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Dioxin disrupts cranial cartilage and dermal bone development in zebrafish larvae

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD or dioxin) disrupts craniofacial development in zebrafish larvae. However, the cellular changes responsible for the decreased jaw size remain poorly understood. We show that smaller jaw size is due to a decrease in both the size and number of chondrocytes in the developing craniofacial cartilages. TCDD was found to decrease ossification of osteoblasts in the perichondrium of craniofacial cartilages. We also discovered that TCDD caused clefting of the parasphenoid, an effect with similarity to TCDD-induced cleft palate in mice. Thus, dermal and perichondrial bone development of the craniofacial skeleton are clearly disrupted by TCDD exposure in the zebrafish larvae. This dysmorphic response of the zebrafish craniofacial skeleton after exposure to TCDD is consistent with findings demonstrating disruption of axial bone development in medaka and repression of *sox9b* in zebrafish.

1. Introduction

Craniofacial malformations are a hallmark of TCDD developmental toxicity in fish, birds, and mammals (Couture et al., 1990; Henry et al., 1997; Thomae et al., 2005; Yeager et al., 2006). Mammals and other vertebrates often develop a cleft palate when exposed early in development to TCDD (Couture et al., 1990; Thomae et al., 2005; Yeager et al., 2006). Teleost fish are among the most sensitive species to TCDD toxicity, and exhibit a more pronounced response. TCDD exposure produces decreased jaw size and other craniofacial malformations in developing lake trout sac fry, and zebrafish embryos (Cook et al., 2003; Henry et al., 1997 Henry et al., 1997). In zebrafish, these malformations can be prevented by mutations in, or knockdown of, the aryl hydrocarbon receptor 2 (Ahr2) (Prasch et al., 2003).

Normal craniofacial development in zebrafish involves numerous signaling pathways. Originating in the pharyngeal endoderm, Runx3, Egr1, and Sox9b form a regulatory cascade that represses follistatin A. This cascade promotes growth and proliferation of cranial neural crest cell (cNCC)-derived chondrocytes (Dalcq et al., 2012). In normal development, the ceratohyal cartilage grows by a process called convergent extension whereby immature chondrocytes at the medial epiphysis of the ceratohyal cartilage comprise a population of cells that divide, intercalate, and elongate the cartilage (Shwartz et al., 2012).

As indicated above, the Sox9b transcription factor is involved in the normal growth and development of craniofacial cartilages in zebrafish (Yan et al., 2005). We know that TCDD repression of *sox9b* is a key element in producing craniofacial malformations (Xiong et al., 2008). We have recently constructed a *sox9b:EGFP* reporter fish, Tg(-2421/+29*sox9b*:*EGFP*) that aids the investigation of jaw development and understanding the effects of TCDD (Plavicki et al., 2014; Hofsteen et al., 2013)

Although fish have been shown to have a decreased jaw size after exposure to TCDD, the cellular cause of this is unknown. In this study we sought to determine whether the reduction in jaw size was due to reduced cell size, or number, and what specific cell types

Abbreviations: TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; sox9b, SRY [sex determining region Y]-box 9b; Ahr, aryl hydrocarbon receptor; cNCC, cranial neural crest cell; EGFP, enhanced green fluorescent protein.

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in the jaw were affected. Since medaka ossification markers are decreased after TCDD exposure in the cell-rich hyaline cartilage of the hypural plate (Dong et al., 2012), we expected to see a decrease in perichondrial ossification of craniofacial cartilages.

TCDD has been shown to cause cleft palate in mice and beak deformities in birds, yet little is known about potential effects of embryonic exposure to TCDD on the developing medial craniofacial dermal bones in fish. Therefore, we were also interested in determining whether the zebrafish could be used as a model for palate clefting caused by TCDD exposure. We focused on effects of TCDD on development of the parasphenoid, a dermal bone lying in a position similar to the palate of mammals (Swartz et al., 2011).

Here we report that TCDD decreased the size of the developing craniofacial cartilages by reducing both size, and number of chondrocytes within the cartilages. We also demonstrate that TCDD-induced craniofacial malformation in zebrafish includes reduced ossification of chondrocytes as they begin to form bone, and clefting of the parasphenoid.

2. Materials and methods

2.1. Zebrafish

All fish were from the AB background. Zebrafish of an AB background expressing the transgene, Tg(−2421/+29*sox9b*:*EGFP*), were used in the perichondrium marker and proliferation assays. Adults were housed in 38 L glass aquaria with recirculating reverse osmosis water lightly buffered with Instant Ocean Sea Salts (60 mg/L; Aquarium Systems, Mentor, OH) and maintained at 27 °C. Embryos were housed in plastic plates with methylene blue added to the water (50 μM). Larvae were anesthetized with 0.8 mM tricaine (MS 222, Sigma) buffered to pH 7 in Tris for *in vivo* staining. Zebrafish were euthanized by tricaine overdose. Adhering to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, all procedures involving zebrafish were approved by the Animal Care and Use Committee of the University of Wisconsin–Madison.

2.2. TCDD exposure of zebrafish embryos

Embryos were exposed to waterborne TCDD (>99% purity; Chemsyn, Lenexa, KS) at 1 ng (3.1 nM)/ml or vehicle (DMSO 0.1%) for 1 h in glass scintillation vials at 10 embryos per ml with gentle rocking (Antkiewicz et al., 2005). Each treatment group was considered an $n = 1$.

2.3. Staining cartilage and bone

Zebrafish cartilage was stained with alcian blue 8GX (Sigma–Aldrich, St. Louis, MO) as previously described (Walker and Kimmel, 2007). Briefly, embryos were anesthetized with Tricaine-S and fixed overnight in 4% paraformaldehyde (USB Corporation, Cleveland, OH). Larvae were dehydrated with graduated concentrations of ethanol, and stained with 0.02% Alcian blue in 70% ethanol with 200 mM $MgCl_2$ in water overnight.

Alizarin red (A-5533; Sigma–Aldrich, St. Louis, MO) was used to stain developing bone within the cartilages. For ossification staining, the cartilage stain was supplemented with a final concentration of 0.007% Alizarin red.

In all cases the stains were neutralized with saturated sodium tetraborate and the embryos were digested with 1 mg trypsin/ml (T4799, Sigma). Stains were cleared with graded concentrations of glycerol in KOH: 20% glycerol/0.25% KOH; 50% glycerol/0.25% KOH; and 80% glycerol/0.1% KOH for storage.

For staining bone in live larvae, two methods were used. Zebrafish larvae, 144 hpf, were immersed in 0.2% calcein (C0875; Sigma) staining solution (adjusted to pH 7.2 with 0.5N NaOH) for 5 min, rinsed 5 times for 5 min with fish water, cleared 10 min in fresh fish water, and euthanized in a method described previously by Du et al. (2001). In a method adapted from DeLaurier et al. (2010), zebrafish larvae, 120 hpf, were transferred to egg water supplemented with 0.01 M HEPES and 0.006% Alizarin red overnight. At 144 hpf, they were rinsed 3 times in fish water (5 min/rinse), anesthetized, and imaged.

2.4. Immunohistochemistry

Euthanized zebrafish were fixed overnight at 4 °C with 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed with PBS, and bleached with 0.9% H_2O_2 and 1% KOH, protected from light with foil, and placed on a rocking table for about 1.25 h, or until clear and rinsed five times for five minutes with PBS.

Fixed samples were immunostained as described previously (Dong et al., 2007; Plavicki et al., 2013). Samples were blocked overnight at 4 °C with PBS containing 0.3% Triton (PT, X-100, Sigma–Aldrich, St. Louis, MO) and 4% bovine serum albumin (PBT). Samples were incubated with the primary antibody in PBT overnight at 4 °C.

The primary rabbit IgG antibody against a mitotic marker, p-histone H3 (Ser-10, Hendzel et al., 1997), was purchased from Santa Cruz Biotechnology, Inc. Dallas, TX (sc-8656) and used at a 1:200 PBT dilution. The secondary antibody for the chondrocyte proliferation study was the goat anti rabbit highly cross absorbed Alexa Fluor 488 (2° Alexa Fluor® 488, Invitrogen, Carlsbad, CA). The secondary antibody for the perichondrial proliferation assay was the goat anti rabbit highly cross absorbed Alexa Fluor 568 (2° Alexa Fluor® 568, Invitrogen). The primary mouse antibody against collagen type II was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (II-II6B3) and used at a 1:20 PBT dilution. The secondary antibody for collagen II was a goat anti mouse Alexa Fluor 633 highly cross absorbed (2° Alexa Fluor® 633, Invitrogen).

Primary antibodies were diluted in PBT and tissue samples were covered in this solution. Samples were rinsed in PT overnight at 4 °C. Secondary antibodies were diluted in PBT (1:200) and covered with this solution overnight at 4 °C. Embryos were placed in 300 nM DAPI (4',6-diamidino-2-phenylindole, 422,801, Biolegend, San Diego, CA) for more than 15 min or immediately mounted. Embryos were mounted in Vectashield with DAPI or Vectashield (Vector Laboratories, Burlingame, CA).

2.5. Imaging

Alcian blue and Alizarin red stains were imaged with a Zeiss Axioskop2 Plus microscope equipped with an Axiocam and differential/Nomarski interference contrast microscopy optics. Calcein stains were imaged with an Olympus DP72 digital camera on an Olympus S2 × 16 microscope with a GFP filter (excite, 470 nm; emit 525 nm). The proliferation assays and immunofluorescent stains were imaged with an Olympus Fluoview FV1000 confocal microscope. For both chondrocyte proliferation studies, about 70 optical sections at 1.5 μm intervals were collected, analyzed, and counted using FV10 ASW 1.7 Fluoview software with the appropriate filters for the secondary antibodies and EGFP expression in Tg(−2421/+29*sox9b*:*EGFP*) zebrafish. Fluorescent Alizarin red was imaged with an Olympus Fluoview FV1000 confocal microscope using a Texas Red filter (excite, 595 nm; emit, 615 nm).

2.6. Chondrocyte analyses

Chondrocyte length was determined by imaging and measuring the lengths of 5–10 chondrocytes per ceratohyal cartilage in Adobe Photoshop that spanned the entire width of the ceratohyal