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Developmental exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin alters DNA methyltransferase (*dnmt*) expression in zebrafish (*Danio rerio*)

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Abstract

DNA methylation is one of the most important epigenetic modifications involved in the regulation of gene expression. The DNA methylation reaction is catalyzed by DNA methyltransferases (DNMTs). Recent studies have demonstrated that toxicants can affect normal development by altering DNA methylation patterns, but the mechanisms of action are poorly understood. Hence, we tested the hypothesis that developmental exposure to TCDD affects *dnmt* gene expression patterns. Zebrafish embryos were exposed to 5 nM TCDD for one hour from 4 to 5 hours post-fertilization (hpf) and sampled at 12, 24, 48, 72 and 96 hpf to determine *dnmt* gene expression and DNA methylation patterns. We performed a detailed analysis of zebrafish *dnmt* gene expression during development and in adult tissues. Our results demonstrate that *dnmt3b* genes are highly expressed in early stages of development, and *dnmt3a* genes are more abundant in later stages. TCDD exposure upregulated *dnmt1* and *dnmt3b2* expression, whereas *dnmt3a1*, *3b1*, and *3b4* are downregulated following exposure. We did not observe any TCDD-induced differences in global methylation or hydroxymethylation levels, but promoter methylation of aryl hydrocarbon receptor (AHR) target genes was altered. In TCDD-exposed embryos, AHR repressor a (*ahr1*) and *c-fos* promoters were differentially methylated. To characterize the TCDD effects on DNMTs, we cloned the *dnmt* promoters with xenobiotic response elements and conducted AHR transactivation assays using a luciferase reporter system. Our results suggest that *ahr2* can regulate *dnmt3a1*,

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dnmt3a2 and *dnmt3b2* expression. Overall, we demonstrate that developmental exposure to TCDD alters *dnmt* expression and DNA methylation patterns.

Keywords

TCDD; zebrafish development; dnmt; aryl hydrocarbon receptor; dioxin; DNA methylation

Introduction

In eukaryotes, DNA methylation is an important mechanism of epigenetic regulation of gene expression. DNA is methylated at cytosine residues within CG dinucleotides. Genomic regions containing a high frequency of CG dinucleotides are termed CpG islands (CGI) (Illingworth and Bird, 2009). Generally, CGIs lack DNA methylation and are associated with the proximal promoter regions of the majority of human genes (Suzuki and Bird, 2008). Methylated CGIs are heritably repressed, and DNA methylation has been considered as a marker for long-term transcriptional inactivation (Jaenisch and Bird, 2003; Suzuki and Bird, 2008). DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which are essential for establishment of DNA methylation patterns and for their maintenance during cell replication, thereby contributing to gene regulation (Goll and Bestor, 2005). During cell division, DNA methylation patterns are faithfully copied to daughter cells, maintaining stable gene expression patterns in subsequent cell lineages.

There are three enzymatically active DNMTs in mammals (DNMT1, DNMT3A and DNMT3B) (Bird, 2002). DNMT1 is a maintenance methyltransferase that ensures inheritance of methylation patterns during cell division by preferentially methylating hemimethylated CG dinucleotides (Goll and Bestor, 2005). DNMT3 enzymes are responsible for *de novo* methylation, establishing DNA methylation patterns in germ cells and in preimplantation embryos – generating cells with broad developmental potential (Reik *et al.*, 2001; Goll and Bestor, 2005; Chedin, 2011; Denis *et al.*, 2011).

Recent studies in mammals have demonstrated that exposure to environmental chemicals during critical windows of early development alters DNA methylation patterns leading to persistent changes in gene expression (Skinner, 2007; Skinner, 2011; Skinner, 2014b). It has been suggested that the DNA methylome is most susceptible to environmental chemicals during periods of epigenetic reprogramming (Skinner, 2014b; Skinner, 2014a). In vertebrates, reprogramming occurs during two different periods of development. It first occurs soon after fertilization, when parental DNA methylation patterns are erased and zygotic methylation patterns are established (Jirtle and Skinner, 2007; Faulk and Dolinoy, 2013). Second, it occurs during germ cell development. Before undergoing sex determination, the primordial germ cells undergo erasure and re-establishment of DNA methylation patterns in a sex-specific manner (Reik *et al.*, 2001; Gabory *et al.*, 2009). These two periods of epigenetic reprogramming are considered to be sensitive to environmental stressors, and any changes to the epigenome could result in transgenerational effects (Jirtle and Skinner, 2007). Even though persistent effects after developmental exposure to toxicants

are widely demonstrated, there is very little understanding of how chemicals alter DNA methylation patterns.

In this study, we hypothesized that environmental chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) affect DNA methylation patterns by altering the expression of *dnmts*. TCDD is a prototypical congener of a large group of halogenated polycyclic hydrocarbons, and its mode of action is well characterized. TCDD is a ligand for the aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix/Per-AHR nuclear translocator (ARNT)-Sim (bHLH-PAS) protein family of transcription factors that play important roles in toxicology and development (Okey, 2007). Upon activation by a ligand, AHR translocates to the nucleus and forms a heterodimer with ARNT, another bHLH-PAS protein. The AHR/ARNT heterodimer recognizes and binds to xenobiotic response elements (XREs) in the promoter regions of a variety of target genes. Zebrafish, a well-established developmental and toxicological model, possess multiple homologs of mammalian *DNMT3A* (*dnmt3a1* and *dnmt3a2*) and *DNMT3B* (*dnmt3b1*, *3b2*, *3b3* and *3b4*) genes due to a genome duplication event in the teleost fish lineage shortly after their divergence from the tetrapod lineage, as well as tandem gene duplications. There often is subfunction partitioning among the duplicated genes providing a unique opportunity for obtaining new mechanistic insights into the multiple functions of a single human gene, adding to the value of the zebrafish model. There is very little known about the expression and function of zebrafish *de novo* DNMTs during development, or tissue-specificity in adults (Goll and Halpern, 2011). Zebrafish express three AHR paralogs (AHR1a, AHR1b, AHR2), but AHR2 plays a major role in mediating the toxic developmental effects of TCDD in fish (Prasch *et al.*, 2003). The mechanism of action of TCDD in zebrafish has been well characterized (Carney *et al.*, 2006a; Carney *et al.*, 2006b). TCDD is also shown to cause transgenerational effects in zebrafish (Baker *et al.*, 2014a; Baker *et al.*, 2014b). More recently, zebrafish are being used to study the effect of toxicants including TCDD on DNA methylation and *dnmt* expression (Fang *et al.*, 2013a; Fang *et al.*, 2013b; Corrales *et al.*, 2014; Olsvik *et al.*, 2014).

In this study, we quantified the expression of *dnmts* during early development and in adult tissues. We determined the effect of developmental exposure to TCDD on the expression of *dnmt* genes and on global and gene-specific DNA methylation. We hypothesized that the XREs in the *dnmt* promoter regions might be involved in the TCDD effects on *dnmt* expression. We generated luciferase reporter constructs under the control of *dnmt* promoters, and characterized the role of AHR in TCDD-mediated regulation of *dnmts*.

Materials and Methods

Experimental animals

The wild-type TL (Tupfel/Long fin mutations) strain of zebrafish were used in this study. The zebrafish were maintained in 28°C system water with a 14-h light, 10-h dark cycle. The procedures used in this study were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution. Fertilized eggs were collected by placing egg collection traps in tanks (containing approximately 30 male and 30 female zebrafish) 30 minutes prior to the start of the light cycle, and removing the traps 30 minutes later.

Developmental profiling of *dnmts*

To determine the expression patterns of *dnmts* during early development, zebrafish embryos were sampled at distinct developmental stages: 0.2 hpf (1-cell), 1 hpf (4-cell), 1.5 hpf (16-cell), 3.3 hpf (high), 4 hpf (sphere), 4.7 hpf (30% epiboly), 6 hpf (shield), and 10 hpf (bud stage) (Westerfield, 2000). The exact developmental stage of the embryos was assessed by microscopic observation prior to sampling. Four biological replicates were collected for each developmental stage, and each replicate consisted of 20 embryos (n=20). Samples were flash-frozen in liquid nitrogen, and stored at -80°C until RNA and DNA was isolated.

Dnmt expression in adult tissues

Adult zebrafish were euthanized with MS-222 buffered with sodium bicarbonate before the dissection of the eye, brain, liver and gonads (ovaries). Tissues were snap frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

Effect of TCDD on *dnmt* gene expression

Zebrafish embryos were exposed to either TCDD (5 nM final concentration) or carrier control (dimethyl sulfoxide (DMSO), 0.01% final concentration) for one hour starting at 4 hpf. We have previously determined that one hour exposure to TCDD concentrations between 0.5-10 nM activate AHR (Jenny *et al.*, 2009). Five nanomolar TCDD was chosen since it does not cause excessive toxicity, but still produces a robust response. After the exposure, the embryos were rinsed thoroughly with fresh system water and incubated at 28°C . Embryos were sampled at five developmental time points (12 hpf, 24 hpf, 48 hpf, 72 hpf, and 96 hpf) to determine the effects of TCDD on the expression of *dnmts*. At each time point, RNA and DNA were isolated from a separate set of four biological replicates. Each replicate consisted of 30 embryos (n=30). This experiment was repeated twice to ensure the consistency of the results.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using the RNA STAT60 method (Tel-Test Inc., TX). Complementary DNA was synthesized from 1 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, CA). Quantitative PCR was performed with iQ SYBR Green Supermix in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, CA). Real-time PCR primers are listed in Supplementary Table 1. The PCR conditions used were 95°C for 3 min (1 cycle) and 95°C for 15 s/ 65°C for 1 min (40 cycles). At the end of each PCR run, a melt curve analysis was performed to ensure that only a single product was amplified. Two technical replicates were used for each sample. A no-template control was included on each plate to ensure the absence of background contamination. Relative expression was normalized to that of beta-actin (*actb*) or elongation factor alpha (*ef1a*) ($2^{-\text{Ct}}$; where $\text{Ct} = [\text{Ct}(\textit{dnmt}) - \text{Ct}(\textit{actb} \text{ or } \textit{ef1a})]$). *Actb* was used to normalize the *dnmt* gene expression in embryos, and *ef1a* was used for adult tissues. The expression data for *actb* and *ef1a* are shown in Supplementary Figure S1. Two-way ANOVA was used to determine the effect of TCDD and developmental stage on *dnmt* mRNA expression levels (GraphPad Prism version 5.3). A probability level of $p < 0.05$ was considered statistically significant.

Developmental expression profiles of dnmts from RNAseq data

Zebrafish embryo RNA samples from the 1.25 hpf (8-cell cleavage stage), 4 hpf (sphere), 6 hpf (shield), 8 hpf (75% epiboly), 10 hpf (bud) and 24 hpf (prim-5) stages were sequenced using the Illumina platform. Two biological replicates were sequenced for each of these samples, except for the 8-cell cleavage stage, which was sequenced in triplicate. Data analysis was done using the Tophat-Cufflinks-Cuffdiff pipeline following default parameters (Trapnell *et al.*, 2013). The sequencing reads were mapped to the zebrafish genome version Zv9 (<http://useast.ensembl.org/info/data/ftp/index.html>). The number of raw reads and the percentage of reads that aligned to the genome are shown in Supplementary Table 2. Fragments per kilobase of transcript per million mapped reads (FPKM) of different *dnmt* genes were obtained using the cummeRbund package. Integrative Genomics Viewer (IGV version 2.3) was used to visualize developmental patterns of *dnmt* gene expression (Thorvaldsdottir *et al.*, 2013).

Genomic DNA isolation and bisulfite conversion of DNA

Genomic DNA was isolated from the embryos using the proteinase K digestion method, followed by phenol-chloroform extraction. The concentration of DNA was determined using a NanoDrop Spectrometer and A260/280 ratios were between 1.9-2.1. The quality of the DNA was confirmed by running 0.5 µg of DNA on a 0.8% agarose gel and visualizing with ethidium bromide stain under UV light.

Bisulfite conversion of DNA was done using the EZ methylation kit (Zymo Research, CA). Briefly, 1 µg of genomic DNA was denatured by the addition of dilution buffer and incubation at 37°C for 15 minutes. Following denaturation, 100 µL of CT conversion reagent was added to the DNA and incubated at 50°C for 15 hours in the dark for bisulfite conversion. Bisulfite-converted DNA (BS-DNA) was purified using spin columns and eluted from the column matrix in a total volume of 10 µL. BS-DNA was stored at -20°C for later use.

Quantification of global DNA methylation and hydroxymethylation levels

Global cytosine methylation and hydroxymethylation levels were determined using established HPLC protocols (Ramsahoye, 2002) with some modifications. Briefly, genomic DNA was hydrolyzed with a combination of RNase A and RNase T1 followed by ethanol precipitation. DNA was digested to nucleosides as described previously (Hashimoto *et al.*, 2014) and separated on a 6490 Triple Quad LC-MS with UV absorbance detector (1290 Infinity UV detector, 6490 Triple Quad Mass detector, Agilent, Santa Clara, CA) equipped with an XSelect™ HSS T3 column (2.1 × 100mm, 2.5 µm, Waters, Milford, MA).

Identification of CpG islands for DNA methylation profiling

We used the UCSC genome browser CpG Tracks to identify CpG islands in the promoter regions of *ahr2*, *ahr2a* and *c-fos* (v-fos FBJ murine osteosarcoma viral oncogene homolog Ab) genes. *Ahr2* and *ahr2a* were chosen because of their role in TCDD-induced toxicity, as well as being AHR-target genes, and *c-fos* was selected based on our previous observation that it is persistently upregulated (2.3 fold compared to DMSO-exposed controls) after

developmental exposure to PCB126, another potent AHR ligand (unpublished). We confirmed that these CpG islands meet the criteria set by Takai and Jones (2002) by analyzing these sequences using the CpG Island Searcher (<http://cpgislands.usc.edu>). This algorithm defines CpG islands as being longer than 500 bp and having a GC content greater than 55% and an [observed CpG]/[expected CpG] ratio > 0.65 (Takai and Jones, 2002).

Bisulfite PCR (BS-PCR)

Methylation analysis of CpG islands was performed by BS-PCR. A 50 µl PCR reaction was carried out in 1X PCR buffer, 5 mM MgCl₂, 1 mM dNTP mix, 1 unit of Taq polymerase, 50 pmol each of the forward and reverse primers and ~50 ng of bisulfite-treated genomic DNA. BS-PCR primers were designed using the sense strand of the bisulfite-converted DNA for *ahr2* and *ahr2a* and anti-sense strand for *c-fos*. The primer sequences are provided in Supplementary Table 1. PCR cycling conditions were 94°C for 10 min, followed by 40 cycles of [94°C for 30 s, 55°C for 30 s and 72°C for 30 s], and a final cycle of 72°C for 8 min. PCR products were electrophoresed on 1% agarose gels, bands excised and gel-extracted using the Gene Clean II kit (MP Biomedical, CA). Purified PCR products were cloned using the pGEM-Teasy cloning kit (Promega, MI) as per the manufacturer's protocol. Mini-preps were prepared using the Pure Yield plasmid miniprep kit (Promega, MI). For each sample, a minimum of 5 clones were sequenced. BS-PCR together with sequencing of several clones provides allele-specific methylation profiles.

Functional analysis of XREs in *dnmt* promoters

In order to explore further the TCDD-inducible expression of DNMTs, we tested the ability of AHR2 in transactivating *dnmt* gene promoters. The DNA sequence of approximately 2 kb promoter region for each *dnmt* gene was retrieved from the zebrafish Ensembl genome browser, and searched for the presence of XREs (core sequence GCGTG). XREs were found in all *dnmt* genes, except *dnmt1* and *dnmt3b4*. We amplified the promoter regions of *dnmts* that contained XREs using primers designed with *KpnI* and *BglII* restriction enzyme sites in forward and reverse primers, respectively. The primer sequences are provided in Supplementary Table 1. The amplified products were cut with *KpnI* and *BglII* and ligated into pGL3.1 basic vector (Promega) to create the pGL3-*dnmt* promoter constructs. The promoter constructs were sequenced to ensure the accuracy of cloning.

Transient transfections were performed as described in Evans et al. (2008). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, MO) supplemented with fetal calf serum (10% final concentration) at 37°C under 5% CO₂. Cells were plated at 3×10^4 cells/well in 48-well plates. pGudLuc6.1 (DRE-luc), a luciferase reporter construct regulated by four XREs from the mouse CYP1A1 promoter was used as a positive control. *Renilla* luciferase (pGL4.74; Promega) was used as the transfection control. Cells were transfected with 50 ng of each pGL-dnmt3 promoter construct along with expression constructs for AHR2 (5 ng), ARNT2 (25 ng), pGudLuc6.1 (50 ng), and pGL4.74 (3 ng). Transfection of DNA with the X-tremeGENE HP DNA transfection reagent (Roche, IN) were carried out in triplicate wells 24 h after plating. The ratio of transfection reagent to DNA was 1:1. The total amount of DNA was kept constant by the addition of empty pcDNA3.1 (Invitrogen, Carlsbad, CA) vector. Six hours post-transfection, cells were

exposed to 0.01% DMSO or TCDD (10 nM final concentration). Cells were lysed 18 h after dosing, and luminescence was measured using the Dual Luciferase Assay kit (Promega) in a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). The final values were expressed as a ratio of firefly luciferase units to *Renilla* luciferase units.

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism 5.3 software package (GraphPad Software Inc., San Diego, CA). To analyze the developmental expression of *dnmts*, one-way ANOVA followed by Tukey post-hoc test was performed. Two-way ANOVA was used to determine the effect of TCDD and time on *dnmt* mRNA expression levels, and a Tukey's range test was used to detect statistical significance. Paired t-test was used to determine the effect of TCDD on *ahr2*, *ahr2a* and *c-fos* gene expression as well as in transient transfection assays. P-values of less than or equal to 0.05 were considered statistically significant.

Results

Dnmt gene expression during zebrafish development

There is some ambiguity in the nomenclature of zebrafish DNMTs in various publicly available databases (Supplementary Table 3). One recent study has proposed a new nomenclature based on molecular evolution and phylogenetic analysis of these orthologs (Campos *et al.*, 2012). We concur with their classification and followed their nomenclature in this study.

Zebrafish *dnmt* gene expression was measured during the first 10 hours post fertilization (hpf), which revealed that all *dnmts*, except *dnmt3a1*, are expressed during this period (Fig. 1). We detected high levels of *dnmt1* mRNA from 0.2 hpf (1-cell) to 4 hpf (sphere stage), suggesting maternal deposition of these transcripts in the embryos (Fig. 1A). After 4 hpf, *dnmt1* levels drop significantly, and relatively low expression levels were observed for the rest of the developmental stages investigated (Fig. 4A). These results were further confirmed by RNAseq where significantly high FPKM values were observed in 1.25 hpf (8-cell stage) embryos compared to later time points (Fig. 2A).

In contrast to *dnmt1*, *dnmt3* genes are expressed at relatively low levels during early development. Using qRT-PCR we were unable to detect *dnmt3a1* expression up to 10 hpf (bud stage). The expression increased by 24 hpf and was relatively stable through 96 hpf (Fig. 4B). RNAseq analysis confirmed very low levels of expression (FPKM values below 1) in the first 10 hours of development, with a slight increase by 24 hpf (Fig. 2B). *Dnmt3a2* expression was observed at all developmental stages (Fig. 1C), with an increase at 24 hpf (Fig. 2C and 4C).

Among the four *dnmt3b* genes, *dnmt3b1*, *3b2* and *3b3* showed relatively low expression in earlier stages following fertilization, and the levels gradually increased by 10hpf (bud stage) (Fig. 1C,D and E). In contrast, *dnmt3b4* had the highest level of expression from 0.2 hpf (1-cell) through 3.3 hpf (high) stages (Fig. 1F). The expression levels of all *dnmt3b* genes gradually decreased by 24 hpf (Fig. 2D-G), and maintained a very low expression level

during 48-96 hpf (Fig. 4D-G). In contrast to qRT-PCR, RNAseq data allowed comparison of *dnmt* expression levels to each other, which showed that *dnmt3a1* and *dnmt3a2* were expressed at lower levels in comparison to other *dnmts* during the first 24 hours of zebrafish development (Fig. 2A-C). *Dnmt3b3* had the highest expression among the other *dnmt3* genes (Fig. 2F).

Dnmt gene expression in adult tissues

In adults, *dnmt1* was most highly expressed in the ovary, followed by the liver (Fig. 3A). In contrast, *dnmt3a* genes were highly expressed in the brain (Fig. 3B-C). All four *dnmt3b* genes showed highest expression in the ovary compared to other tissues (Fig. 3D-G).

Effect of TCDD on dnmt gene expression

TCDD exposure caused significant upregulation of *dnmt1* expression at 12 and 24 hpf. At later time points, there was no significant effect of TCDD on *dnmt1* expression (Fig. 4A). TCDD exposure selectively altered the expression of *dnmt3* genes. At 24, 48 and 96 hpf, TCDD exposure downregulated the expression of *dnmt3a1* (Fig. 4B), but had no significant effect on the expression of *dnmt3a2* (Fig. 4C). Among the four *dnmt3b* genes, TCDD exposure downregulated the expression of *dnmt3b1* and *dnmt3b4* at 24 and 12 hpf, respectively, whereas it upregulated *dnmt3b2* expression at 12 hpf (Fig. 4D, E, G). There was no effect of TCDD on *dnmt3b3* expression (Fig. 4F). We observed classical TCDD-induced phenotypes that included elongated heart, pericardial, and yolk sac edema in a small proportion of embryos between 72 and 96 hpf.

Effect of TCDD on global and gene-specific DNA methylation levels

TCDD did not affect 5-methylcytosine or hydroxymethylcytosine levels at any of the time points investigated. Methylcytosine levels showed an increasing trend during development, but the changes were not statistically significant (Fig. 5A). However, hydroxymethylcytosine levels showed significant increases during development, with maximum levels observed at 96 hpf (Fig. 5B).

Among the AHR-target genes, TCDD treatment did not affect the methylation of the CpG island in the *ahr2* promoter, where only one out of 24 CpG sites in the CpG island was hypomethylated (Fig. 6A). However, TCDD exposure hypomethylated the CpG island in the *c-fos* promoter, and hypermethylated the *ahrra* promoter. In the *c-fos* promoter, 11 out of 22 CpG dinucleotides were hypomethylated, and 14 out of 34 CpG sites were hypermethylated in the *ahrra* promoter (Fig. 6B, C). The methylation patterns in the *c-fos* and *ahrra* promoters were significantly altered by TCDD exposure (Fig. 6D). All three genes were induced in response to TCDD. The fold-induction values were 3, 2.5, and 5-fold, for *ahr2*, *c-fos*, and *ahrra*, respectively (Fig. 6E-G).

Functional analysis of XREs in dnmt genes

Among the five *dnmt* promoters tested, three of them caused transactivation of the luciferase reporter by AHR2 and ARNT2 in the presence of TCDD. Cells transfected with *dnmt3a1*, *3a2* and *3b2* promoter constructs resulted in 5, 6, and 4-fold induction of luciferase activity in the TCDD-treated cells, respectively (Fig. 7). The positive control transfection of AHR2,

ARNT2, and pGudLuc6.1 resulted in 13-fold induction of luciferase in TCDD-treated cells over the DMSO control.

Discussion

In this study we performed a detailed analysis of zebrafish *dnmt* gene expression throughout embryonic development and in selected adult tissues. We demonstrated that TCDD exposure during early development alters *dnmt* gene expression, and these effects are developmental stage-specific. TCDD exposure resulted in changes in methylation levels in the promoters of AHR target genes, *c-fos* and *ahr*. In luciferase reporter assays with *dnmt3* promoters, we observed that AHR could regulate *dnmt3a1*, *3a2* and *3b2* expression.

Dnmt expression during early development

Our results indicate that zebrafish *dnmt* genes exhibit unique expression patterns during embryonic development, which concur with previous studies where spatio-temporal expression patterns were analyzed using RT-PCR and whole mount *in situ* hybridization (Smith *et al.*, 2005; Rai *et al.*, 2006; Rai *et al.*, 2010; Smith *et al.*, 2011; Campos *et al.*, 2012; Takayama *et al.*, 2014). We observed high levels of *dnmt1* expression in early stage embryos soon after fertilization, suggesting that *dnmt1* is a maternally stored mRNA and is essential for directing early embryonic development prior to activation of the zygotic genome. As a maintenance methyltransferase, *dnmt1* is essential for maintaining the methylation of transposable elements during early embryogenesis in mice, thereby suppressing potential retrotransposition (Hashimshony *et al.*, 2003; Gaudet *et al.*, 2004). This is consistent with the findings that *DNMT1*-knockout mice show delayed development and fail to survive past mid-gestation, possibly due to aberrant expression of imprinted genes and chromosomal instability (Li *et al.*, 1992; Brown and Robertson, 2007). Similarly, morpholino knockdown of *dnmt1* in zebrafish caused increased mortality during gastrulation. Any surviving embryos showed organ-specific developmental effects such as defects in differentiation of the retina, exocrine pancreas, and intestine (Rai *et al.*, 2006). These results suggest that maternal *dnmt1* transcripts protect early embryo viability, organ specification, and differentiation.

In mammals, *DNMT3B* is highly expressed in early stages of development, whereas *DNMT3A* is more prevalent in later stages (Okano *et al.*, 1999; Watanabe *et al.*, 2004; Watanabe *et al.*, 2006; Hirasawa and Sasaki, 2009). Our results demonstrate similar patterns of expression in zebrafish, suggesting distinct roles for *dnmt3a* and *3b* genes during development. We were unable to detect *dnmt3a1* expression in early stage embryos using qRT-PCR, and consequently, RNAseq data revealed very low expression (FPKM values less than 1 upto 10 hpf). *Dnmt3a2* had low levels of expression during the first 10 hpf and the levels rose significantly by 24 hpf. Using *in situ* hybridization, Takayama et al (Takayama *et al.*, 2014) have shown tissue-specific expression of *dnmt3a* genes during later stages of development (72 hpf) in zebrafish, suggesting a role for these genes in tissue differentiation. We observed high levels of *dnmt3b* gene expression during early development and the expression gradually decreased by 24 hpf. Similar results have been observed in previous studies (Shimoda *et al.*, 2005; Rai *et al.*, 2010; Takayama *et al.*, 2014), which reported

localization of these transcripts to specific regions of the developing zebrafish. For instance, *dnmt3b1* is localized to aorta-gonad-mesonephros and caudal hematopoietic tissue regions (Takayama *et al.*, 2014) and *dnmt3b2* is localized to the brain, particularly in the tectum (Shimoda *et al.*, 2005), suggesting important roles in hematopoietic stem cell (HSC) differentiation and neurogenesis, respectively. Interestingly, *dnmt3a* genes are highly expressed in the adult brain, as opposed to *dnmt3b* genes whose expression is highest in the ovaries, suggesting distinct functions for these orthologs. It remains to be determined what the specific roles of these genes are in establishing *de novo* methylation patterns.

Corresponding to the changes in the expression of these genes, we also observed a moderate increase in global DNA methylation during development. Recent studies have characterized the DNA methylation profiles in the early embryos (up to 10 hpf) using whole genome bisulfite sequencing, and these results suggest that the embryonic genome is rapidly methylated soon after the midblastula transition (MBT) (Jiang *et al.*, 2013; Potok *et al.*, 2013; McGaughey *et al.*, 2014). Similar studies need to be conducted in embryos after knocking down individual *de novo* *dnmts* or in knockout animals in order to determine the role of these genes in *de novo* DNA methylation. 5-hydroxymethylcytosine (5hmC) is another important epigenetic modification of DNA that is shown to play an important role in gene regulation. 5hmC is an intermediate product of active DNA demethylation, where methylcytosine (5mC) is converted to 5hmC, and is catalyzed by the Ten Eleven Translocation (TET) family of enzymes (Coppieters *et al.*, 2014; Wen and Tang, 2014). We observed increasing levels of 5hmC during development with significantly higher levels at 96 hpf compared to 24 hpf. The physiological role of 5hmC in zebrafish development is unclear, but recent studies suggest that it is involved in several cellular processes and emerges as an important player in brain development (Kato and Iwamoto, 2014; Rudenko and Tsai, 2014).

Effect of TCDD on *dnmt* expression patterns

Recent studies in mammals (Wu *et al.*, 2004; Okino *et al.*, 2006; McClure *et al.*, 2011; Singh *et al.*, 2011; Manikkam *et al.*, 2012a; Manikkam *et al.*, 2012b; Takeda *et al.*, 2012; Somm *et al.*, 2013; Takeda *et al.*, 2014) and zebrafish (Olsvik, *et al.*, 2014) have demonstrated that exposure to TCDD alters DNA methylation patterns not only in the exposed generation, but also in subsequent generations. However, the mechanism of action by which toxicants affect DNA methylation is unknown. We hypothesized that toxicant-induced changes in DNA methylation patterns are due to altered expression of *dnmts*. There are very few studies that have investigated the effect of toxicants on *dnmt* expression (Desaulniers *et al.*, 2009; Fang *et al.*, 2013b; Kundakovic *et al.*, 2013; Huumonen *et al.*, 2014). In rodents, reduced expression of *dnmt* genes was observed in prepubertal livers in response to polychlorinated biphenyl (PCB) mixture exposure (Desaulniers *et al.*, 2009), whereas TCDD did not show any effect on *dnmt* gene expression in murine embryonal fibroblasts (Huumonen *et al.*, 2014). We observed TCDD-induced upregulation of *dnmt1* and *dnmt3b2* expression, and downregulation of *dnmt3a1*, *dnmt3b1* and *dnmt3b4* genes at certain developmental time points. Developmental stage-specific effects on *dnmt1* expression were also observed in zebrafish embryos exposed to benzo[a]pyrene (BaP) (Fang *et al.*, 2013b). It is unclear why TCDD and benzo[a]pyrene effects occur only at specific

time points during development, but one potential reason could be the spatial and temporal differences in *dnmt*, *ahr*, and *ahrra* expression in the developing embryos (Andreasen *et al.*, 2002; Seritrakul and Gross, 2014; Takayama *et al.*, 2014). It remains to be determined whether *dnmts*, *ahr*, and its regulators are co-localized in developing tissues. Further studies using *in situ* hybridization are necessary to determine the effect of TCDD on *dnmt* expression in developing tissues.

TCDD effects on *dnmt* gene expression observed in this study suggest that both establishment and maintenance of methylation patterns could potentially be impacted. Establishment of genomic methylation patterns is accomplished by the concerted action of *de novo* and maintenance methyltransferases. In zebrafish embryos, DNA methylation patterns are established during the midblastula transition, coinciding with the activation of zygotic transcription (Jiang *et al.*, 2013; Potok *et al.*, 2013). *Dnmt3* genes, expressed in early embryos, have been shown to methylate unmodified DNA *de novo*, resulting in hemi-methylated DNA (Lyko *et al.*, 1999; Okano *et al.*, 1999). *Dnmt1*, in turn, recognizes the hemi-methylated CpG sites and methylates the complementary CpG. Exposure to toxicants during this critical developmental period is shown to affect epigenetic processes essential for normal development (Faulk and Dolinoy, 2013).

We did not see any significant changes in global cytosine and hydroxymethylation levels in TCDD exposed embryos as measured by HPLC. However, the proximal promoters of two of three AHR target genes we examined revealed alterations in their methylation patterns in response to TCDD exposure. AHRR is an important negative regulator of AHR function (Haarmann-Stemmann *et al.*, 2007; Hahn *et al.*, 2009). Zebrafish possess two AHRR paralogs, both of which are AHR target genes. AHRRa regulates constitutive AHR signaling during development, whereas AHRRb regulates polycyclic aromatic hydrocarbon-induced gene expression (Jenny *et al.*, 2009). We observed hypermethylation of the *ahrra* promoter in TCDD exposed embryos. Epidemiological studies in humans have demonstrated a strong correlation between exposure to cigarette smoke, which contains AHR ligands, and hypomethylation of two distinct CpG sites in the AHRR loci (Breitling *et al.*, 2011; Monick *et al.*, 2012; Philibert *et al.*, 2012; Philibert *et al.*, 2013). However, there are no experimental studies investigating the effect of PCBs or TCDD on AHRR methylation. The zebrafish *ahr2* gene itself exhibits a modest but consistent induction by exposure to AHR ligands (Andreasen *et al.*, 2002; Hahn *et al.*, 2014), suggesting that it may regulate its own expression. However, we did not observe a direct effect of TCDD on the methylation pattern of the *ahr2* promoter within the proximal region tested. *C-fos* is a proto-oncogene and is one of the immediate early response genes induced in response to TCDD exposure (Hoffer *et al.*, 1996; Lensu *et al.*, 2006; Tang *et al.*, 2008). *C-fos* was found to be hypomethylated in zebrafish embryos exposed to BaP at 3.3 hpf and 96 hpf (Corrales *et al.*, 2014). We also observed hypomethylation of the *c-fos* promoter region in TCDD-exposed embryos. Irrespective of the TCDD effect on the methylation patterns, mRNA levels were induced as shown in previous studies (Hoffer *et al.*, 1996; Andreasen *et al.*, 2002; Jenny *et al.*, 2009; Hahn *et al.*, 2014), suggesting that gene expression is regulated by many factors including proximal promoter DNA methylation.

Overall, these studies demonstrate that TCDD alters *dnmt* expression and gene-specific DNA methylation patterns. *In vitro* functional analysis of XREs in *dnmt3* promoters suggests that *ahr2* can regulate *dnmt3a1*, *3a2* and *3b2* expression. However, these results do not completely concur with the *in vivo* gene expression patterns. The reduced expression of *dnmts* in response to TCDD exposure could be regulated by negative response elements that are located outside the 2kb proximal promoter. Therefore, we may not have reproduced the full *in vivo* expression pattern in the reporter assay. On the other hand, TCDD-induced expression of *dnmt3b2* was captured by reporter gene expression under the control of *dnmt3b2* proximal promoter.

Further studies need to be conducted to determine the effects of TCDD and other toxicants on genome-wide DNA methylation patterns. Recent advances in sequencing technologies have enabled characterization of genome-wide changes using whole genome methylation profiling or reduced representation bisulfite sequencing (RRBS) of CpG rich regions of the genome (Chatterjee *et al.*, 2013; Jiang *et al.*, 2013; Potok *et al.*, 2013; McGaughey *et al.*, 2014). These methods allow us to explore the effects of toxicants not only in CpG islands, but also in gene bodies and CpG shores (Portela and Esteller, 2010; Kulis *et al.*, 2013; Edgar *et al.*, 2014). Furthermore, mechanistic studies need to be conducted in order to delineate the molecular mechanisms associated with altered *dnmt* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- TCDD altered the *dnmt* expression in a gene and developmental time-specific manner.
- TCDD hypermethylated *ahrra* and hypomethylated *c-fos* proximal promoter regions.
- Functional analysis suggests that *ahr2* can regulate *dnmt3a1*, *3a2* and *3b2* expression.
- *Dnmt3b* genes are expressed early whereas *dnmt3a* are abundant later in development.

Figure 1. *Dnmt* expression profiles during early zebrafish development measured using quantitative real-time PCR

Beta-actin (*actb*) was used as an internal standard. The delta Ct method (2^{-Ct} ; $Ct = (dnmt - actb)$) was used to calculate the relative expression of mRNA. We were unable to detect *dnmt3a1* expression at these developmental time points. All values represent mean + standard error of mean (S.E.M; n = 4).

Figure 2. *Dnmt* expression profiles as determined by RNA-sequencing

Normalized Fragments per Kilobase per Million Reads (FPKM) for each *dnmt* gene are plotted at each time point. All values represent mean + standard error of mean (S.E.M; n = 2-3).

Figure 3. *Dnmt* expression in adult tissues measured using quantitative real-time PCR
Elongation Factor 1-alpha (*ef1a*) was used as an internal standard. The delta Ct method (2^{-Ct} ; $Ct = (dnmt-ef1a)$) was used to calculate the relative expression of mRNA. All values represent mean + standard error of mean (S.E.M; n = 4).

Figure 4. Effect of TCDD on *dnmt* expression

Beta-actin was used as an internal standard. All values represent mean + standard error of mean (S.E.M; n = 4). The delta Ct method (2^{-Ct} ; $Ct = (dnmt-actb)$) was used to determine the relative expression of mRNA. (Two-way ANOVA; n=4). Two-way ANOVA was used to determine the effect of treatment and time on *dnmt* expression. Tukey post-hoc test was used for determining statistical significance. * denotes statistical significance from the control group at that particular time point (p<0.05).

Figure 5. Global DNA methylation and hydroxymethylation patterns in TCDD-exposed embryos measured using the high performance liquid chromatography method

All values represent mean + standard error of mean (S.E.M; n = 4). Two-way ANOVA was used to determine the effect of treatment and time on 5-methylcytosine (A) or hydroxymethylcytosine (B) levels. Tukey post-hoc test was used for determining statistical significance ($p < 0.05$). Asterisks denote effect of time on global hydroxymethylation levels. No TCDD effect was observed.

Figure 6. Effect of TCDD on gene-specific DNA methylation patterns

Lollipop diagram showing the (A) *ahr2*, (B) *c-fos* and (C) *ahrra* promoter methylation profiles of each CpG dinucleotide. Filled and open circles represent methylated sites and unmethylated sites, respectively. Methylation profiles were determined in DMSO and TCDD-exposed 96 hpf larvae. Five clones were sequenced from each gene. (D) Bar diagram showing DNA methylation changes as percent methylation. It is calculated by dividing the number of methylated and unmethylated CpG dinucleotides by total number of CpG sites investigated. (E-G) Quantitative RT-PCR of *ahr2*, *c-fos* and *ahrra* gene expression. Beta-actin was used as an internal standard. All values represent mean + standard error of mean (S.E.M; n = 4). The delta Ct method ($2^{-\Delta Ct}$; $\Delta Ct = (GOI-actb)$; GOI- gene of interest) was

used to determine the relative expression of *ahr2*, *c-fos* and *ahr2a* mRNA. * denotes statistical significance from the DMSO group for that particular gene (paired t-test; $p < 0.05$).

Figure 7. Functional analysis of XREs in *dnmt* gene promoter regions
COS-7 cells were transfected with the constructs shown, and exposed to DMSO or TCDD (10 nM). Luciferase activity was measured and normalized to the transfection control *Renilla* luciferase. Results are representative of two independent experiments. * denotes statistical significance compared to the DMSO-exposed group (paired t-test; $p<0.05$).