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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Upregulates *FoxQ1b* in Zebrafish Jaw Primordium

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Abstract

Vertebrate jaw development can be disrupted by exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)—a potent activator of the aryl hydrocarbon receptor (AHR) transcription factor required for transducing the toxic effects of TCDD. We used zebrafish (*Danio rerio*) embryos to investigate transcriptional responses to TCDD with the goal of discovering novel, jaw-specific genes affected by TCDD exposure. Our results uncovered a novel target of TCDD-activated Ahr belonging to the evolutionarily conserved family of forkhead box transcription factors. Quantitative real-time polymerase chain reaction analysis demonstrated that *FoxQ1b* was upregulated by TCDD 7- and 10-fold at 24 and 48 h postfertilization (hpf), respectively. The rate of TCDD-induced *FoxQ1b* expression was more rapid than that of *Cyp1a*, a known direct target of TCDD-activated Ahr. TCDD-mediated induction of *FoxQ1b* was suppressed in the presence of an Ahr antagonist, α -naphthoflavone, as well as following knockdown of Ahr2 expression using an Ahr2-specific morpholino antisense oligonucleotide. In situ hybridization analysis of *FoxQ1b* expression at 48 hpf demonstrated that *FoxQ1b* is specifically expressed in the jaw primordium where it discretely outlines a developing jaw structure known as Meckel's cartilage—a conserved structure in all jawed vertebrates that develops abnormally in the presence of TCDD. These results identify a novel target of TCDD-activated Ahr and suggest that *FoxQ1b* may play a role in craniofacial abnormalities induced by developmental exposure to TCDD.

Introduction

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during embryogenesis causes severe craniofacial abnormalities in diverse model organisms, consistent with an evolutionarily conserved mechanism regulating TCDD action and craniofacial development. For example, TCDD exposure is associated with cleft palate in mice (1–3), and it induces beak malformations representative of severe cleft lip and palate in chicken (4).

Popularly referred to as dioxin, TCDD is the archetype of a family of related polychlorinated compounds found ubiquitously in the environment. It is a known carcinogen and teratogen produced during commercial processes including waste incineration, pulp and paper manufacturing, petroleum refining, and production of specific chlorinated chemicals (5). Dioxins are also produced in common combustion products including automobile and diesel exhaust and cigarette smoke (5).

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Supporting Information Available: Figures showing chromosomal regions containing FoxQ1 genes that exhibit conserved synteny, *FoxQ1b* induction that occurs after exposure to TCDD at later developmental stages, TCDD-mediated disruption of Meckel's and ceratohyal cartilages, and distribution of AhREs in the promoter regions of *FoxQ1a*, *FoxQ1b*, and *Cyp1A1*. Tables of genes and primer sequences analyzed by qPCR and *FoxQ1* nucleotide and protein sequence similarities. This material is available free of charge via the Internet at <http://pubs.acs.org>.

TCDD acts via the aryl hydrocarbon receptor (AHR)—a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) superfamily (6,7). In the absence of activation, AHR resides in the cytoplasm in a complex consisting of two molecules of the HSP90 chaperone and one molecule each of the cochaperones ARA9 and p23 (8–10). Upon ligand binding, AHR translocates to the nucleus where it forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT), thus reconstituting a functional transcriptional activator. The AHR:ARNT heterodimer recognizes and binds to specific sequences called AHR response elements (AHREs) in the promoter region of target genes (reviewed in ref 11). Zebrafish possess three orthologs of human AHR, Ahr1a, Ahr1b, and Ahr2 (12,13), and two orthologs of ARNT, Arnt1 and Arnt2 (14,15). The toxic effects of TCDD are mediated by the Ahr2:Arnt1 heterodimer (15–17). Transcriptional activation by AHR is subject to a negative feedback regulatory loop involving AHRR, the AHR repressor (18). AHRR is induced by TCDD-activated AHR and competes with AHR for binding to ARNT. In addition, the AHRR:ARNT heterodimer functions as a suppressor and competes with AHR: ARNT heterodimer for AHRE binding (19).

In mice, Ahr function is critical during development and required for normal hepatic, cardiovascular, and immune functions (20,21). Currently, we know of only a few genes that are regulated directly by AHR despite its importance in development and homeostasis and its function as a regulator of gene expression (11). The exceptions are a number of genes activated as part of the adaptation to environmental contaminants that are unlikely to play a significant role in development (11,22,23).

Zebrafish embryos are particularly sensitive to TCDD toxicity. Embryos exposed to TCDD exhibit heart looping malformations, diminished caudal blood flow, edema, and defects in swim bladder inflation (24). Exposure to TCDD during embryogenesis or at early larval stages also causes malformations of the lower jaw and surrounding cartilaginous structures, and the severity is proportional to the time of onset of exposure. Defects can be seen in Meckel's cartilage, palatoquadrate cartilage, and hyosymplectic cartilage—all of which have homologs in mammals (25–27).

Many genes critical for proper vertebrate craniofacial development have been identified; yet, only one—*Sox9b*—is known to be affected by TCDD exposure (27). We used microarray technology to identify genes encoding transcription factors whose expression is affected by exposure to low levels of TCDD and that may be involved in craniofacial development. We report the identification of a member of the Q subfamily of forkhead box genes as one such potential direct target of activated Ahr. The spatiotemporal expression of this gene in relation to craniofacial development suggests that *FoxQ1b* may play a crucial role in modulating TCDD-mediated craniofacial abnormalities.

Experimental Procedures

Zebrafish

Wild-type AB zebrafish (*Danio rerio*) embryos were obtained from natural spawnings of adult fish maintained at 28.5 °C on a 14/10 h light/dark cycle. Embryos were staged according to Kimmel et al. (28).

TCDD Exposures

Zebrafish adults were allowed to spawn, and embryos were collected, scored for viability, and sorted by developmental stage. For microarray analysis, zebrafish embryos were exposed to 0.1% v/v DMSO (vehicle control) or 1 nM TCDD in DMSO (Cerilliant CIL, Inc.) from 1 to 24 h postfertilization (hpf). For most other experiments, unless otherwise specified, embryos

were exposed to 0.1% v/v DMSO or 1 nM TCDD in DMSO for 1 h from 6 to 7 hpf. For assays designed to determine induction rates, embryos were exposed to 0.1% DMSO or 1 nM TCDD at 24 or 48 hpf for up to 90 min. For inhibition assays, the AHR antagonist, α -naphthoflavone (29) (ANF; Sigma-Aldrich), was dissolved in DMSO and used at a final concentration of 500 nM. Exposure conditions for each replicate were conducted at 28.5 °C and involved no more than 10 embryos per 1 mL volume of DMSO or TCDD solutions. Exposures were terminated by triple washes in 0.5× E2 media (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM MgSO₄, 0.075 mM KH₂PO₄, 0.0025 mM Na₂HPO₄, 0.5 mM CaCl₂, and 0.35 mM NaHCO₃), and embryos were reared in 100 mm Petri dishes.

Microarray Analysis

RNA from two pools each of DMSO or TCDD-exposed embryos (described above) was sent to the Microarray Core Facility at the University of Wisconsin—Madison (directed by Dr. Christopher Bradfield) where microarray hybridizations were performed on Agilent zebrafish genome V2 4 × 44K arrays. After they were scanned and normalized, the data sets were statistically analyzed according to the MicroArray Quality Control (MAQC) consortium recommendations and as previously described (30,31). Approximately 65 genes undergoing an absolute fold-change difference in expression level ≥ 2.0 in control versus TCDD-treated embryos were identified (Table 1). Data are available at <http://edge.oncology.wisc.edu/datafiles/planchartarchive.zip>.

Morpholino Injections

A previously described translation-blocking morpholino antisense oligonucleotide (MO; Gene-Tools) designed against zebrafish *Ahr2* was used (32). Approximately 5 nL of a 0.1 mM solution of the *Ahr2* MO or Gene-Tool's standard control MO was injected into the yolk sac of AB wild-type zebrafish embryos at the 1–4 cell stage. Injections were performed in triplicate groups of approximately 50 embryos each. Each replicate was further divided into two groups—one for RNA extraction and reverse transcription and another for rearing and phenotypic observation.

RNA Extraction and Reverse Transcription

Total RNA was recovered from pooled whole embryos using TRIzol (Invitrogen) according to the manufacturer's protocol. Fifty embryos per replicate were used for the microarray experiment, and 10 embryos per replicate were used for quantitative polymerase chain reaction (qPCR) experiments. RNA quality was assessed by microchip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies) and used for qPCR experiments if the RNA integrity number (RIN) exceeded 8.0. First-strand cDNA was synthesized from 2 μ g of total RNA using MMLV reverse transcriptase (RT) from a RETROscript kit (Ambion) according to the manufacturer's protocol. Negative controls (no RT-control) were included.

Alcian Blue Staining

Zebrafish larvae were euthanized in Tricaine and fixed overnight in 4% paraformaldehyde at 4 °C. Alcian blue staining was performed as previously described (33) using a low-acid protocol (34).

Quantitative Polymerase Chain Reaction (qPCR)

qPCR analysis was performed on an Mx3000P Real-Time PCR system (Stratagene) using the Brilliant SYBR Green qPCR reagent (Stratagene) according to the manufacturer's protocol. The cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s. Threshold cycles (C_t) and dissociation curves were determined with MxPro software (Stratagene). Expression levels were normalized to *D. rerio*

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which was invariant in every experiment performed regardless of the age of embryos or exposure condition. Fold-change was calculated using $FC = 2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_{t,GOI} - \Delta C_{t,norm}$ (GOI, gene of interest; norm, *Gapdh*). All analyses were performed in triplicate. Statistically significant fold changes were determined by Student's *t* test. Primer sequences are reported in Table S1 of the Supporting Information.

In Situ Hybridization

Whole-mount in situ hybridization was performed as previously described (35). A 609 bp *FoxQ1b* probe corresponding to nucleotides 667–1275 of BC139522, the transcript from which the *FoxQ1b* microarray probe was derived, was PCR amplified from zebrafish cDNA, subcloned into the dual-promoter pCR II-TOPO vector (Invitrogen), and transcribed using Sp6 or T7 polymerase in the presence of DIG-UTP (Roche) to generate antisense and sense probes, respectively. Alkaline phosphatase-conjugated anti-DIG antibody (Roche) was used for the detection of the hybridized probe.

Results

FoxQ1b Is a Novel Target of TCDD-Induced Transcriptional Upregulation

Analysis of an Agilent V2 4 × 44K zebrafish microarray data set identified approximately 65 genes undergoing an absolute fold-change difference in expression level ≥ 2.0 in control versus TCDD-treated embryos (Table 1). Within this data set, we observed robust expression increases in several genes known to be regulated by ligand-activated Ahr such as cytochrome P4501A, cytochrome P4501B1, and the aryl hydrocarbon receptor repressor (*Ahr*) as well as many genes not previously associated with TCDD-activated Ahr induction or repression. (In several cases, redundant probes for some of these genes exhibited a wide range of expression changes, possibly reflecting differential splice variants of the genes or variable efficacy in probe design.) Of particular interest among this data set was the 16-fold induction of Agilent probe A_15_P199746 observed in TCDD-exposed zebrafish. Computational analysis identified several zebrafish cDNA sequences containing the probe sequence, including BC115154, all mapping to zebrafish chromosome 2. We analyzed the predicted amino acid sequence and determined that the gene encodes one of two zebrafish paralogs, orthologous to mouse *Foxq1* and human *FOXQ1*, members of the Forkhead box family of transcription factors (Figure S1 and Table S2 of the Supporting Information). The NCBI *Entrez Gene* database contains an entry for a related yet different zebrafish ortholog of *FOXQ1* (see accession ID NM_212907); thus, we refer to the gene that we uncovered as *FoxQ1b* and its NCBI—curated paralog as *FoxQ1a*. *FoxQ1a*, which is not represented on the Agilent array—is on zebrafish chromosome 20.

Zebrafish chromosomes 2 and 20, containing *FoxQ1b* and *FoxQ1a*, respectively, exhibit conserved synteny with mouse chromosome 13 and human chromosome 6 (Figure S1 of the Supporting Information). Included in these conserved regions are two other members of the *Fox* family of transcription factors, *FoxC1* and *FoxF2*. Conservation of synteny is lost between mammals and zebrafish just outside these two genes. In mammals, *Edn1* and *Gli3* flank the *Fox* subfamily members, whereas in zebrafish these genes dispersed to chromosomes 19 (*Edn1*) and 24 (*Gli3*). Interestingly, in mouse *Edn1*, *Foxc1*, *Foxf2*, *Foxq1*, and *Gli3* are critical for normal craniofacial development (36–41).

FoxQ1b Is Selectively and Rapidly Induced by TCDD

To validate the microarray observation that *FoxQ1b* is upregulated by TCDD, we exposed zebrafish embryos at 6 hpf for 1 h to either 0.1% DMSO or 1 nM TCDD. Following exposure, RNA was extracted at 12, 24, and 48 hpf, reversed-transcribed, and analyzed by qPCR.

Expression values for four genes were measured as follows: cytochrome P4501A (*Cyp1a*; positive control for TCDD induction), *FoxQ1a*, *FoxQ1b*, and glycerol-dehyde-3-phosphate dehydrogenase (*Gapdh*, normalizer). Induction of *FoxQ1a*, *FoxQ1b*, or *Cyp1a* was not observed at 12 hpf. However, by 24 hpf, TCDD-mediated induction of *FoxQ1b* and *Cyp1a* expression was readily apparent (Figure 1A). Although by 48 hpf *FoxQ1b* levels were not as high as those of *Cyp1a*, levels continued to rise from 7- (24 hpf) to 10-fold (48 hpf) relative to DMSO controls. In contrast, *FoxQ1a* expression was unaffected by exposure to TCDD. *Gapdh* was invariant under all conditions.

The time-dependent rate of TCDD-mediated induction of *FoxQ1b* was also assessed. Embryos were exposed to TCDD at 24 or 48 hpf, and induction of *FoxQ1b* and *Cyp1a* transcription was determined 30, 45, 60, and 90 min following the initiation of exposure. The results indicated that TCDD-mediated induction occurs more rapidly for *FoxQ1b* than for *Cyp1a* at both stages of embryogenesis with 24 hpf embryos being more responsive (Figure 1B). At 24 hpf, *FoxQ1b* expression was upregulated ≥ 2 -fold 30 min after initiating exposure, whereas a comparable level of *Cyp1a* induction was not observed until 45 min after initiating exposure. By comparison, neither gene was significantly induced at 48 hpf until 45 (*FoxQ1b*) and 60 min (*Cyp1a*). Expression of *FoxQ1b* and *Cyp1a* increased over the course of the experiment, reaching approximately 16- and 32-fold induction levels, respectively, by 90 min postexposure, regardless of developmental stage.

Lower jaw growth and development in zebrafish larvae remain sensitive to TCDD after 48 hpf (27). To test if TCDD-mediated induction of *FoxQ1b* was a persistent phenomenon at later stages, we exposed 96 hpf zebrafish larvae to TCDD for 1 h according to the protocol of Xiong and colleagues, except that we used 1 nM TCDD instead of 3 nM (27). RNA was extracted at 48 h postexposure and reversed-transcribed. TCDD-mediated induction of *FoxQ1b*, as measured by qPCR, occurred even at this later stage of larval development (144 hpf). Interestingly, steady-state transcript levels are apparent at or before 48 h postexposure, peaking at approximately 8–10- (*FoxQ1b*) and 200-fold (*Cyp1a*) higher than DMSO controls (Figure S2 of the Supporting Information).

TCDD-Induced FoxQ1b Expression Localizes to Jaw Structures That Develop Abnormally in the Presence of TCDD

Zebrafish embryos were exposed at 6 hpf to 0.1% DMSO or TCDD as described. At approximately 144 hpf, larvae were euthanized, fixed, and subsequently stained with Alcian blue to reveal cartilaginous structures. Before larvae were euthanized, embryos were observed for hallmark effects of TCDD exposure. Consistent with the published literature, we observed craniofacial deformities; edematous pericardial sac, yolk, and blebs in the head region; diminished caudal blood flow by 48 hpf with complete loss of apparent flow by 72 hpf; growth suppression; and overall loss of vigor (data not shown) (25–27,42,43). Significant changes in the morphology of cartilaginous structures comprising the jaw were apparent by Alcian blue staining particularly in Meckel's and ceratohyal cartilages (Figure S3 of the Supporting Information).

To determine whether *FoxQ1b* was expressed in these structures, we performed in situ hybridization with a probe derived from the *FoxQ1b* open reading frame. Embryos were exposed at 6 hpf for 1 h to either 0.1% DMSO or 1 nM TCDD and then fixed at 24 and 48 hpf. At 24 hpf, we observed a general increase in the intensity of *FoxQ1b* expression in TCDD-exposed embryos, consistent with our qPCR analysis, but expression was not discretely localized. In contrast, at 48 hpf, strong *FoxQ1b* expression in TCDD-exposed embryos was clearly seen in structures of the lower jaw, particularly Meckel's cartilage and associated structures (Figure 2). Individual cells reminiscent of condensing chondrocytes staining positive for *FoxQ1b* can be seen at higher magnification (data not shown). No other expression was

apparent, indicating that TCDD-induced *FoxQ1b* expression is specific to the developing jaw at this point in development. Expression was not observed in DMSO-exposed control embryos by in situ, consistent with low levels of expression observed by qPCR.

FoxQ1b Promoter/Enhancer Region Contains Aryl Hydrocarbon Response Elements (AhREs)

We searched the region upstream of the putative translational start site (TSS) of zebrafish *FoxQ1b* and comparable regions for zebrafish *FoxQ1a*, human *FOXQ1*, and mouse *Foxq1* genes for putative AhREs. Approximately 7 kilobases (kb) upstream of the TSS were analyzed using MatInspector (Genomatrix), a program that searches promoter regions using position weight matrices, rather than a single consensus sequence, thus accounting for known variability in transcription factor binding sites such as the AhRE (44). Six putative AhREs [core binding site: 5'-CGTG-3' (45)] were identified in the zebrafish *FoxQ1b* region, indicating that this gene may be regulated directly by AhR (Figure S4 of the Supporting Information). In contrast, an equivalent region of *FoxQ1a*, a gene that was not responsive to TCDD (Figure 1A), contained three AhREs. Notably, analysis of the promoter/enhancer region of human *FOXQ1* identified nine consensus AHREs within the first 3.5 kb from the TSS, whereas in mouse, three AhREs were found within 4.5 kb from the TSS.

TCDD-Induced FoxQ1b Expression Is Ahr-Dependent

To determine if TCDD-mediated induction of *FoxQ1b* required ligand-activated Ahr, we exposed 48 hpf embryos to 0.1% DMSO or TCDD in the presence or absence of the Ahr antagonist, ANF (29), and measured induction at 60 min postexposure. As seen in Figure 3A, induction of *FoxQ1b* and *Cyp1a* was reduced approximately 5-fold in the presence of ANF, demonstrating that Ahr plays a critical role in TCDD-mediated induction of *FoxQ1b*. ANF alone had no effect on *FoxQ1b* or *Cyp1a* (data not shown).

To further explore whether Ahr2 activation is necessary for *FoxQ1b* induction, we blocked translation of its mRNA using a morpholino antisense oligonucleotide (32). Embryos at the 1–4 cell stage were injected with the *Ahr2* or a control MO and exposed to 0.1% DMSO or 1 nM TCDD at 6 hpf for 1 h. At 48 hpf, total RNA was extracted from a subset of injected embryos and reverse-transcribed, and the *Gapdh*-normalized levels of *Cyp1a* and *FoxQ1b* were measured by qPCR. *Cyp1a* and *FoxQ1b* transcript levels were approximately 70 ($p = 0.03$) and 90% ($p = 0.05$) lower, respectively, in MO-injected embryos as compared to control MO-injected embryos (Figure 3B). The remaining embryos were allowed to develop to the stage where jaw development could be assessed. TCDD exposure had no effect on jaw development in MO-injected embryos, whereas control MO-injected embryos displayed severe jaw developmental defects. Other TCDD toxic end points were observed in control MO-injected embryos but not in *Ahr2* MO-injected embryos, including edema, reduced caudal blood flow, and death.

Discussion

Here, we provide the first report that activation of Ahr by TCDD significantly upregulates the forkhead box gene, *FoxQ1b*, in conserved structures of the jaw primordium that develop abnormally in the presence of TCDD. The forkhead box gene family, first identified in *Drosophila melanogaster* (46), encodes transcription factors that bind DNA as monomers via a helix-loop-helix domain known as the winged-helix motif (46–50). In mammals, there are 19 subfamilies (Fox A through Fox S) whose members are important for multiple processes, including embryonic development, organogenesis, and homeostasis (51). Several Fox genes are required for craniofacial development in mice, including *Foxc1*, *Foxc2*, *Foxe1*, *Foxf2*, *Foxg1*, *Foxi1*, *Foxo1*, *Foxp2*, *Foxp3*, and *Foxq1* (38,41,52–61). *FoxQ1a* and *FoxQ1b* are

paralogs that probably arose from a whole-genome duplication event in the evolutionary history of zebrafish (62). Our results demonstrate that *FoxQ1b* is significantly induced by TCDD, whereas its paralog, *FoxQ1a*, is refractory. A TCDD effect on *FoxQ1* was reported previously in a microarray study of exposed mammalian thymic epithelial cells; however, this observation was neither featured nor validated by the authors (63).

The differential response to TCDD by the *FoxQ1* paralogs may reflect results from computational analyses of the promoter regions in which we identified fewer putative AhREs in *FoxQ1a* than *FoxQ1b*. Studies are underway to determine if the relative abundance of AhREs explains the differential response of these genes to TCDD or whether other factors may be involved (e.g., chromatin structure, presence of other enhancer/repressor sites). A similar analysis of the human and mouse orthologs uncovered multiple AhREs consistent with the hypothesis that the effect of TCDD on the expression of this gene may be an evolutionarily conserved feature.

The onset and level of TCDD-mediated transcriptional upregulation are more rapid and higher, respectively, for *FoxQ1b* than for *Cyp1a*, consistent with the hypothesis that, like *Cyp1a*, *FoxQ1b* may be a direct target of Ahr2 activation. This hypothesis is strongly supported by our data demonstrating that TCDD-mediated induction of *FoxQ1b* is suppressed significantly in the presence of the Ahr antagonist, ANF, and nearly absent in Ahr2 morpholino knockdown experiments.

In situ hybridization at 24 hpf showed that *FoxQ1b* upregulation was not localized to any particular structure but rather was seen throughout the embryo. However, at 48 hpf, *FoxQ1b* was discretely and strongly upregulated in the jaw primordium of TCDD-exposed zebrafish. Significantly, this expression localized to the developing Meckel's cartilage, which is one of the jaw structures most severely compromised in zebrafish exposed to TCDD. This time point also coincides with the condensation of prechondrogenic neural crest-derived cells in the first and second pharyngeal arches (64). The lower jaw structures, including Meckel's, palatoquadrate, hyosymplectic, and ceratohyal cartilages, are derived from these two embryonic tissues (65). Interestingly, Goering and colleagues observed genetic background-specific craniofacial abnormalities resulting from the knock out of mouse *Foxq1* (55), consistent with our hypothesis that misregulation of *FoxQ1b* by TCDD plays a role in zebrafish craniofacial abnormalities. Expression of *FoxQ1b* was also observed in more posterior arches, suggesting that TCDD-mediated *FoxQ1b* induction might also adversely affect structures that descend from these arches.

In mammals, *Foxq1* is a single-copy gene that functions in the regulation of gastric acid secretion from parietal cells (55), mucin expression from stomach foveolar cells (66), differentiation and development of the hair shaft (67), and craniofacial development (55). Transgenic mice expressing a ligand-independent, constitutively activated form of Ahr have high incidences of glandular stomach tumor formation due to hyperproliferation of parietal and chief cells (68). In light of the observation that *Foxq1* knock out mice have defective parietal cells (55), it is possible that constitutive activation of Ahr leads to overexpression of *Foxq1* and subsequently to loss of regulatory control over parietal cell proliferation. Interestingly, human *FOXQ1* is overexpressed in colorectal adenocarcinoma and lung carcinoma cell lines, as well as in primary cultures from patient-derived colorectal tumors (69), supporting a role for *Foxq1* in regulating cell proliferation. On the basis of these data and our observations of TCDD induction of *FoxQ1b*, we are also evaluating its expression in the digestive system of zebrafish embryos and larvae exposed to TCDD.

Lastly, the observation that craniofacial abnormalities in *Foxq1*^{-/-} mice arise in a background-dependent context (55) implies that background-specific modifiers affect *Foxq1* function.

Modifiers coupled with mutations altering *FOXQ1* expression or function could be segregating in human populations given the large degree of genetic variability, thereby making it potentially feasible to identify distinct populations more susceptible to environmental toxicants that alter craniofacial development. It will be interesting to pursue investigations in zebrafish that lead to the discovery of gene networks involving *FoxQ1b* that affect craniofacial development and are susceptible, directly or indirectly, to TCDD exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TCDD selectively and rapidly induces *FoxQ1b*, but not *FoxQ1a*, transcript expression in zebrafish embryos. (A) Zebrafish embryos were exposed at 6 hpf for 1 h to either 0.1% DMSO or 1 nM TCDD. Following exposure, RNA was extracted at 12, 24, and 48 hpf, reversed-transcribed, and analyzed by qPCR. Statistically significant induction was observed for *Cyp1a* and *FoxQ1b* at all time points except for 12 hpf. No significant induction was observed for *FoxQ1a* at any time point. Each time point is the average of at least three biological replicates. Error bars are standard deviations of the mean (** indicates $p \leq 0.01$). (B) Zebrafish embryos were exposed to 0.1% DMSO or 1 nM TCDD at 24 or 48 hpf. Embryos were sacrificed for RNA extraction at 30, 45, 60, 75, and 90 min after the initiation of exposure. At 24 hpf, *FoxQ1b* expression was significantly induced by TCDD (2.5-fold) within 30 min and up to 16-fold by 90 min. *Cyp1a* was not significantly induced until 45 min (3.4-fold) after exposure was initiated. Induction initiation was delayed for both genes at 48 hpf with *Cyp1a* responding less rapidly than *FoxQ1b*, although by 90 min postexposure, induction levels were similar to those observed for 24 hpf.

Figure 2.

TCDD induces localized *FoxQ1b* expression in the zebrafish jaw primordium by 48 hpf. Zebrafish embryos were exposed at 6 hpf for 1 h to either 0.1% DMSO or 1 nM TCDD. Embryos were fixed at 48 hpf and analyzed using whole mount in situ hybridization. (A) A 0.1% DMSO-exposed embryo. (B and C) A 1 nM TCDD-exposed embryo. Arrowheads: *FoxQ1b* is also expressed in several posterior branchial arches. Ey, eye; Y, yolk sac.

Figure 3.

Inhibition of Ahr activation decreases TCDD-mediated induction of *FoxQ1b*. The Ahr antagonist ANF and a translation-blocking morpholino antisense oligonucleotide designed against zebrafish *Ahr2* were used to determine if inhibition of Ahr2 would suppress TCDD-mediated *FoxQ1b* induction. (A) Zebrafish embryos (48 hpf) were exposed to 0.1% DMSO or 1 nM TCDD in the presence and absence of 500 nM ANF. Embryos were sacrificed for RNA extraction at 60 min after the initiation of exposure, and transcript levels were measured by qPCR. ANF inhibited induction of *FoxQ1b* and *Cyp1a* approximately 5-fold ($p = 0.001$ and $p = 0.02$, respectively). ANF alone had no effect on *FoxQ1b* or *Cyp1a* (data not shown). (B) Zebrafish embryos were injected with an *Ahr2* or control MO (approximately 5 nL of a 0.1 mM solution) at the 1–4 cell stage and exposed to 0.1% DMSO or 1 nM TCDD at 6 hpf for 1 h. At 48 hpf, total RNA was extracted, and transcript levels were measured by qPCR. TCDD-mediated induction of *Cyp1a* and *FoxQ1b* was reduced by approximately 70 ($p = 0.03$) and 90% ($p = 0.05$), respectively, in MO-injected embryos as compared to control MO-injected embryos.

Table 1

TCDD-Responsive Genes Identified by Microarray Analysis

gene symbol	probe ID	fold change ↑	gene symbol	probe ID	fold change ↓
CYP1A	A_15_P100578	75.06	ANK3	A_15_P581392	-2.03
CYP1A	A_15_P131751	67.65	NTRK3	A_15_P106521	-2.03
CYP1A	A_15_P167441	14.62	ZNF235	A_15_P339440	-2.04
CYP1B1	A_15_P554522	30.48	NT5C1A	A_15_P182621	-2.13
CYP1B1	A_15_P171581	28.64	-	A_15_P452380	-2.20
CYP1B1	A_15_P148661	28.64	SCMH1	A_15_P361595	-2.25
CYP1B1	A_15_P131961	12.13	-	A_15_P584987	-2.30
CYP1B1	A_15_P491107	2.06	DDHD2	A_15_P232496	-2.31
AHR	A_15_P172711	25.81	DPP6	A_15_P480545	-2.33
FOXQ1	A_15_P199746	16.56	SALL1	A_15_P277171	-2.35
SULF6B1	A_15_P153096	5.94	ANKRD11	A_15_P434015	-2.41
ZNF595	A_15_P501442	4.41	HLA-DRB1	A_15_P581792	-2.55
OPN1MW	A_15_P110493	4.08	CADM2	A_15_P329451	-2.57
-	A_15_P154321	3.92	GJD2	A_15_P388950	-2.57
IMP2	A_15_P472975	3.76	RNF213	A_15_P223046	-2.57
ZPLD1	A_15_P565127	3.53	BMPRIA	A_15_P524332	-2.58
KCNH2	A_15_P474960	3.20	-	A_15_P395180	-2.60
CLDN4	A_15_P238176	3.16	NR1D2	A_15_P160171	-2.62
CALCRL	A_15_P362595	3.03	IL11RA	A_15_P114635	-2.71
ZBTB16	A_15_P524707	2.91	CHRM5	A_15_P221666	-2.79
-	A_15_P106218	2.64	TRIM37	A_15_P453300	-2.87
MEAP4	A_15_P188721	2.50	GPR22	A_15_P242246	-2.93
PER2	A_15_P102459	2.46	LOC390988	A_15_P389815	-3.05
PXDN	A_15_P481545	2.46	-	A_15_P119164	-3.56
FOXJ1	A_15_P542892	2.25	NR5A2	A_15_P100569	-3.81
-	A_15_P311746	2.22	LAMP3	A_15_P583562	-3.92
FOXF2	A_15_P571567	2.19	SELP	A_15_P207751	-4.96
FOXF2	A_15_P142441	2.17	-	A_15_P134381	-5.46
BAZ2A	A_15_P336915	2.16	FBXO38	A_15_P527062	-5.82

gene symbol	probe ID	fold change ↑	gene symbol	probe ID	fold change ↓
GLIPR2	A_15_P575052	2.11	ZNF180	A_15_P586572	-6.50
ATP1A1	A_15_P103650	2.10			
DTX1	A_15_P188771	2.08			
TMEM86B	A_15_P187026	2.08			
MLL3	A_15_P521602	2.06			
CPA1	A_15_P117784	2.04			