −1969 to −1 bp and was 2~3-fold higher compared to that from the promoter-less construct (Fig. 3C and D). Consequently, the activation region for the *erβ* gene ranged from −1969 to −1491 bp. Finally, we demonstrated that the promoter sequences for the *gper* gene from −1998 to −1 bp and those of the *erβ* gene from −1969 to −1 bp (constructed in Section 3.2) satisfied the testing requirements for EDC regulatory effects.

Transcription activities of genes are always influenced by the binding and regulation of transcription factors in their activation regions. To explore which transcription factor regulated GPER and ERβ expression in zebrafish embryos, we utilized JASPAR web service (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) using a relative profile score threshold of 99%. JASPAR identified several AT Rich Interactive Domain 3A (BRIGHT-Like) proteins (ARID3A) (Fig. 4A), growth factor independent 1 transcription repressors (Gfi1) and JunD Proto-Oncogene (JUND) putative binding sites at the 5′-upstream promoter region (−986 to −488 bp) of *gper* genes (Fig. 4B). One ARID3A, two SRY-related HMG-box 10 proteins (Sox10) and one Forkhead Box L1 (FoxL1) binding site were identified between −1969 and −1491 bp of *erβ* (Fig. 4C). Except for a common transcription factor of ARID3A, the transcription factors for ERβ and GPER were different, suggesting that they elicited distinct responses to EDC stress.

3.4. Effects of TCS, E2 and POP exposure on the expression of GPER and ERβ

The classic and novel estrogen receptors, ERβ and GPER, exhibited different expression modes in luciferase experiments when exposed to different EDCs. To further probe the differential effects of TCS, E2 and POP on ERβ and GPER expression, we conducted WIHC experiments on larval zebrafish. While minimal fluorescence appeared in the absence of

Table 1
Percentages of developmental malformations in 96-hpf zebrafish larvae after EDC exposure.

EDCs/ Malformation	Cardiac edema (mean ± SD)%	Pigment reduction (mean ± SD)%	Spinal malformation (mean ± SD)%	Cranial hemorrhage (mean ± SD)%	Yolk sac deformity (mean ± SD)%
Control	–	–	–	–	–
TCS	9.7 ± 2.1	–	8.8 ± 1.8	–	9.7 ± 2.1
E3	100	7.0 ± 1.4	11.7 ± 3.3	–	26.1 ± 11.0
EE2	100	3.0 ± 1.7	–	3.6 ± 2.4	3.6 ± 2.2
POP	56.0 ± 7.0	–	–	3.7 ± 1.7	12.0 ± 6.4
BPA	18.6 ± 2.2	–	3.28 ± 1.1	–	9.9 ± 1.2
BHPF	6.1 ± 1.3	–	2.5 ± 0.7	–	2.5 ± 1.1
E1	100	3.3 ± 1.7	8.7 ± 1.7	–	18.0 ± 5.4
E2	100	–	10.6 ± 3.6	2.5 ± 0.4	9.7 ± 1.7

Notes: (1) Embryos for acute exposure experiments were reared in 96-well plates with one embryo per well containing 200 mL of EDC solution from 6 to 96 hpf; (2) Embryos were exposed to EDC concentrations of TCS (200 μg/L), E1 (10 μg/L), BPA (200 μg/L), BHPF (200 μg/L), E3 (10 μg/L), E2 (10 μg/L), EE2 (10 μg/L) and POP (125 μg/L); and (3) Mean ± SD (n = 3); each biological replicate includes 96 zebrafish; SD denotes abbreviation of standard deviation.

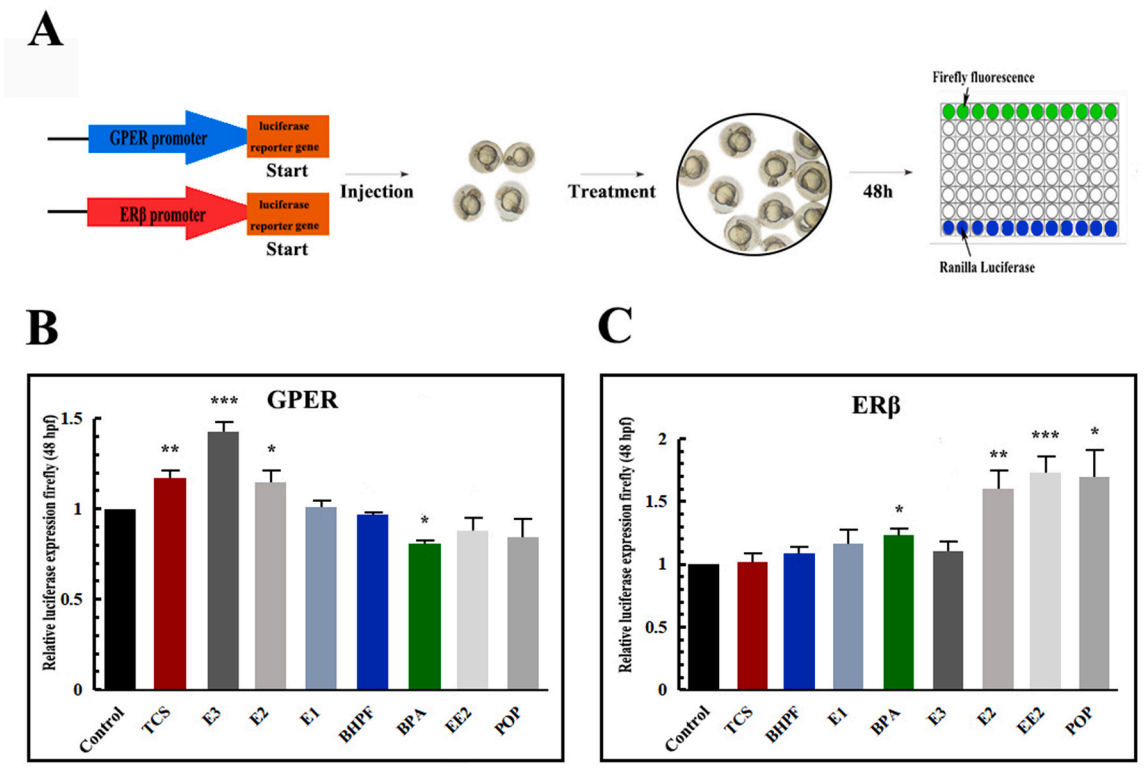

Fig. 2. Expression of GPER and ERβ by luciferase reporter analysis after EDC exposure in 48-hpf embryos. **Note:** (1) A, construction scheme for luciferase reporter analysis in the promoter sequences of *gper* and *erβ* genes; (2) B and C, relative luciferase expression of *gper* and *erβ* upon EDC exposure; and (3) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

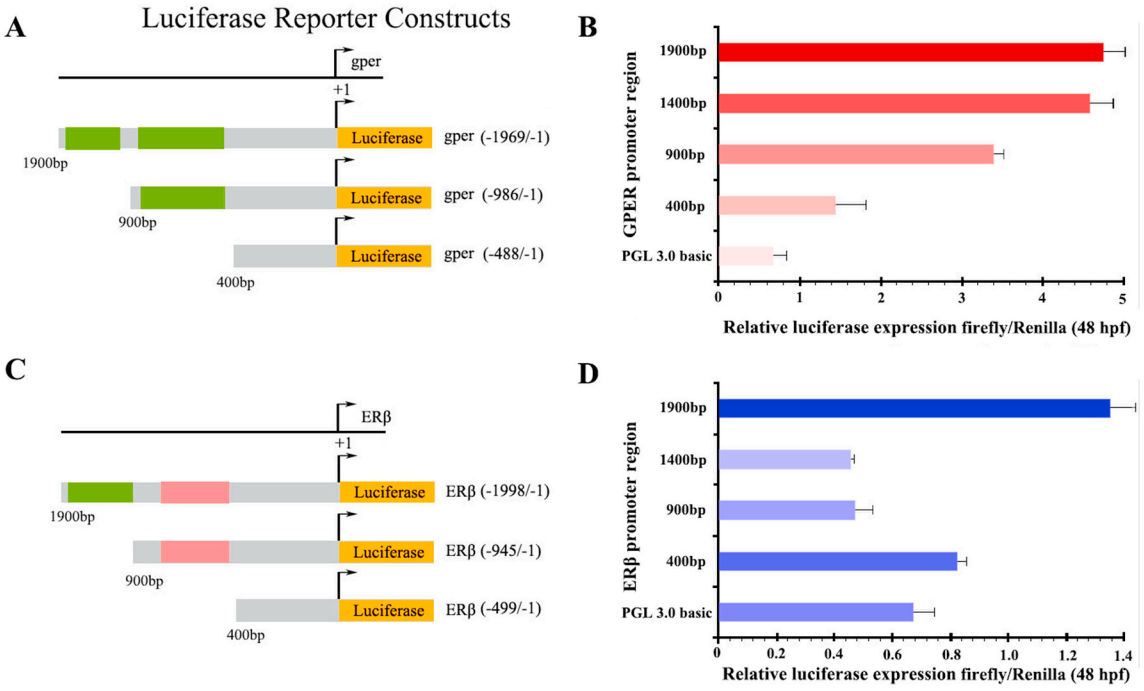

Fig. 3. Promoter characteristics and activity analyses of GPER and ERβ. **Note:** (1) A and C, Schematic of key transcription-factor binding sites in the promoter region of GPER and ERβ; (2) Green region: Putative key transcription-factor binding sites; (3) Red region: putative transcription-factor repressor action sites; (4) B and D, The activities of promoter fragments in GPER and ERβ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

EDC exposure, strong fluorescence occurred with EDC exposure (Fig. 5). The strongest fluorescence intensity was found in the E2-induced GPER-expression group, followed by the POP-induced ERβ-expression group.

In the TCS-exposure treatment, GPER expression in zebrafish forebrain and trunk showed a ~2.8-fold ($p < 0.001$) increase in green fluorescence protein (GFP) compared to the control group; however, this

Fig. 4. Prediction for binding sites of transcription factors. **Note:** (1) A, Prediction scheme of transcription factor; (2) B, The binding sites for transcription factors are in box; (3) Red font for Arid3a binding site, purple font for JUND, blue font for Gfi1, yellow font for Sox10 and green font for Foxl1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

response was not found in other tissues (Fig. 5A and B). As for E2 exposure, stronger GPER fluorescence occurred in the forebrain and trunk with an enhanced expression of ~1.6-fold ($p < 0.01$) for GFP; meanwhile, ERβ expression increased ~3-fold ($p < 0.001$) in the pericardial region compared to the control group (Fig. 5A and B). POP exposure induced a ~3.2-fold ($p < 0.001$) higher expression of ERβ in the pericardial region than the control group (Fig. 5A and C), but no change in GPER expression was observed in any other tissues. Expression changes for GPER and ERβ induced by TCS, POP and E2 were in general agreement with those documented by luciferase activity. Integration of these results provides compelling evidence that TCS and POP act on GPER and ERβ, respectively, while E2 acts on both GPER and ERβ.

3.5. Expression of transcription factors and luciferase analysis after binding site mutation

For the key transcription sites, we predicted three transcription factors for each receptor: Arid3a, JUND and Gfil for GPER, and Arid3a, Sox10 and Foxl1 for ERβ. To verify the relationship between the expression of GPER and ERβ and regulation of transcription factors in the promoter, we exposed TCS, E2 or POP to zebrafish from 6-hpf embryos to 96-hpf larvae and characterized these transcription factors by qRT-PCR (Fig. 6A–I). In general, the trends identified for changes in expression of *gper* and *erβ* by qRT-PCR were consistent with those of luciferase activities. The expression of GPER increased ~11-fold ($p < 0.001$) upon TCS exposure and 6~7-fold ($p < 0.001$) for E2 exposure. In contrast, no changes in GPER expression were observed upon POP exposure (Fig. 6F). TCS exposure slightly decreased ERβ expression, but both E2 and POP exposure increased ERβ expression by 2~4-fold ($p < 0.001$) (Fig. 6G).

With regard to changes in key transcription factors (Arid3a, Gfi1 and JUND for GPER; Arid3a, Foxl1 and Sox10 for ERβ), TCS induced a prominent increase in JUND expression (Fig. 6B) and a slight decrease in Gfil expression (Fig. 6C), but no obvious changes for the other three transcription factors (Fig. 6A, D and 6E). In contrast, E2 exposure resulted in increased expression of Arid3a, Foxl1 and Sox10, but no apparent changes in expression of JUND and Gfil. POP exposure led to increased expression of Foxl1 and Sox10, but no detectable changes in the other three transcription factors. These findings provide strong evidence that TCS acts on GPER by mainly regulating the expression of JUND with two binding sites at its key transcription sites. POP activates ERβ by means of regulating Foxl1 and Sox10, especially for Foxl1 having

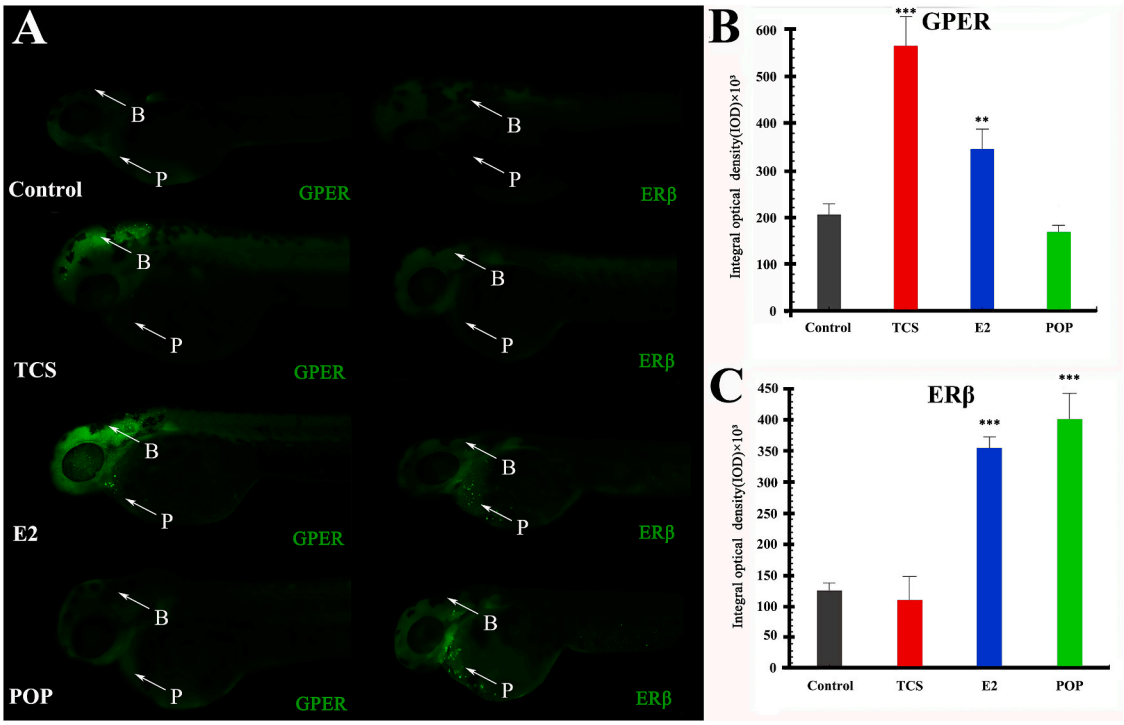

Fig. 5. Immunofluorescence of GPER and ERβ. **Note:** (1) A, Immunofluorescence of GPER and ERβ in 96-hpf zebrafish; (2) Capital letters in 5A: B, brain; P, pericardium liver; (3) B and C, Quantitative analysis of immunofluorescence; (4) "**" and "***" indicate significance levels at $p < 0.01$ and $p < 0.001$, respectively.

many binding sites at its key transcription sites (Fig. 4C). Although Arid3a is a common transcription factor for both GPER and ERβ, no change was found in Arid3a expression when exposed to TCS or POP (Fig. 6A), providing evidence that Arid3a is not the main target of the two receptors.

Subsequently, we mutated the binding sites of these transcription factors in the key active sites of the promoters to demonstrate the regulatory relationship between receptors and five transcription factors. Sequencing of PCR products after site-directed mutation showed that the binding sites for transcription factors were successfully mutated (Fig. S3). We then inserted the mutated fragments into pGL3.0 plasmids for luciferase activity analysis. After mutation of binding sites for Arid3a and JUND in the GPER promoter sequences, fluorescence intensities (FIs) decreased by 18.6% and 23.8% ($p < 0.05$), respectively; however, no changes in FIs were observed for Gfil mutation (Fig. 6H). Similarly, after mutation of binding sites for Arid3a and Foxl1 in the ERβ promoter sequences, FIs decreased by 21.2% and 61.1%, ($p < 0.01$ or $p < 0.001$), respectively; however, no changes in FIs were observed for Sox10 mutation (Fig. 6I).

4. Discussion

Environmental risk assessment for EDCs is a crucial issue in toxicological, biological and epidemiological disciplines (Bedoya-Ríos et al., 2017). EDCs are exogenous pollutants that enter the environment through human activities, and they have the potential to bioaccumulate in humans and wildlife (Gorelick et al., 2014). These compounds (e.g., phthalates, bisphenols, parabens) are commonly used in detergents, pesticides and personal care products providing a worldwide contaminant source. When released to the environment, EDCs can bioaccumulate in organisms through inhalation, diet or skin contact to adversely affect organism health (Singh et al., 2018). Organisms secrete several hormones to regulate metabolism, such as thyroid hormones, estrogen and androgen (Zhao et al., 2018). EDCs interact with these metabolic processes causing endocrine dysfunction, thereby resulting in potentially severe harm to the nervous, reproductive and endocrine systems of organisms (Mostafavi and Hosseini, 2014). Further, EDCs destroy/inhibit the synthesis and metabolism of hormones, or interfere with the synthesis of hormone receptors, contributing to a myriad of adverse health effects.

Our previous research using zebrafish demonstrated that the estrogenic effect of TCS was not attributable to classical estrogen receptors (ERα and/or ERβ), but rather through action on the novel estrogen receptor GPER (Lin et al., 2017). Elucidating how different types of EDCs produce estrogenic effects by action on varying receptors provides important information for understanding the molecular toxicological pathways associated with these pollutants and their analogues. Using a SKBR3 cell-based fluorescence competitive binding assay (a molecular docking method), Cao et al. (2017) found that six BPA analogues bound to GPER directly, with bisphenol AF (BPAF) and bisphenol B (BPB) showing much higher (~9-fold) binding affinity than BPA. Consistent with these binding results, BPAF and BPB exhibited higher agonistic activity than BPA with the lowest effective concentration of 10 nM. We also confirmed that at sublethal doses, BPA produces a significant inhibitory effect on GPER activity, which was in general agreement with Cao et al. (2017). Cao et al. (2018) also provided evidence that hydroxylated polybrominated diphenyl ethers (OH-PBDEs), rather than PBDEs, could bind to GPER, activating subsequent signaling pathways and promoting SKBR3 cell migration via GPER pathways. Both reports utilized E2 as a positive control to verify the effect of E2 on GPER, which was similar to our approach in this study. Future research needs to enrich the estrogen-receptor types of zebrafish when exposed to various EDCs, to provide important new information for assessing environmental risk and toxicity evaluation of contaminants.

The molecular action modes of EDCs mainly arise from the structural similarity between exogenous chemicals and endogenous hormones. Through binding to extracellular receptors, EDCs are transported into the nucleus where they combine with promoter sites to activate the expression of target genes. Therefore, EDCs can stimulate, inhibit or interfere with endocrine processes associated with androgen, estrogen and thyroid hormones (Hampl et al., 2016). However, the chemical structures of many EDCs are distinctly different from endogenous

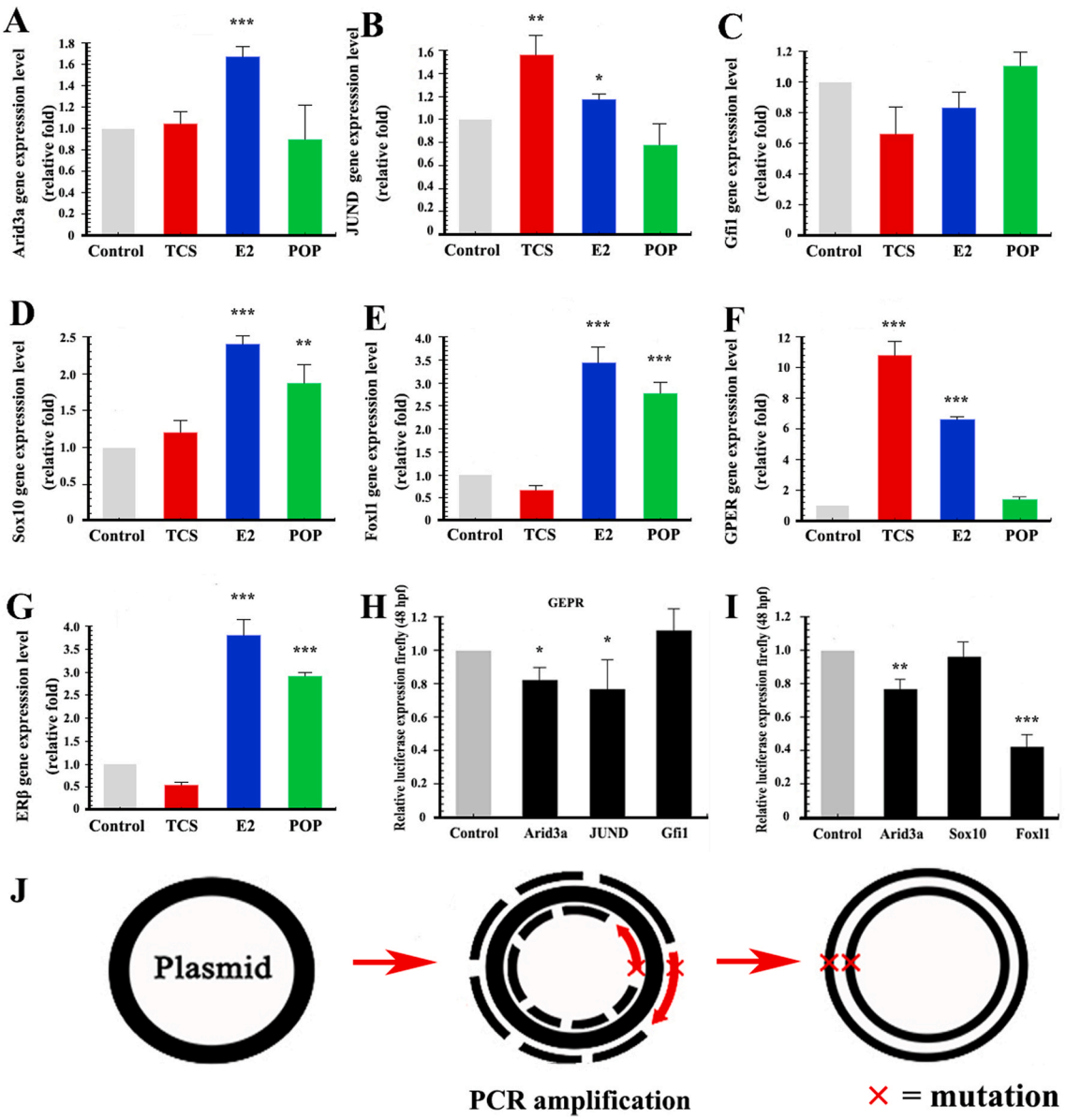

Fig. 6. qRT-PCR of the related transcription factors. **Notes:** (1) A, B, C, D and E, Expression of transcription factors under EDC exposure; (2) F and G, Expression of GPER and ERβ; (3) H and I, Luciferase analysis after mutation of binding sites; (3) J, Schematic diagram of site-directed mutation in promoter regions; (4) "*", "**" and "***" indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

hormones, yet they still induce endocrine disrupting effects. We ascribe this divergence to EDCs directly affecting the activities of enzymes related to hormone synthesis, thereby inhibiting the production, metabolism, transport and cell signal transduction of endogenous hormones and their receptors. For example, many EDCs are known to affect gene expression, enzyme activity and the content of sex hormones (Lombo et al., 2019). The different molecular structures among EDCs result in their contrasting transcription factors, estrogen receptors and signaling pathways. Consequently, their exposure targets different organs and produces various phenotypic malformations (Haggard et al., 2018).

Transcription factors are an important class of nuclear proteins related to the regulation of gene expression (Zou et al., 2020). Transcription factors are divided into two functional categories: basic transcription factors and regulatory transcription factors. Regulatory transcription factors combine with enhancer sequences at different sites where they interact with transcription procedures to realize regulatory functions for gene transcription. Regulatory transcription factors control complex and diverse biological processes, and thus dictate the response of organisms to environmental and physiological stimuli, and the differential development of organisms (Levin, 2018). To probe the mechanism(s) of action for TCS, POP and E2 on two types of estrogen receptors, we constructed a series of double luciferase systems in the key transcription regions. Integrated with qRT-PCR, we deduced the transcription regulatory factors and their underlying molecular mechanisms for our representative EDCs.

The dual luciferase assay is widely applied in cell lines for rapid and accurate determination of the activity for a given promoter (Dao et al., 2017). However, this technique does not allow for analysis of the promoter and gene function in the context of the whole organism. Therefore, we adopted an experimental protocol based on the dual luciferase system to examine zebrafish embryos. The luciferase reporter DNA plasmids were injected into zebrafish embryos at the one-cell developmental stage (Kunkel et al., 2018), together with expression constructs of interest, and luciferase activity was determined in the time window of promoter activity (24–48 hpi).

Given that exposure to different EDC species induces different

estrogen receptors, an important scientific query arises: Why do estrogen receptors have different expression patterns? This question was addressed by examining the upstream pathway of estrogen receptor genes. The promoter regions of GPER and ERβ were analyzed by construction of a series of reporter plasmids containing different regions (Lin et al., 2017). After luciferase activity analysis, we detected the most critical promoter regions defining GPER and ER activities, and further identified the active sites for key transcription factors in these regions (Siersbak et al., 2014). In the promoter sequences of GPER, we located three transcription-factor binding sites with the highest similarity (Arid3a, JUND and Gfi1). Likewise, three transcription-factor binding sites with the highest similarity were observed in the ERβ promoter sequences (Arid3a, Sox10 and Foxl1).

Based on our experimental results, we posit a model of EDCs acting on estrogen receptors (Fig. S4). First, three EDCs (TCS, POP and EE2) act on GPER and/or ERβ to activate the MAPK/ERK pathway, which further results in changes in the expression and phosphorylation of a series of transcription factors. Then, the uncoupled or phosphorylated transcription factors in the cytoplasm are translocated into the nucleus where they bind to the key transcription activity regions of GPER/ERβ to regulate their transcription levels (Lin et al., 2017; Wang et al., 2020). JUND and Arid3a, as well as Arid3a and Foxl1, are coupled transcription factors that bind to the GPER and ERβ transcription activation regions, respectively. JUND and Arid3a are “switch buttons” that initiate GPER gene transcription, while Arid3a and Foxl1 facilitate ERβ gene expression. GPER transcription levels increase when JUND is “turned on” under TCS exposure (Fig. S4A). Correspondingly, POP acts on ERβ by regulating the transcription factor of FOXL1 (Fig. S4B), and E2 promotes the up-regulation of both GPER and ERβ transcription levels by activating the transcription factor of Arid3a (Fig. S4C).

5. Conclusions

Herein, a series of *in vivo* zebrafish experiments assessed the potential estrogenic effects of eight common EDCs and their underlying molecular mechanisms. Following acute exposure, we identified five major malformation types induced by the eight EDCs and the specific malformations originating from each EDC species. Using a dual luciferase system of GPER and ERβ, we categorized the main estrogen-receptor types for the eight EDCs. TCS, E2 and E3 mainly activated the expression of GPER; BPA, POP, EE2, E2 and E3 activated the expression of ERβ; E2 and E3 acted on both GPER and ERβ; and in sharp contrast, E1 and BHPF had little or no effect on the two receptors. We provide compelling evidence that *gper* and *erβ* activities were mainly regulated by transcription factors that bound to their key active regions. TCS promoted GPER expression by acting on the JUND transcription factor, while POP promoted ERβ expression by activating the Foxl1 transcription factor. In contrast, E2 mainly regulated transcription of GPER and ERβ by Arid3a. Overall, the integration of methodologies utilized in this investigation provides a powerful approach to detect the estrogenic effects of contaminants, to identify the main types of estrogen receptors, and to elucidate the mechanism(s) of action. Consequently, these findings enhance our understanding of toxicological mechanisms involving EDCs, and provide targets for the diagnosis and control of EDC-induced diseases.

Credit author statement

All authors have seen and approved the final version of the manuscript being submitted. We warrant that the article is the authors’ original work, hasn’t received prior publication and isn’t under consideration for publication elsewhere. Their individual contributions are as follows: Wenhao Huang: Writing-original draft. Weiming Ai: Data curation. Weiwei Lin: Data curation. Fang Fang, Methodology. Xuedong Wang: Data curation. Haishan Huang Methology. Randy A. Dahlgren: Review & editing. Huili Wang: Conceptualization, Project administration, Supervision. Huili Wang, on behalf of all authors.

Declaration of competing interest

Wenhao Huang declares no conflict of interest; Weiming Ai receives the foundation of the National Science Foundation of Zhejiang Province (LY20C030005); Weiwei Lin declares no conflict of interest; Fang Fang receives the foundation of the National Science Foundation of Zhejiang Province (LY19H260005); Xuedong Wang declares no conflict of interest; Haishan Huang declares no conflict of interest; Randy A. Dahlgren declares no conflict of interest; Huili Wang receives the foundation of the National Natural Science Foundation of China (31770552), and the Natural Science Foundation of Jiangsu Province (BK20191455).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111068.

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