



## Cyp1a reporter zebrafish reveals target tissues for dioxin

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### ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the unintentional byproduct of various industrial processes, is classified as human carcinogen and could disrupt reproductive, developmental and endocrine systems. Induction of *cyp1a1* is used as an indicator of TCDD exposure. We sought to determine tissues that are vulnerable to TCDD toxicity using a transgenic zebrafish (*Danio rerio*) model. We inserted a nuclear enhanced green fluorescent protein gene (EGFP) into the start codon of a zebrafish *cyp1a* gene in a fosmid clone using DNA recombineering. The resulting recombinered fosmid was then used to generate *cyp1a* reporter zebrafish, embryos of which were exposed to TCDD. Expression pattern of EGFP in the reporter zebrafish mirrored that of endogenous *cyp1a* mRNA. In addition, exposure of the embryos to TCDD at as low as 10 pM for 72 h, which does not elicit morphological abnormalities of embryos, markedly increased GFP expression. Furthermore, the reporter embryos responded to other AhR ligands as well. Exposure of the embryos to TCDD revealed previously reported (the cardiovascular system, liver, pancreas, kidney, swim bladder and skin) and unreported target tissues (retinal bipolar cells, otic vesicle, lateral line, cloaca and pectoral fin bud) for TCDD. Transgenic *cyp1a* reporter zebrafish we have developed can further understanding of ecotoxicological relevance and human health risks by TCDD. In addition, they could be used to identify agonists of AhR and antidotes to TCDD toxicity.

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### 1. Introduction

TCDD is a polychlorinated dibenzo-*p*-dioxin (PCDD) and is an unintentional byproduct of various industrial processes and incineration activities. TCDD is “the most toxic anthropogenic substance ever identified,” was classified in 1997 as a human carcinogen by the International Agency for Research on Cancer (IARC) and has been reported to disturb reproductive, developmental, immunological and endocrine systems. Due to its toxicity, stability and

bioconcentration (or bioaccumulation), TCDD is an environmental and public health threat (National Research Council, 2006). Of the many incidents of TCDD-mediated toxicity, two incidents are noteworthy. The first is the massive use of agent orange in 1965–1971 as a defoliant during the Vietnam war. The second is the poisoning in 2004 of Victor Yushchenko, then a candidate for the Ukraine presidency (Hites, 2011; Mandal, 2005; Sorg et al., 2009).

The mechanism of TCDD toxicity commences with its binding in the cytosol to AhR, a ligand-activated nuclear transcription factor. Exogenous ligands for AhR are TCDD, benzo[a]pyrene (B[a]P), 3-methylcholanthrene (3-MC) and β-naphthoflavone (β-NF). However, endogenous ligands for AhR have been elusive (Nguyen and Bradfield, 2008). Several molecules related to tryptophan were proposed to be the endogenous ligands. For example, tryptamine ( $EC_{50} = 0.2$  mM), indole acetic acid ( $EC_{50} = 0.5$  mM) (Heath-Pagliuso et al., 1998) and 6-formylindolo[3,2-*b*]carbazole (FICZ) ( $EC_{50} = 34$ –830 pM) that is a tryptophan photooxidation product (Rannug et al., 1987), were reported to bind to AhR. In

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addition, Platten and colleagues reported in 2011 that kynurenone ( $EC_{50} = 12.3 \mu M$ ), a tryptophan catabolite, is an endogenous ligand of human AhR, and inhibits antitumor immune response and increases tumor cell survival and migration (Opitz et al., 2011). Once AhR binds to TCDD, the resulting AhR-TCDD complex translocates into the nucleus, binds to AhR nuclear translocator (Arnt) and then associates with dioxin-responsive elements (DREs) of TCDD-responsive genes, one of which is the *cyp1a* gene that encodes cytochrome P450 1A, a xenobiotic metabolizing enzyme. Mammals have two isoforms of *cyp1a*, *cyp1a1* and *cyp1a2*, whereas fish have only one kind of *cyp1a* gene. Expression levels of *cyp1a* gene have been used as an indicator of TCDD exposure (Goldstone and Stegeman, 2006; King-Heiden et al., 2012; Mandal, 2005; Mimura and Fujii-Kuriyama, 2003; Yamazaki et al., 2002). Therefore, transgenic animals harboring *cyp1a* regulatory elements fused to a reporter gene could be used as sentinels for aquatic contamination with dioxin and other AhR ligands (Jones et al., 1991; Operana et al., 2007).

Bioassays are essential to assess the effect of TCDD on living organisms, and cultured animal cells and animals have been used for the bioassays. Because bioassays using cultured animal cells are convenient to perform, they have been widely used (Carvan et al., 2000; Postlind et al., 1993; Wiebel et al., 1996; Yueh et al., 2005; Zhang et al., 2006). However, this approach has several limitations. First, the cell culture model does not reflect the bioconcentration effect, which is a key player in the chronic toxicity of TCDD in organisms. Second, metabolism of toxic chemicals in the liver sometimes plays an important role in the toxicity of the chemicals, but is not accounted for in the cell culture model. Lastly, pharmacokinetics study is not plausible in the cell culture model. These limitations make an animal bioassay system more attractive. Traditionally, mouse, rabbit and guinea pig have been used for the animal bioassay.

Zebrafish (*Danio rerio*) is a suitable model organism to study the metabolite effects of xenobiotic chemicals because zebrafish liver is very similar to mammalian liver (Chu and Sadler, 2009). Moreover, transgenic zebrafish embryos can be exploited as sentinels for aquatic pollution by chemicals including estrogen, retinoic acid, polychlorinated biphenyl and TCDD (Carvan et al., 2000).

To carry out risk assessment of toxic substance, it is critical to determine in organisms target tissues for the substance (Slotkin et al., 1987). The target tissues can be identified by detecting protein product of target genes (in the case of TCDD, *Cyp1a* protein) in organisms by immunohistochemistry. For example, *Cyp1a* protein was detected in the dorsal aorta, lower jaw primordial and cardiac ventricle of zebrafish embryos (Yamazaki et al., 2002), and the proximal tubules of the kidney, hepatocytes and the intestinal epithelium of adult zebrafish (Zodrow et al., 2004). Moreover, *Cyp1a* protein was detected in the tissues of other fish species: the brain of gilthead seabream (*Sparus aurata*) (Ortiz-Delgado et al., 2002); the endothelial cells, hepatocytes and the intestinal epithelium of the lake trout (*Salvelinus namaycush*) embryos (Guiney et al., 1997); and hepatocytes, exocrine pancreas, gut and mesonephros of European flounder (*Platichthys flesus*) (Grinvis et al., 2000). The target tissues can be revealed also by detection of target genes (in the case of TCDD, *cyp1a* mRNA) in organisms by either *in situ* hybridization with *cyp1a* riboprobes on zebrafish embryos (Andreasen et al., 2002; Yamazaki et al., 2002) or fluorescence microscopy of zebrafish embryos injected with *cyp1a* reporter plasmid (Mattingly et al., 2001). Both methods are, however, labor-intensive and the results vary, which can be circumvented by transgenic *cyp1a* reporter zebrafish. For example, Krone et al. identified target tissues of cadmium toxicity using transgenic *hsp70* reporter zebrafish (Blechinger et al., 2002). In addition, Kudoh and colleagues uncovered target tissues for environmental estrogen using estrogen-responsive transgenic zebrafish (Lee et al., 2012).

Therefore, we set out to generate a zebrafish *cyp1a* reporter line that recapitulates the endogenous expression of *cyp1a* and that increases expression of GFP when exposed to TCDD.

## 2. Materials and methods

### 2.1. Zebrafish

Wild type zebrafish (TL strain) were obtained from the Zebrafish International Resource Center (Eugene, OR, USA), maintained using standard procedures (Westerfield, 2007) and staged in hours post-fertilization (hpf) or days post-fertilization (dpf) as per standard criteria (Kimmel et al., 1995).

### 2.2. General reagents

Pfu DNA polymerase and restriction endonucleases were purchased from Bioneer (Daejeon, Korea) and New England Biolab (Ipswich, MA, USA), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

### 2.3. Bacterial artificial chromosome (BAC) recombineering

A fosmid clone (zFD385m15; aka CH1073-385m15) harboring a zebrafish *cyp1a* gene was purchased from BACPAC Resources Center (Oakland, CA, USA). The fosmid recombineering was performed as described previously (Lee et al., 2001) with modification. In brief, a 393-bp region 5' to the start codon of *cyp1a* and a 1054-bp region 3' to the start codon of *cyp1a* were amplified by PCR from the fosmid and cloned into the Acc65I/AflII and SpeI/SacII sites of NLS-EGFP-FRT-Kan<sup>®</sup>-FRT plasmid (Shin et al., 2003), respectively, where NLS, FRT and Kan indicate nuclear localization sequence, flipase recognition site and kanamycin resistance gene, respectively (see Supplemental Material, Fig. S1). Expression of EGFP fused to NLS is limited to the nucleus in cells, which makes it easier to count cells expressing EGFP. Primers (Bioneer) used to amplify the 393-bp region by PCR are forward 5'-TCA GCG GTA CCT GCA GCG TAA ATC AGC AGA TAC-3' (underlined are introduced nucleotides) and reverse 5'-TGC GGC TTA AGT GCT GAT TTA ATA ACC TGT TTA G-3'. Primers used to amplify the 1054-bp region by PCR are forward 5'-TAG TCA CTA GTA TTA CGA TAC GTT CGA TAA GGT G-3' and reverse 5'-TAC TAC CGC GGA ATC GGA TTA GAC CAT TAG CAT C-3'. Subsequently, the resulting construct was linearized, transformed into the fosmid-harboring EL250 cells (Lee et al., 2001) and recombined with the fosmid. The FRT-Kan<sup>®</sup>-FRT cassette was then removed by 0.1% L-arabinose induction. The final recombined construct was verified by PCR and DNA sequencing (Macrogen; Daejeon, Korea).

### 2.4. Microinjection and generation of a zebrafish *cyp1a* reporter line

The recombined construct was amplified by HiSpeed Plasmid Midi Kit (Qiagen; Valencia, CA, USA) and purified by ethanol precipitation. Seventy five picograms of the construct were microinjected into one-cell stage embryos along with phenol red. The embryos expressing GFP at high levels in skin were selected at 24 hpf and then raised to adulthood. To screen germline-transformed founders, we incrossed the adult fish and screened their progeny for GFP expression. The progeny with GFP expression was then raised to adulthood to establish a *cyp1a* reporter line. F3 generation transgenic zebrafish larvae were used for the experiments.

### 2.5. In situ RNA hybridization

Digoxigenin (DIG)-labeled antisense *cyp1a* RNA probes from zebrafish *cyp1a* plasmid were generated with a T7 DIG RNA

labeling Kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. Embryos at 32 hpf were fixed with 4% paraformaldehyde, digested with proteinase K (10 µg/ml) for 20 min, washed in phosphate-buffered saline containing 0.1% Tween 20 (PBST), pre-hybridized with hybridization buffer (50% formamide, 5× SSC pH 7.0, yeast tRNA [500 µg/ml], heparin [50 µg/ml] and 0.1% Tween 20), hybridized overnight at 70 °C with the hybridization buffer harboring the DIG-labeled *cyp1a* probes (2 ng/µl), washed sequentially at 65 °C with wash buffer I (50% formamide, 2× SSC and 0.1% Tween 20), wash buffer II (2× SSC and 0.1% Tween 20) and wash buffer III (0.2× SSC and 0.1% Tween 20), blocked with 2% blocking reagent (Roche), incubated at room temperature with anti-DIG antibody conjugated with alkaline phosphatase (1:2000), washed with PBST, incubated with BM Purple (Roche) in NTMT buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl<sub>2</sub> and 0.1% Tween 20) until staining appeared, washed with PBST, post-fixed with 4% paraformaldehyde, and imaged with a SteREO Discovery V20 stereomicroscope (Carl Zeiss, Jena, Germany).

## 2.6. Exposure of zebrafish embryos to TCDD and other AhR ligands

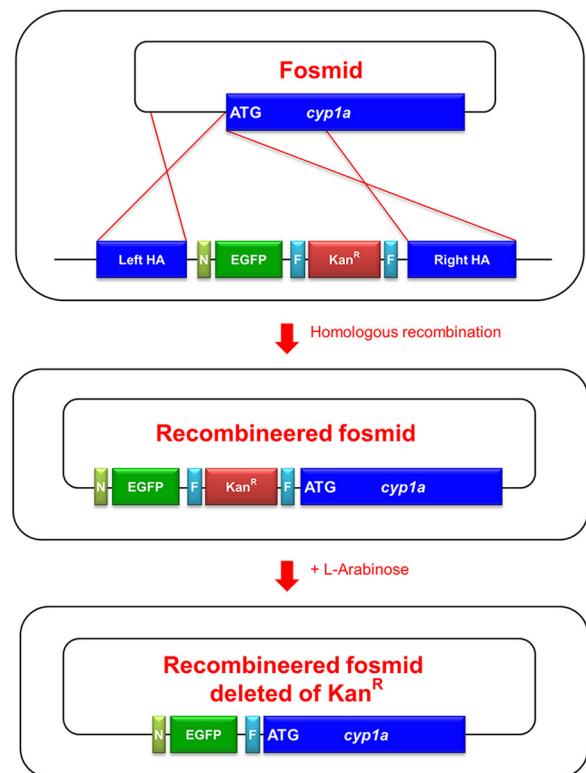
Forty zebrafish embryos were placed in a polystyrene petri dish (90 mm × 20 mm; SPL Life Sciences, Pocheon, Korea) harboring 40-ml egg water (60 µg/ml sea salt [Instant Ocean] in distilled water). 1-Phenyl-2-thiourea (0.2 mM; PTU; Sigma-Aldrich) was added to the embryos from the 75%-epiboly stage (8 hpf) onwards. The embryos at 30 hpf were statically exposed for 24–72 h to either dimethyl sulfoxide (DMSO; 0.1%) or TCDD (31 µM) in toluene (AccuStandard, New Haven, CT, USA; catalog # M-613) diluted to various concentrations. PTU-containing egg water with DMSO or TCDD was replenished daily. In addition, the embryos at 30 hpf were statically exposed for 24 h to B[a]P (1 µM; Sigma-Aldrich; # B1760), 3-MC (1 µM; Sigma-Aldrich; # 46434) or β-NF (10 µM; Sigma-Aldrich; # N3633), dissolved or diluted in DMSO. Finally, the embryos were imaged with an MZ16 FA fluorescent stereomicroscope (Leica; Wetzlar, Germany). The resulting images were then assembled using Adobe Photoshop (San Jose, CA, USA).

## 2.7. Western blotting (WB)

Zebrafish embryos were lysed by M-PER mammalian protein extraction reagent (Thermo Scientific, Pittsburgh, PA, USA), separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (Pall Corporation, New York, NY, USA). Subsequently, the membrane was probed with anti-EGFP antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog # sc-9996), washed with TBST (0.2 M Tris, 1.37 M NaCl and 0.1% Tween-20, pH7.6), and probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:4000, Santa Cruz Biotechnology, catalog # sc-2005). The bound antibody was detected by enhanced chemiluminescence (AniGen, Suwon, Korea) and then exposed to X-ray film (AGFA, Mortsel, Belgium).

## 2.8. Serial sectioning and confocal microscopy of embryos

Upon exposure to TCDD, embryos at 54 hpf were anesthetized by tricaine, fixed with 4% paraformaldehyde at 4 °C overnight, embedded in 1.5% agar with 5% sucrose blocks and equilibrated by 30% sucrose solution. Frozen blocks were sliced into 10 µm sections on glass slides using Cryostat Microtome (Carl Zeiss). The sections were imaged with a confocal laser scanning microscope (LSM 510 Pascal; Carl Zeiss). The resulting images were then assembled using Adobe Photoshop.



**Fig. 1.** Schematic diagram depicting the generation of a zebrafish *cyp1a* reporter construct using DNA recombination. HA, N, EGFP, F and Kan<sup>R</sup> indicate homologous arm, nuclear localization sequence, enhanced green fluorescent protein, flipase recognition site and kanamycin resistance gene, respectively. Not drawn to scale.

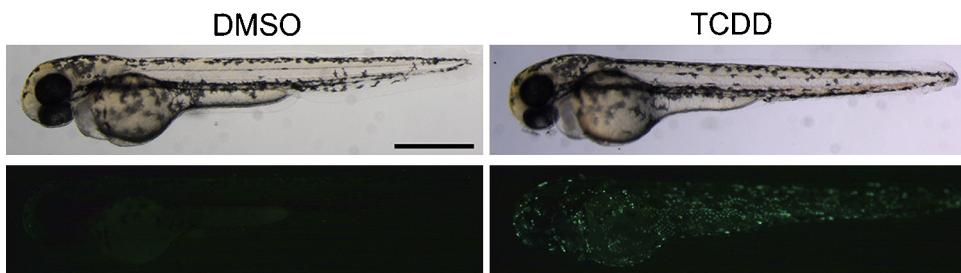
## 3. Results

### 3.1. Construction of *cyp1a* reporter construct

ZeRuth and Pollenz identified a –2699/+71 region (+1 represents a transcription start site) of a zebrafish *cyp1a* gene as a *cis*-regulatory region responsible for dioxin-mediated induction of *cyp1a* in mouse hepatoma cell line Hepa-1 (ZeRuth and Pollenz, 2005, 2007). Although this region has dioxin-responsive elements (DREs), it might not harbor all DREs and *cis*-regulatory elements required to direct endogenous expression of *cyp1a* in zebrafish, let alone a reporter construct encoding GFP gene driven by 333-bp zebrafish *cyp1a* promoter, which was used to generate *cyp1a* reporter zebrafish by Yung et al. (Hung et al., 2012). Hence, we sought to generate a zebrafish *cyp1a* reporter construct harboring all DREs and *cis*-regulatory elements required for endogenous expression of *cyp1a*.

To this end, we first searched the Ensembl database (Zv9) for BAC clones spanning a zebrafish *cyp1a* gene only. Unfortunately, there was no such BAC clone. There were, however, fosmid clones spanning just zebrafish *cyp1a* (Fig. 1). Of these clones, zFD385m15 covers from 8225 bp upstream to 30,061 bp downstream of the start codon.

A 301 bp region 5' to the start codon of *cyp1a* and a 1054 bp region 3' to the start codon of *cyp1a* were amplified by PCR from fosmid zFD385m15 (Fig. 1), inserted into a targeting construct as homologous arms, and then used for DNA recombination with a fosmid zFD385m15 (see Supplemental Material, Fig. S1) The recombination event was verified by PCR with two primers, complementary to the fosmid and the targeting construct, respectively (see Supplemental Material, Fig. S2 lanes 1–4). Subsequently, an FRT-Kan<sup>R</sup>-FRT cassette was removed by L-arabinose induction,



**Fig. 2.** A recombined fosmid has regulatory elements sufficient for TCDD induction of a zebrafish *cyp1a* gene. One-cell stage zebrafish embryos were injected with the recombined constructs, exposed to either DMSO (used as a vehicle) or TCDD (10 nM) during 24–48 hpf and imaged by either transmitted (upper) or fluorescent (lower) light microscopy. Note pigmentation in the embryos, which was caused by the absence of PTU in the egg water. The images are representative of three independent experiments. Lateral views anterior to the left are shown. Scale bar = 500  $\mu$ m.

which was verified by PCR with two primers complementary to the flanking region of the cassette (see Supplemental Material, Fig. S2, lanes 5 and 6).

### 3.2. Generation of transgenic *cyp1a* reporter zebrafish

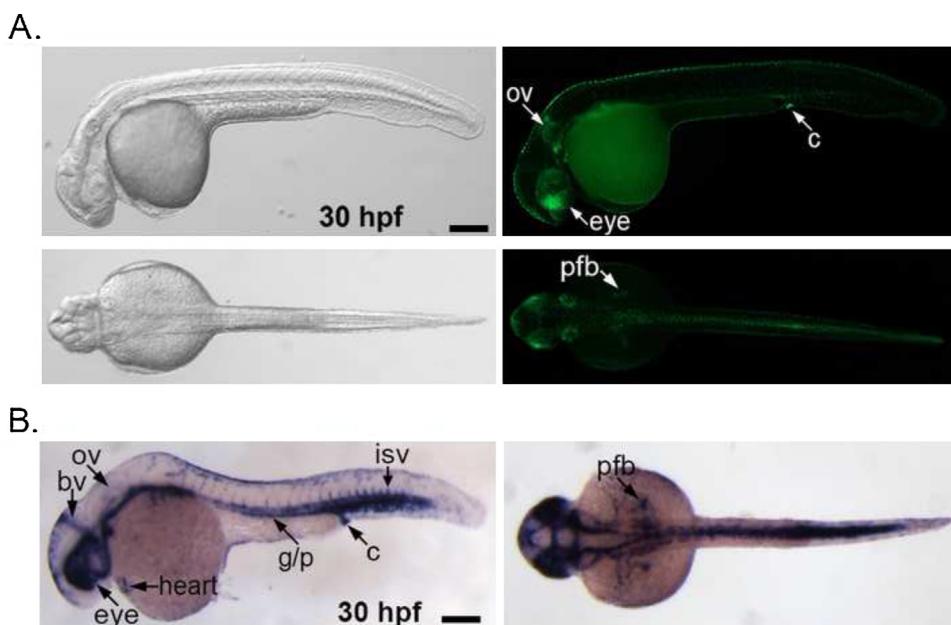
The resulting recombined fosmid was microinjected into one-cell stage zebrafish embryos. The embryos were then exposed to either DMSO (used as vehicle) or TCDD during 24–48 hpf. GFP signals in the embryos were indicative of *cyp1a* promoter activities. DMSO treated embryos showed basal expression of GFP signals at 48 hpf. TCDD treatment, however, markedly increased GFP signals throughout the embryos (Fig. 2), demonstrating that the recombinant fosmid contains DREs.

Subsequently, we generated transgenic *cyp1a* reporter zebrafish using the recombined fosmid. This transgenic zebrafish is hereafter referred to as *Tg(cyp1a:nls-egfp)* line. *Tg(cyp1a:nls-egfp)* embryos at 30 hpf displayed GFP expression primarily in skin, eye, otic vesicle, pectoral fin bud and cloaca (Fig. 3A). To test if this expression pattern in the transgenic line mirrors that of endogenous *cyp1a* in wild type zebrafish embryos, we carried out *in situ* RNA hybridization on 30 hpf *Tg(cyp1a:nls-egfp)* embryos with zebrafish *cyp1a* riboprobes. Strong expression of *cyp1a* mRNA

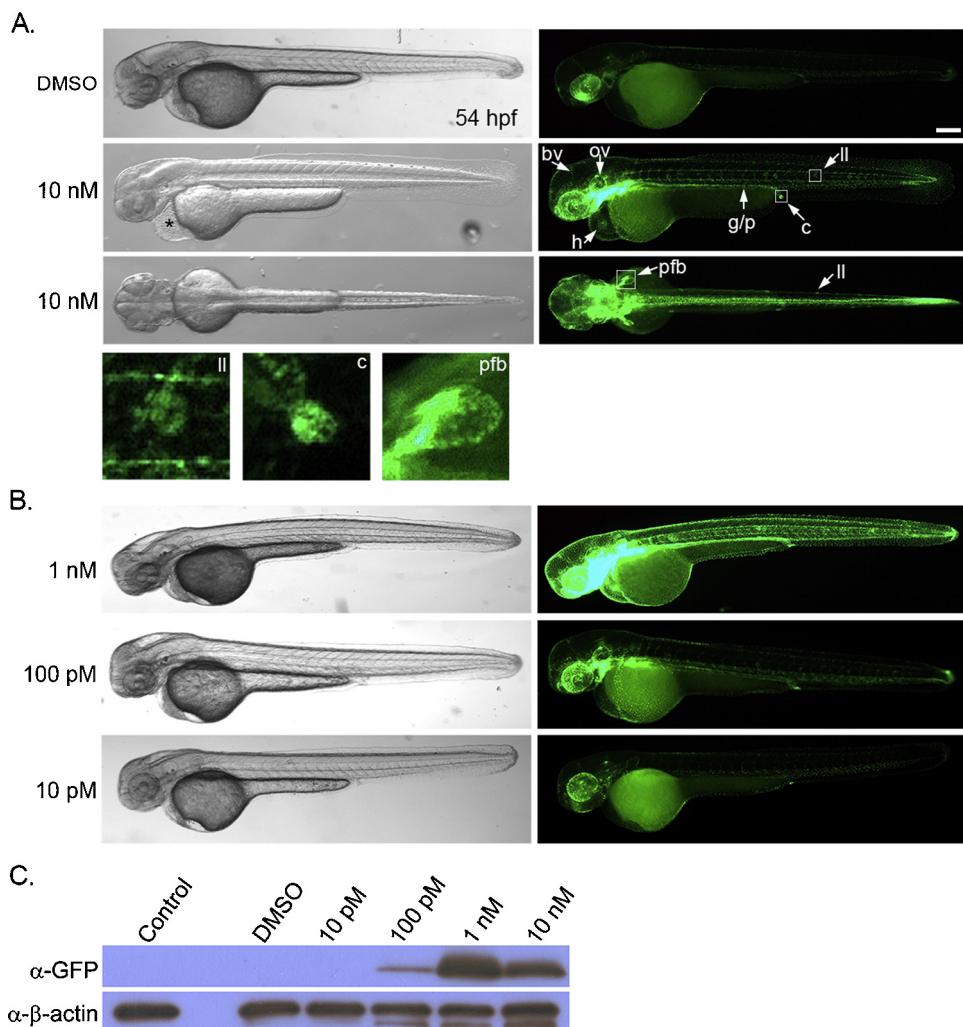
was detected in the skin, eye, heart, pectoral fin bud, gut, cloaca and intersegmental vessels, and faint expression in the otic vesicle, paralleling the GFP expression pattern in *Tg(cyp1a:nls-egfp)* embryos, with the exception of the heart, gut and intersegmental vessels (Fig. 3B). This finding indicates that *Tg(cyp1a:nls-egfp)* harbors almost all cis-regulatory elements required to direct endogenous expression of *cyp1a* in zebrafish.

### 3.3. TCDD increases GFP expression in *Tg(cyp1a:nls-egfp)* embryos in a dose-dependent manner

To test if *Tg(cyp1a:nls-egfp)* responds to TCDD, 30 hpf *Tg(cyp1a:nls-egfp)* embryos were exposed to TCDD for 24 h and then assessed for GFP expression. Significant increase in GFP expression was noted throughout the embryos exposed to 10 nM TCDD compared to those exposed to the DMSO, especially in the skin, otic vesicle, pectoral fin bud, cardiovascular system, gut, pronephros, cloaca and lateral line (Fig. 4A). To determine the limit of detection (LOD) of TCDD for *Tg(cyp1a:nls-egfp)*, 30 hpf *Tg(cyp1a:nls-egfp)* embryos were exposed for 24 hr to various concentration of TCDD ranging from 1 nM to 10 pM. The LOD by 24-hr exposure seemed to lie somewhere between 100 pM and 10 pM, as demonstrated by fluorescent microscopy (Fig. 4B, right) and WB with anti-GFP antibody



**Fig. 3.** *Tg(cyp1a:nls-egfp)* zebrafish embryos express GFP in the skin, eye, otic vesicle, cloaca and pectoral fin bud, which almost parallels endogenous expression pattern of *cyp1a*. (A) *Tg(cyp1a:nls-egfp)* embryos at 30 hpf were imaged by fluorescent microscopy. Scale bar = 200  $\mu$ m. Lateral (upper) and dorsal (lower) views anterior to the left are shown. (B) *In situ* hybridization was performed on *Tg(cyp1a:nls-egfp)* embryos at 30 hpf with zebrafish *cyp1a* riboprobe. Lateral (left) and dorsal (right) views anterior to the left are shown. Scale bar = 200  $\mu$ m. bv: brain vessel; ov: otic vesicle; pfb: pectoral fin bud; g: gut; p: pronephros; c: cloaca; isv: intersegmental vessel.



**Fig. 4.** TCDD increases GFP expression in *Tg(cyp1a:nls-egfp)* embryos in a dose-dependent manner. (A and B) *Tg(cyp1a:nls-egfp)* embryos at 30 hpf were exposed to DMSO (as vehicle) or TCDD at indicated concentration for 24 h and imaged by fluorescent microscopy. Insets in A are magnified below. Transmitted light (left) and fluorescent images (right). Lateral views anterior to the left are shown, except the lower panels of A, which are dorsal views anterior to the left. Asterisk indicates pericardial edema. bv, brain vessel; ov, otic vesicle; h, heart; pfb, pectoral fin bud; g, gut; p, pronephros; c, cloaca; II, lateral line. Scale bar = 250  $\mu$ m. (C) The embryos in A and B were subject to WB using anti-GFP antibody. To prevent saturation of band intensity in a 1-nM lane, the membrane was exposed to the film very briefly. As a result, no band is observed in DMSO and 10 pM lanes, although GFP<sup>+</sup> cells were noted by fluorescence microscopy in *Tg(cyp1a:nls-egfp)* treated with DMSO or 10 pM TCDD.

(Fig. 4C). Of note, 1 nM TCDD induced more GFP in the embryos than did 10 nM TCDD, as demonstrated by fluorescence microscopy and WB (Fig. 4). It is conceivable that 10 nM TCDD is more toxic to the embryos, thereby suppressing expression of GFP, at least to some extent. Exposure of zebrafish embryos to TCDD was reported to induce “early life stage toxicity,” such as craniofacial malformation, pericardial and yolk sac edema (Henry et al., 1997; Prasch et al., 2003), which we observed for zebrafish embryos exposed to 10 nM TCDD for 24 hr (data not shown). Meanwhile, embryos treated with 1 nM or 100 pM TCDD for 24 hr did not exhibit the toxicity, yet expressed GFP (Fig. 4B and C).

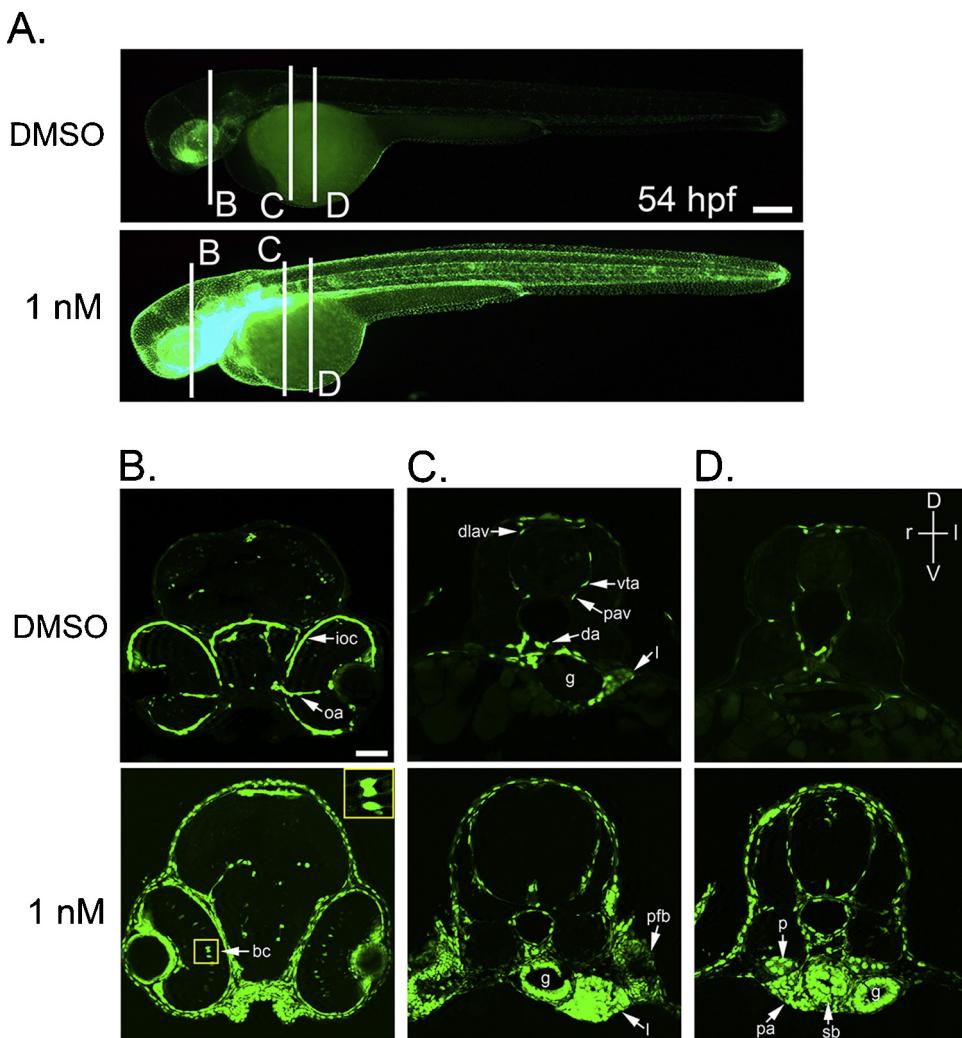
### 3.4. *Tg(cyp1a:nls-egfp)* embryos reveal target tissues for TCDD

To further explore TCDD target tissues that are not discernible by surface morphology, we cross-sectioned embryos at 54 hpf exposed to either DMSO or 1 nM TCDD for 24 h and then observed the sections with confocal microscopy. In DMSO treated embryos, various blood vessels expressed GFP (Fig. 5B–D upper panel). In 1 nM TCDD treated embryos, however, high levels of GFP were induced in the bipolar cells in the retina, gut, liver, pancreas, swim bladder and

pronephros as well (Fig. 5B–D lower panel). Of note, we did not observe GFP expression in the brain, spinal cord and somite, indicating specificity of target tissues for TCDD.

### 3.5. Extended exposure time enhances sensitivity of *Tg(cyp1a:nls-egfp)* embryos to TCDD

In order for *Tg(cyp1a:nls-egfp)* embryos to act as a sentinel for aquatic dioxin pollution, they should have the LOD as low as possible. It is plausible that lengthening the exposure time might lower the LOD of TCDD in *Tg(cyp1a:nls-egfp)*. Therefore, we tested if exposure of *Tg(cyp1a:nls-egfp)* embryos to 10 pM TCDD, which does not induce GFP expression in the embryos upon 24 h exposure, for an extended period of time could induce GFP expression. To this end, we exposed *Tg(cyp1a:nls-egfp)* embryos to 10 pM TCDD for 3 days (30–102 h). Indeed, lengthening the exposure time to 72 h induced GFP expression, as demonstrated by fluorescent microscopy and WB (Fig. 6). Therefore, we conclude that lengthening the exposure time lowers the LOD of TCDD for *Tg(cyp1a:nls-egfp)*.



**Fig. 5.** *Tg(cyp1a:nls-egfp)* embryos reveal target tissues for TCDD. (A) Embryos shown in Fig. 4A were fixed with paraformaldehyde and cross-sectioned as indicated. Lateral views anterior to the left are shown. Scale bar = 250  $\mu$ m. (B–D) The embryos cross-sectioned as indicated in (A) were imaged by confocal laser microscopy. Ventral views anterior to the top are shown. Inset represents magnification of the boxed area. ioc: inner optic circle; oa: optic artery; bc: bipolar cell; dlav: dorsal longitudinal anastomotic vein; vta: vertebral artery; pav: parachordal vessel; da: dorsal aorta; g: gut; sb: swim bladder; l: liver; pfb: pectoral fin bud; p: pronephros; pa: pancreas. Scale bar = 50  $\mu$ m.

### 3.6. *Tg(cyp1a:nls-egfp)* embryos can detect other AhR ligands

Human *CYP1A1* can be also induced by other AhR agonists such as benzo[a]pyrene (B[a]P), 3-methylcholanthrene (3-MC) and  $\beta$ -naphthoflavone ( $\beta$ -NF) in a mouse hepatoma cell line Hepa-1 and transgenic mice (Operana et al., 2007; Ovesen et al., 2011). To examine if this is the case with *Tg(cyp1a:nls-egfp)* embryos, we exposed the 30 hpf embryos to B[a]P, 3-MC and  $\beta$ -NF for 24 h and then assessed GFP expression in the embryos. All three AhR agonists markedly increased GFP expression in the embryos (Fig. 7), demonstrating that *Tg(cyp1a:nls-egfp)* responds to other AhR agonists as well. Of note, the concentrations of B[a]P, 3-MC and  $\beta$ -NF (1  $\mu$ M, 1  $\mu$ M and 10  $\mu$ M, respectively) used in the exposure were 3 orders of magnitude higher than that of TCDD. Taken together, this finding suggests that *Tg(cyp1a:nls-egfp)* might respond to all endogenous and exogenous ligands of AhR, raising the possibility that *Tg(cyp1a:nls-egfp)* zebrafish could function as a biosensor of AhR ligands.

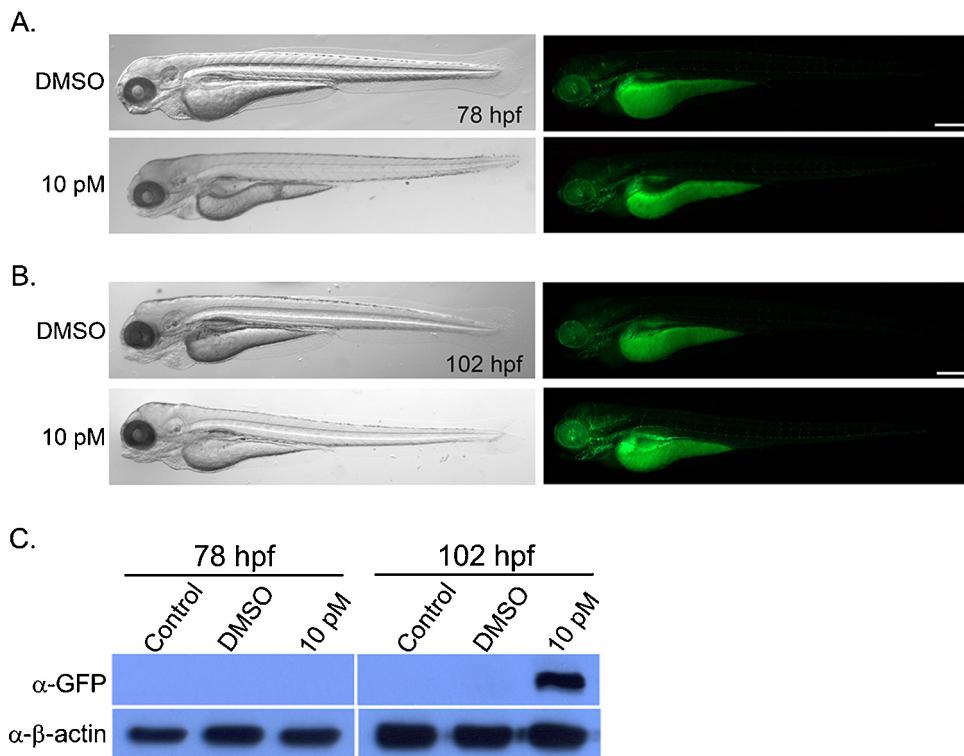
## 4. Discussion

Here, we report the following tissues to be targets for TCDD using transgenic zebrafish expressing *Tg(cyp1a:nls-egfp)*

constructed by DNA recombineering: the cardiovascular system, liver, kidney, swim bladder, skin, retinal bipolar cells, otic vesicle, lateral line, pancreas, cloaca and pectoral fin bud. We show in *Tg(cyp1a:nls-egfp)* embryos that expression pattern of GFP and endogenous *cyp1a* are comparable, and that GFP expression is increased upon exposure to TCDD, even at 10 pM when the exposure period is lengthened to 3 days. In addition, *Tg(cyp1a:nls-egfp)* responds to other AhR ligands as well, such as B[a]P, 3 MC and  $\beta$ -NF.

Toscano and colleagues microinjected a -1612/+292 region (+1 represents a transcription start site) of a human *CYP1A* gene fused to a *gfp* gene into one-cell stage zebrafish embryos and assessed GFP signals in the absence or presence of TCDD (Mattingly et al., 2001). Whereas they did not note GFP signals in the embryos in the absence of TCDD, we did in the *Tg(cyp1a:nls-egfp)* embryos. In addition, they observed limited expression of GFP in the *Tg(cyp1a:nls-egfp)* embryos exposed to TCDD as opposed to widespread expression of GFP in our experiments. This discrepancy might reflect the difference in the origin (human vs. zebrafish) of the *CYP1A* gene, in the size of the regulatory region fused to the GFP gene (2 kb vs. 30 kb), or in the nature (transient vs. stable) of expression of a reporter gene.

How do target tissues uncovered by this study translate into assessment of ecotoxicological relevance and human health risks

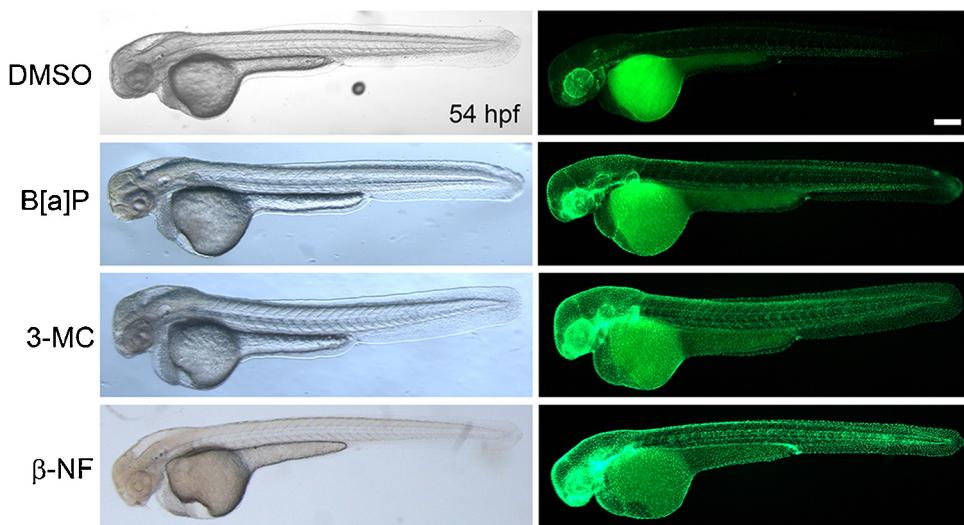


**Fig. 6.** Extended exposure time lowers the LOD of TCDD for *Tg(cyp1a:nls-egfp)* embryos. (A and B) *Tg(cyp1a:nls-egfp)* embryos at 30 hpf were exposed to DMSO (as vehicle) or TCDD at 10 pM for 48 h (A) or 72 h (B) and imaged by fluorescent microscopy. Lateral views anterior to the left are shown. Scale bar = 250  $\mu$ m. (C) The embryos in A and B were subject to WB using anti-GFP antibody. To prevent saturation of band intensity in a 10 pM lane, the membrane was exposed to the film very briefly. As a result, no band is observed in DMSO lanes, although GFP<sup>+</sup> cells were noted by fluorescence microscopy in *Tg(cyp1a:nls-egfp)* treated with DMSO.

by TCDD? First, otic vesicle, lateral lines and retina (especially bipolar cells), all showing increased expression of *cyp1a* upon TCDD exposure, have sensory neurons, suggesting vulnerability of sensory neurons to TCDD exposure. This is supported by a previous report showing that TCDD exposure elicited a dose-dependent decrease in sensory conduction velocities in rats (Grehl et al., 1993). Second, TCDD induction of *cyp1a* in pectoral fin bud suggests susceptibility of limb development to TCDD. This notion is reinforced by a Kojuma et al. report that a Drosophila homolog of AhR is involved in the Drosophila leg development (Kozu et al., 2006). Furthermore, Zodrow and Tanguay demonstrated that caudal fin regeneration is suppressed by TCDD (Zodrow and Tanguay, 2003).

Lastly, we showed that cloaca is target tissue of TCDD in zebrafish. As cloaca is a common gut and urogenital opening (Pyati et al., 2006), it is tempting to speculate that TCDD might hamper development of gut and urogenital openings.

The lowest concentration of TCDD that *Tg(cyp1a:nls-egfp)* could detect was 10 pM when embryos were exposed to TCDD for 3 days. Given the maximum contaminant level (MCL) of TCDD in drinking water set by the United States Environmental Protection Agency, 93 fM or 30 ppq (part per quadrillion) (United States Environmental Protection Agency, 2013) it would be desirable to lower LOD of TCDD for *Tg(cyp1a:nls-egfp)* embryos. How could we then lower the LOD? First, extending the exposure time could do, given the



**Fig. 7.** B[a]P, 3-MC and β-NF increases GFP expression in *Tg(cyp1a:nls-egfp)* embryos. *Tg(cyp1a:nls-egfp)* embryos at 30 hpf were exposed to 1  $\mu$ M B[a]P, 1  $\mu$ M 3-MC and 10  $\mu$ M β-NF for 24 h and imaged by fluorescent microscopy. Transmitted light (left) and fluorescent (right) images. Lateral views anterior to the left are shown. Scale bar = 250  $\mu$ m.

bioconcentration of TCDD in living organisms. In fact,  $10^{-17}$  M TCDD can be bioconcentrated to  $10^{-12}$  M in fish (Frakes et al., 1993). Second, TCDD-triggered GFP transcription from *Tg(cyp1a:nls-egfp)* can be amplified using genetic manipulations such as GAL4-UAS system (Scheer and Campos-Ortega, 1999). For example, Kudoh et al. employed the GAL4-UAS system to amplify estrogen-mediated induction of GFP reporter (Lee et al., 2012). Hence, generation of double transgenic zebrafish carrying both *gal4* fused to *cyp1a* promoter+enhancer and *uas* fused to nuclear GFP [termed *Tg(cyp1a:gal4; uas-nls-egfp)*] could amplify the TCDD-triggered GFP induction, thereby enabling detection of TCDD at MCL. Third, increase in copy number of *Tg(cyp1a:nls-egfp)* integrated into the zebrafish genome could lower the LOD.

It is conceivable to generate transgenic zebrafish expressing DRE sequences fused to a reporter gene, i.e., GFP, luciferase or *lacZ*, to use as sentinels for aquatic TCDD pollution. What are then the advantages of *Tg(cyp1a:nls-egfp)* over DREs fused to a reporter gene? First, *Tg(cyp1a:nls-egfp)* has lower LOD of TCDD than DREs fused to a reporter gene. *Tg(cyp1a:nls-egfp)* can detect as low as 10 pM TCDD, as opposed to 100 pM TCDD in the human hepatoma cell line, HepG2, by DREs fused to luciferase (Yueh et al., 2005) and 50 pM TCDD in the mouse hepatoma cell line, Hepa, by DREs fused to *lacZ*. Given an amplification effect of luciferase and  $\beta$ -galactosidase, *Tg(cyp1a:nls-egfp)* is much more sensitive than DRE-luciferase or DRE-*lacZ*. Second, GFP expression in *Tg(cyp1a:nls-egfp)* embryos parallels endogenous expression of *cyp1a*, which is not the case with DREs fused to a reporter gene, implying that *Tg(cyp1a:nls-egfp)* is a TCDD biosensor with more physiological relevance (Willey et al., 1998).

Wolf, Tukey et al., generated transgenic mice expressing a human CYP1A1 reporter (Campbell et al., 1996; Galijatovic et al., 2004; Operana et al., 2007). However, *Tg(cyp1a:nls-egfp)* zebrafish embryos have several advantages over the transgenic CYP1A1 reporter mice. First, *Tg(cyp1a:nls-egfp)* is more sensitive. Only 10 pM TCDD (1.3 ng of TCDD per 40 ml of egg water that carries 40 embryos) is required for induction of GFP in zebrafish, as opposed to intraperitoneal injection or oral administration of at least 1  $\mu$ g/kg TCDD (20 ng of TCDD per 20 g of a mouse) in mice. Second, induction of *Tg(cyp1a:nls-egfp)* is assessed by fluorescent microscopy without dissection, whereas dissection is required for the assessment in the mice. Third, zebrafish are cheaper to maintain and easier to breed compared to mice. Taken together, *Tg(cyp1a:nls-egfp)* zebrafish are suitable for quick screen for the presence of minuscule amount of TCDD, which does not elicit morphological abnormalities in zebrafish.

The target tissues we report here will further understanding of ecotoxicological relevance and human health risks by TCDD, thereby facilitating risk assessment of TCDD. We believe that not only could *Tg(cyp1a:nls-egfp)* zebrafish embryos be used as a sentinel for aquatic dioxin pollution, but they can also be used to screen for inhibitors of TCDD toxicity, thereby providing therapeutic modalities against TCDD toxicity and furthering the understanding of the molecular mechanism underlying TCDD toxicity. Finally, *Tg(cyp1a:nls-egfp)* zebrafish embryos would be of help to identify Ahr ligands, both endogenous and exogenous.

## Conflict of interest

No competing financial interest is declared.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2013.03.010>.

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