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Aryl Hydrocarbon Receptor Activation Inhibits Regenerative Growth

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ABSTRACT

There is considerable literature supporting the conclusion that inappropriate activation of the aryl hydrocarbon receptor (AHR) alters cellular signaling. We have established previously that fin regeneration is specifically inhibited by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in adult zebrafish and have used this in vivo endpoint to evaluate interactions between AHR and growth-controlling pathways. Because there are experimental limitations in studying regeneration in adult animals, we have developed a larval model to evaluate the effect of AHR activation on tissue regeneration. Two-day-old zebrafish regenerate their amputated caudal fins within 3 days. Here, we demonstrate that TCDD specifically blocks regenerative growth in larvae. The AHR pathway in zebrafish is considerably more complex than in mammals, with at least three zebrafish AHR genes (zfAHR1a, zfAHR1b, and zfAHR2) and two ARNT genes (zfARNT1 and zfARNT2). Although it was presumed that the block in regeneration was mediated by AHR activation, it had not been experimentally demonstrated. Using antisense morpholinos and mutant fish lines, we report that zfAHR2 and zfARNT1 are the in vivo dimerization partners that are required for inhibition of regeneration by TCDD. Several pathways including fibroblast growth factor (FGF) signaling are essential for fin regeneration. Even though impaired FGF signaling and TCDD exposure both inhibit fin regeneration, their morphometric response is distinct, suggesting that the mechanisms of impairment are different. With the plethora of molecular and genetic techniques that can be applied to larval-stage embryos, this in vivo regeneration system can be further exploited to understand cross-talk between AHR and other signaling pathways.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a ubiquitous environmental contaminant, causes a wide variety of toxicities, including reproductive and developmental toxicity, immunotoxicity, cardiotoxicity, teratogenicity, and neurotoxicity at low exposure levels. TCDD elicits toxicity by acting as a ligand for the aryl hydrocarbon receptor (AHR) (Schmidt and Bradfield, 1996). The AHR pathway has been studied in various disparate vertebrates, including several species of fish. The AHR signal transduction pathway in fish species is akin to that of mammals, except fish possess at least two AHR genes and the mammals have only one. AHR isoforms are designated as AHR1 and AHR2 (Hahn et al., 1997). zfAHR1a, zfAHR1b, and zfAHR2 have been identified in zebrafish (Tanguay et al., 1999; Andreasen et al., 2002a; Karchner et al., 2005). In vivo antisense knockdown studies in zebrafish embryos have established that zfAHR2, and not zfAHR1, mediates the multiple end points of TCDD developmental toxicity in zebrafish (Prasch et al., 2003). Four splice variants of zfARNT2 denoted as zfARNT2a, b, c, and x have been cloned and characterized (Tanguay et al., 2000; Wang et al., 2000). In vitro molecular and biochemical studies suggest that zfARNT2b functionally heterodimerizes with zfAHR2 to enhance the dioxin response element-driven transcription in the presence of TCDD (Tanguay et al., 1999, 2000). However, neither morpholino knockdown of zfARNT2 in zebrafish embryo nor zebrafish ARNT2 mutants (*zfarnt2*$^{-/-}$) prevented TCDD-mediated developmental toxicity (Prasch et al., 2004). This contradiction led to further investigation and identification of zebrafish ARNT1. Functional characterization by morpholino approach delineated that zfARNT1 is the functional heterodimer of zfAHR2 in zebrafish (Prasch et al., 2006).

Although AHR-driven transcriptional regulation has been

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; FGF, fibroblast growth factor; hpa, hours postamputation; hpf, hours postfertilization; BNF, β-naphthoflavone; ANF, α-naphthoflavone; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; bp, base pair; WT, wild type; HET, heterozygous; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline and Tween 20; SE, intersegmental vessel; FGFR1, fibroblast growth factor receptor 1; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; SU5402, 3-[3-(2-carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone; GM6001, butanediamide.

extensively studied, the mechanism by which TCDD causes toxicity is not fully understood. The development of in vivo models to explore the complexity of AHR signal transduction is essential. Adult zebrafish have the remarkable capacity to regenerate their caudal fins completely within 14 days after amputation (Geraudie et al., 1995), and it was previously demonstrated that TCDD inhibits this complex process (Zodrow and Tanguay, 2003). Recently, it was reported that the caudal fin primordia of zebrafish larvae are also capable of tissue regeneration in a process remarkably similar to that observed in adults (Kawakami et al., 2004). In the larval fin, within 10 min of amputation, epithelial cells surrounding the amputation plane begin to migrate over the wound site. These epithelial cells accumulate to form a compact wound epithelium by 24 h postamputation (hpa). Actively proliferating mesenchymal cells denoted as blastema cells are evident in the area adjacent to the amputation plane by 24 to 48 hpa. After blastema formation, both the adult and larval regenerating fins exhibit a common cell proliferation profile, with the proliferation starting at the distal area (posterior to the amputation plane). The distal-most cells do not proliferate during the late phase of repair; instead, drastic cell proliferation occurs in the proximal (anterior to the amputation plane) region. In addition to the similar regenerative events between adults and larvae, fibroblast growth factor (FGF) signaling is necessary during fin regeneration, suggesting a common regenerative molecular mechanism (Poss et al., 2000b; Kawakami et al., 2004).

Studies in adult fin regeneration were limited by barriers in molecular and genetic techniques, which motivated us to develop a larval model to evaluate the consequence of AHR activation on early life stage regeneration. The objectives of this study were to first determine whether TCDD impairs larval fin regeneration and then to determine which AHR pathway members mediate the response. Our observations demonstrate that TCDD specifically impedes larval fin regeneration. Activation of the AHR pathway was confirmed by immunohistochemical localization of induced cytochrome P4501A (zfCYP1A), a well-studied AHR-responsive gene. Antisense knockdown and mutant zebrafish lines demonstrate that zfAHR2 and zARNT1 are both required for the TCDD-dependent block in regenerative growth. We also demonstrate that the inhibitory effects of TCDD exposure and FGF receptor antagonism on regeneration are distinct. In addition to the inherent advantages of zebrafish (i.e., rapid development and fecundity), the larval zebrafish model allows the use of many additional molecular and genetic techniques, such as transient and stable transgenics, mutant screens, and antisense gene repression. Thus, the larval zebrafish is an outstanding model to unravel tissue regeneration mechanisms.

Materials and Methods

Zebrafish Lines and Embryos. Fertilized AB strain embryos (University of Oregon, Eugene, OR) were used for all of the experiments. *zfarnt2*$^{-/-}$ mutants (ARNT2^{hi1715}) in the TAB-14 background (Tubingen/AB cross number 14) was a gift of Nancy Hopkins (Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA). All embryos were raised in our laboratory according to standard procedures. Each experimental group consisted of 12 larvae.

Amputation of Zebrafish Larval Fin Primordia and Chemical Exposure. Embryos were dechorionated and anesthetized with 0.008% 3-amino benzoic acid ethylester (tricaine) in fish water. At 48-h postfertilization (hpf), larvae were placed on an agar plate, and the caudal fin primordia was amputated with a surgical razor blade just posterior to the notochord and transferred to a 24-well plate containing chemical-free fish water. TCDD (>99% pure) was purchased from Chemsyn (Lenexa, KS); β-naphthoflavone (BNF, >99% pure) was obtained from Acros Organics (Morris Plains, NJ) and α-naphthoflavone (ANF) from Sigma-Aldrich (St. Louis, MO). The amputated larvae (48 hpf) were exposed to vehicle (0.3% DMSO) or TCDD in vehicle (0.5 ng/ml of fish water) in a 24-well plate for 1 h. After incubation, the embryos were rinsed multiple times and allowed to develop for 3 days in vehicle/TCDD-free water at 27°C. Because BNF and ANF (both 0.3 μg/ml) were not soluble in DMSO, these ligands were dissolved in dimethyl formamide. ANF and BNF exposures were conducted in 24-well plates for 24 h, followed by multiple rinses in water. After exposure to ANF and BNF, the larvae were reared for 3 days at 27°C in ANF- and BNF-free water. The FGFR1 inhibitor (SU5402) was purchased from Calbiohem (San Diego, CA). The amputated larvae were exposed to SU5402 at a final concentration of 5 μM for 1 day, followed by multiple rinses with fish water.

Reverse Transcription Polymerase Chain Reaction. The caudal fin primordia of 48-hpf larvae were amputated, and the animals were exposed to vehicle or TCDD in vehicle (0.5 ng/ml of fish water) for 1 h. After multiple rinses in TCDD-free water, the embryos were reared at 27°C. Regenerating fins were surgically amputated again at 3 dpa just posterior to the notochord. The amputated regenerating fins were directly immersed into TRI reagent (Molecular Research Center, Cincinnati, OH), and RNA was isolated as described previously (Tanguay et al., 1999). Reverse transcription reactions were carried out using 100 ng of total RNA, 500 ng of Oligo $dT_{12–18}$ primer, and 1 mM dNTPs. The mixture was heated to 65°C for 5 min and quick-chilled on ice. The 20-μl reaction contained 1× first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 0.01 M dithiothreitol, 40 units of RNaseOUT, and 200 units of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). This reaction was incubated at 42°C for 50 min followed by inactivation at 70°C for 15 min. Each 50-μl PCR reaction contained a 2-μl aliquot of cDNA as the template; 0.2 mM each of dNTPs; 10× PCR buffer; 1 mM $MgSO_4$; 0.2 μM forward and reverse primers for either AHR1, AHR2, ARNT1, ARNT2b/c, CYP1A, or β-actin (Table 1); and 1.0 unit of KOD Hot Start DNA polymerase (Novagen, San Diego, CA). The reactions were run in a PTC-100 Peltier thermal cycler (MJ Research, South San Francisco, CA) at the following conditions: 94°C for 20 s, 58°C for 30 s, and 72°C for 80 s, for a total of 35 cycles. The PCR products were resolved by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining.

TABLE 1
Oligonucleotides used for PCR

Name of Primer	Sequence of the Primer (5′–3′)
zfAHR1F	TAGACAGCGATATACAGCAG
zfAHR1R	TCTCTCCAACACCATTCATG
zfAHR2F2	ACGGTGAAGCTCTCCCATA
zfAHR2R2	AGTAGGTTTCTCTGGCCAC
zfARNT1F	ATCCTGCGCATGGCCGTATC
zfARNT1R	GATGTAGCCTGTGCAGTGGAC
zfARNT2b/cF	GACTGAATTCCTTTCGCGCCAC
zfARNT2b/cR	CTGGAGCTGCTTGACGTTG
zfCYP1AF	ACAACATCAGAGACATCACC
zfCYP1AR	TCTCTTTGCAGTCGCTCCTGG
zfβ-actinF	AAGCAGGAGTACGATGAGTC
zfβ-actinR	TGGAGTCCTCAGATGCATTG
zfARNT2F1	CGGAAATGTCGCTGTTGTTAGTTGTG
zfARNT2R1	GAACTGAGTTTGCGCGTTTGAGAC
TranspoR	TGCGATGCCGTCTACTTTGA

Morpholinos. Zebrafish aryl hydrocarbon receptor 2 morpholino (*zfahr*2-MO) (Gene Tools, Corvallis, OR) targeted the translation start site beginning 4 bp upstream of the AUG codon to 18 bp downstream of the sequence. The sequence of the *zfahr2*-MO was 5′TGTACCGATACCCGCCGACATGGTT3′ and the 3′-end was fluorescein tagged to assess microinjection success. Morpholinos were diluted to 2.8 mM in 1× Danieau's solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES, pH 7.6], as described previously (Nasevicius and Ekker, 2000). Zebrafish aryl hydrocarbon receptor nuclear translocator 1 morpholino (*zfarnt1*-MO) (5′-GGATTAGCTGATGTCATGTCCGACA-3′) overlapped the translation start site of zfARNT1 mRNA, starting 8 bp upstream of the AUG start codon to 14 bp downstream of the sequence. The morpholinos were diluted to 1.5 mM in 1× Danieau's solution before microinjection. A standard control morpholino (Gene Tools) (5′-CTCTTACCTCAGTTACAATTTATA-3′) was used as a control morpholino (Control-MO). The embryos were injected at the one- to two-cell stage with approximately 1 to 3 nl of the appropriate morpholino solution. Embryos were screened for fluorescence at 24 hpf to reveal successful injection. The caudal fin of selected embryos were amputated and exposed to TCDD or vehicle for 1 h at 48 hpf. The morpholino injected embryos (morphants) were raised for 3 days after amputation, and the regeneration of fin tissue was observed.

Genotyping of zfarnt2 Mutants. Embryonic DNA was extracted from individual embryos in extraction buffer (0.01 M Tris, 2 mM EDTA, 0.2% Triton-X, and 0.2 mg/ml proteinase-K) incubated at 55°C for 2.5 h. The extract was heated at 100°C for 10 min before centrifugation, and the supernatant was used as the DNA template for PCR. The primers used were zfARNT2F1, zfARNT2R1, and TranspoR. zfARNT2F1 and zfARNT2R1 flank the knockout viral insertion, and TranspoR lies within the transposon insert. zfARNT2F1 and zfARNT2R1 were designed to distinguish *zfarnt2*$^{-/-}$ mutants from wild-type (WT) and from heterozygous (HET) larvae, whereas zfARNT2F1 and TranspoR were used to distinguish WT from HET larvae. PCR was performed to amplify the appropriate sequence using KOD Hot Start DNA polymerase, and the PCR products were examined by gel electrophoresis and ethidium bromide staining.

Whole-Mount Immunolocalization of zfCYP1A and α-Acetylated Tubulin. The distribution of zfCYP1A protein in zebrafish larval fin tissue was assessed using the monoclonal antibody C107 (mouse anti-CYP1A, 1:500; Biosense Laboratories, Bergen, Norway). Monoclonal antibodies generated against acetylated tubulin (mouse anti-AT, 1:1000; Sigma) label most axons and major peripheral processes in the developing embryo. On 3 dpa, TCDD exposed and control larvae were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) and washed in PBS + 0.1% Tween 20 (PBST). The larvae were permeablized with 0.005% trypsin (4°C) in PBS on ice for 5 min, rinsed in PBST, and postfixed in 4% paraformaldehyde. Permeablized larvae were blocked in 10% normal goat serum in PBS + 0.5% Triton X-100 for an hour at 22°C and incubated with the primary antibody overnight at 4°C in 1% normal goat serum-PBS + 0.5% Triton X-100. After 4 × 30-min washes in PBST, the larvae were incubated with a secondary antibody (1:1000) (Alexa-546 conjugated goat anti-mouse; Invitrogen) for 5 h at 22°C. The larvae were then washed 4× for 30 min in PBST and visualized by epifluorescence microscopy.

Statistical Analysis. Each experiment was composed of 12 larvae per group. The larvae were exposed to vehicle or chemical with two larvae in each well. Significant difference in the area and length between the control and TCDD-exposed animals was assessed by Student's *t* test ($p < 0.05$) using SigmaStat 2.03 software (SPSS Inc., Chicago, IL).

Results

TCDD Inhibits Larval Zebrafish Caudal Fin Regeneration. Larval zebrafish caudal fin primordia have the remarkable ability to regenerate by a mechanism similar to that observed in adult fin regeneration (Kawakami et al., 2004). These studies were designed to determine whether AHR activation would impact larval fin regeneration similar to adult zebrafish (Zodrow and Tanguay, 2003). The caudal fin primordia of the 48-hpf larvae were partially amputated just posterior to the notochord (Fig. 1). A range of TCDD concentrations was used (0.01 ng to 1 ng/ml) to determine the sublethal concentration that inhibits fin regeneration (data not shown). From these initial dose-response studies, 0.5 ng/ml TCDD was determined as the most effective concentration that impairs fin regeneration without causing mortality before 120 hpf and was used for all of the described studies. The amputated larvae were exposed to DMSO or TCDD and were allowed to develop for 3 days. Zebrafish exposed to vehicle, regenerated their fin tissue in 3 days, whereas the process of regeneration was impaired in animals exposed to TCDD (Fig. 1). Although not the focus of these studies, exposed larvae also developed the typical TCDD toxicological signs, including pericardial edema, yolk sac edema, reduced blood flow, and impairment of the swim bladder (Henry et al., 1997; Prasch et al., 2004) at 3 dpa. This study suggests that larval and adult fin regeneration may be impaired by TCDD potentially through a common molecular mechanism. It is noteworthy that these results allow full

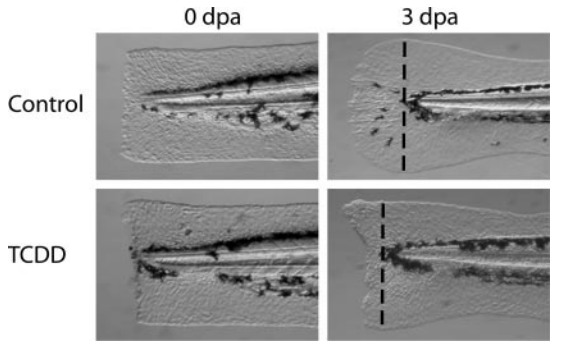

Fig. 1. TCDD inhibits the regeneration of zebrafish fin primordia. The caudal fin primordia of zebrafish larvae (48 hpf) were surgically ablated just posterior to the notochord. After amputation, larvae were exposed to TCDD (0.5 ng/ml) or vehicle control (DMSO). The fin regeneration images were captured immediately after amputation (0 dpa) and 3 dpa. The broken line indicates the plane of amputation at 0 dpa.

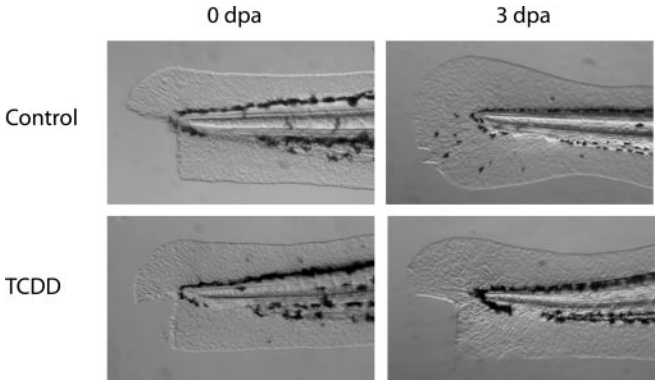

Fig. 2. The suppression of fin regeneration by TCDD is specific to the regenerating tissue. The ventral half of the caudal fin was amputated, followed by the waterborne exposure to TCDD (0.5 ng/ml) or control (DMSO). The embryos were allowed to develop further to analyze whether TCDD preferentially inhibits the regenerating tissue. Fin regeneration images were acquired on 0 at 3 dpa.

exploitation of the larval zebrafish model to elucidate the role of AHR activation and tissue regeneration.

Inhibition of Fin Growth by TCDD Is Specific to the Regenerated Tissue. To determine whether the effects of TCDD are specific to the regenerating fin, we performed partial fin amputations. The ventral halves of the caudal fins were amputated, leaving the dorsal half of the developing fin intact to serve as a control, and the larvae were exposed to vehicle or TCDD (Fig. 2). Images were taken immediately after partial amputation (0 dpa) and 3 days later (3 dpa). In the presence of vehicle, embryos completely regenerated their fins in 3 days. However, fin regeneration was significantly inhibited by TCDD (Fig. 2). Even though TCDD-exposed larvae displayed classic signs of toxicity and regeneration of the ventral portion of the fin was inhibited, growth of the nonamputated dorsal half of fin was not affected (Fig. 2). This suggests that AHR activation specifically interferes with the regenerative process and not just with growth.

To quantify the growth response, the length of maximum outgrowth, the area of regenerated fin tissue (%), and the area of nonamputated fin tissue (%) were measured as depicted in the schematic diagram (Fig. 3A). The length of maximum outgrowth is defined as the distance from the plane of amputation to the tip of the regenerating fin. The

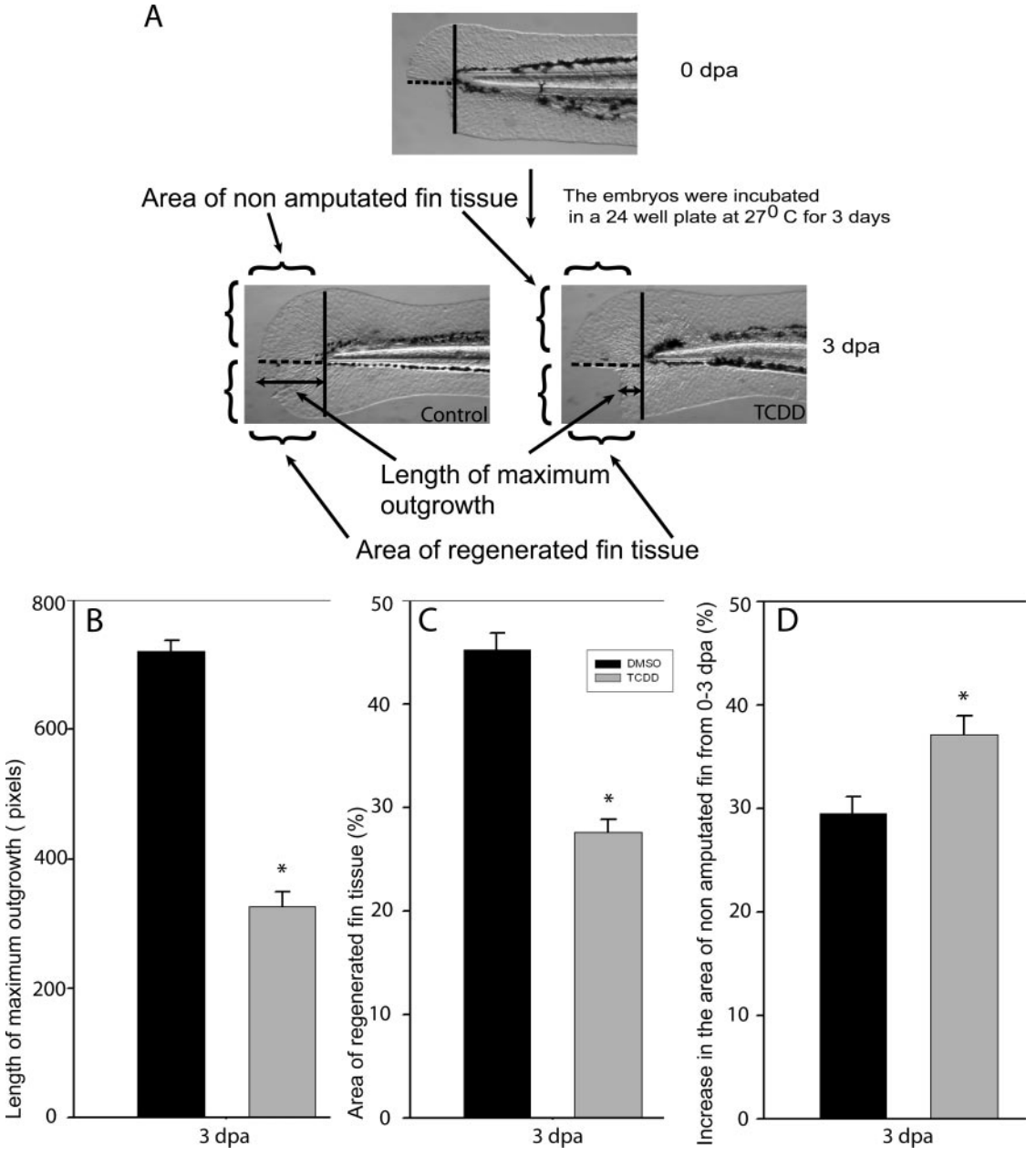

Fig. 3. TCDD preferentially affects the regenerating fin. The length of maximum outgrowth, the area of regenerated fin tissue and the area of nonamputated fin tissue at 0 and 3 dpa were measured in vehicle- and TCDD-exposed embryos as described in the schematic diagram (A). The length of maximum ventral caudal fin outgrowth (1 μm = 1.54 pixels) (B) and the area of regenerated fin tissue (%) in control larvae (C) were significantly greater than the TCDD-exposed embryos ($p < 0.001$). D, the measurement of increase in the area of nonamputated tissue from 0 to 3 dpa will delineate whether TCDD affects the normal growth of fin tissue. The area of the dorsal half of the caudal fin in TCDD-exposed larvae was greater than control larvae ($p < 0.004$). The broken line indicates the plane of amputation. The respective values represent the mean ± S.E.M. (t test, $n = 11$). All three parameters were measured using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD).

area of regenerated fin tissue represents the newly grown ventral fin tissue after partial amputation. The area of the intact dorsal half of the fin was used to determine whether TCDD impacts nonregenerative fin development. The length of maximum outgrowth and the area of regenerated fin tissue (%) in TCDD-exposed larvae were significantly lower compared with the control larvae (Fig. 3, B and C). Surprisingly, the increase in the area of nonamputated fin tissue (%) from 0 to 3 dpa in TCDD-exposed larvae was significantly greater than the control larvae (Fig. 3D). Together, these results establish that TCDD specifically inhibits the growth of regenerating tissue. It is also important to emphasize that normal fin development and growth are not impeded by TCDD unless the fin is amputated.

Detection of AHR Pathway Members in the Regenerating Fin Tissue. To determine the expression profile of AHR members and regulated genes in the larval regenerating fin tissue, RNA was isolated from the regenerating fin tissue of DMSO- or TCDD-exposed larvae at 3 dpa. Reverse transcriptase-polymerase chain reaction was performed using gene-specific primers for zfAHR1, zfAHR2, zfARNT1, zfARNT2b/c, zfCYP1A, and β-actin as a control. The transcripts of *zfahr1, zfahr2, zfarnt1,* and *zfcyp1a* were expressed in both DMSO- and TCDD-exposed larval regenerating fin tissue, but the expression of *zfarnt2b/c* was faint (Fig. 4). Even though this method is nonquantitative, *zfcyp1a* transcript level observed in the TCDD-exposed fin tissue was apparently increased over DMSO levels, suggesting that AHR pathway is active in regenerating fin tissue.

Whole-Mount Immunolocalization of zfCYP1A. A common biomarker to evaluate the activation of AHR pathway by TCDD is to observe CYP1A expression. The temporal and spatial pattern is often associated with the toxicity (Henry et al., 1997; Tanguay et al., 1999; Andreasen et al., 2002b). Immunohistochemical staining of zfCYP1A protein was conducted with larvae exposed to vehicle or TCDD at 48 hpf. Specific immunolocalization of zfCYP1A was detected in the fin tissue of the TCDD-exposed larvae but was not observed in the control larvae (Fig. 5). zfCYP1A was consistently abundant at the distal tip of the caudal fin. TCDD-induced expression of CYP1A in the intersegmental vessels (SE) was used to ensure the positive detection of zfCYP1A between the TCDD- and vehicle-exposed larvae (Fig. 5). Elevated expression of zfCYP1A protein in the fin tissue of TCDD-exposed larvae confirms that the AHR pathway is activated

BNF, Another AHR2 Ligand, Inhibits Regeneration. To further test the hypothesis that TCDD blocks regeneration by inappropriately activating the AHR pathway, BNF was used as an alternative AHR ligand. BNF has been shown to bind and activate the zebrafish AHR2 (Wentworth et al., 2004). When amputated larvae were exposed to BNF (0.3 μg/ml), regeneration was completely blocked as we observed in larvae exposed to TCDD. However, when zebrafish were exposed to the AHR2 antagonist, ANF (0.3 μg/ml), or vehicle, regeneration progressed and was completed in 3 days (Fig. 6). These results strongly suggest that ligand activation of AHR2 is necessary to block the regenerative process.

AHR2 Is Necessary for TCDD to Impede Fin Regeneration. With the availability of the nearly complete zebrafish genomic sequence and the ability to use antisense modified oligonucleotides (morpholinos), the role of any protein in a biological process can be rapidly evaluated in vivo using zebrafish embryos or larvae (Nasevicius and Ekker, 2000). Morpholinos have been shown to be most effective between 0 and 96 hpf. Higher concentrations of morpholino (2.8 mM) can be used to prolong the repression of target genes up to 120 hpf (data not shown). The zfAHR2 and control morphants were amputated and exposed to vehicle or TCDD at 48 hpf and were grown for 3 days. The control and zfAHR2 morphants exposed to vehicle regenerated completely, indicating that the endogenous functions of zfAHR2 are not required for regeneration. As expected, the control morphants exposed to TCDD failed to regenerate (Fig. 7A). The caudal fins in zfAHR2 morphant animals exposed to TCDD were capable of complete regeneration, indicating that inhibition of fin regeneration by TCDD is mediated through zfAHR2 (Fig. 7A). In other words, in the absence of zfAHR2, TCDD has no effect on tissue regeneration. It is noteworthy that the zfAHR2 morphants also failed to develop other signs of TCDD toxicity, including pericardia edema, yolk sac edema, and reduced blood flow, as previously detailed (Prasch et al., 2003). Immunohistochemical analysis was performed on the control and zfAHR2 morphants exposed to vehicle and TCDD to monitor in situ zfCYP1A protein expression. zfCYP1A was not detected in either control or zfAHR2 morphants exposed to vehicle (data not shown). Control morphants had significant vascular and extravascular

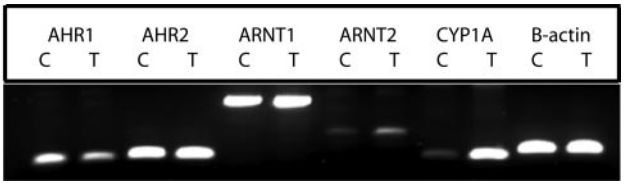

Fig. 4. Identification of AHR pathway members in the regenerating zebrafish fin tissue by reverse transcriptase-polymerase chain reaction. The 48-hpf larvae were exposed to DMSO (C) or TCDD (T) after amputation, and the regenerating fin tissues were harvested for RNA isolation (n = 100). Reverse transcription was conducted followed by PCR using zfAHR1, zfAHR2, zfARNT1, zfARNT2b/c, zfCYP1A, and β-actin specific primer pairs. The PCR products were resolved by agarose gel electrophoresis and were visualized with ethidium bromide staining.

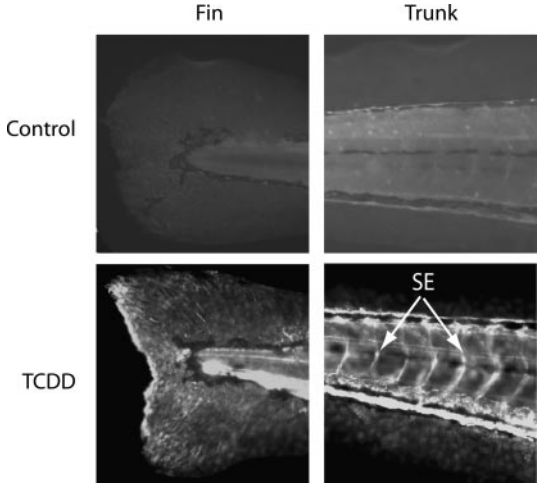

Fig. 5. Whole-mount immunolocalization of zfCYP1A. The immunohistochemical localization of zfCYP1A in control and TCDD-exposed larvae at 3 dpa using monoclonal antibody C107. Depicted are the images of fin tissue and the trunk of the larvae with positive CYP1A localization in the SE.

zfCYP1A expression, and in zfAHR2 morphants, zfCYP1A protein was not detected, indicating complete and persistent knockdown of AHR2 (Fig. 7B). These results confirm that AHR pathway cannot be activated by TCDD in the absence of AHR2.

ARNT1, Not ARNT2, Is Required for Inhibition of Fin Regeneration by TCDD. Because there are at least two ARNT genes expressed in the early embryo, zfARNT1 and zfARNT2, it was important to determine which ARNT is the in vivo partner for zfAHR2 that is necessary to block tissue regeneration. A genetic approach was used to specifically evaluate the role of zfARNT2. Insertional mutagenesis screens had identified previously zebrafish mutants that lacked ARNT2 expression (Amsterdam et al., 1999). These mutants were generously provided by Dr. Nancy Hopkins (Massachusetts Institute of Technology, Cambridge, MA). Embryos collected from *zfarnt2*$^{+/-}$ (HET) parents were raised to 48 hpf, and 40 larvae were amputated and exposed to TCDD. In this pool of larvae, 25% should be *zfarnt2*$^{+/+}$ (WT), 50% should be *zfarnt2*$^{+/-}$ (HET), and 25% should be *zfarnt2*$^{-/-}$ mutants. TCDD completely inhibited the fin regeneration in all of the larvae, irrespective of the ARNT2 genetic status (Fig. 8A). Genotyping of individual larvae were conducted with PCR to determine the larval genetic makeup (Fig. 8A).

To determine the role of zfARNT1 in fin regeneration, morpholinos were used to knockdown early life stage zfARNT1 protein expression. Control and zfARNT1 morphants were amputated at 48 hpf, followed by a waterborne exposure to vehicle or TCDD. The amputated larvae were allowed to regenerate their fins for 3 days. The control morphants exposed to TCDD displayed impaired fin regeneration, whereas the zfARNT1 morphants were able to completely regenerate their fins in the presence of TCDD (Fig. 8C). In situ immunolocalization of zfCYP1A protein confirms that zfCYP1A is highly induced in control morphants exposed to TCDD, whereas the zfARNT1 morphants had a significant decrease in the CYP1A expression (Fig. 8D). Together, these results indicate that ARNT1 is a necessary in vivo dimerization partner for AHR2, which together initiate a process that inhibits tissue regeneration.

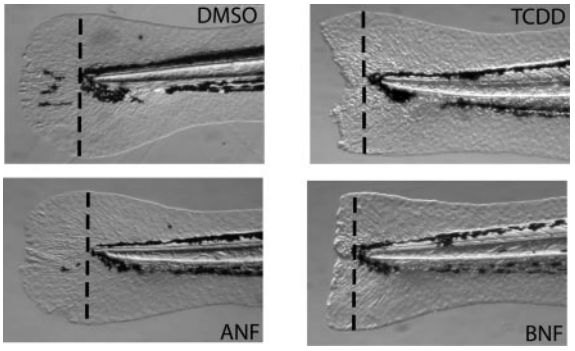

Fig. 6. Activation of AHR2 by BNF inhibits fin regeneration similarly to TCDD. The caudal fin of 48-hpf larvae were surgically amputated and exposed to control (DMSO), an AHR antagonist ANF, or AHR agonists BNF and TCDD. The images were taken at 3 dpa, and the broken line indicates the plane of ablation of fin tissue.

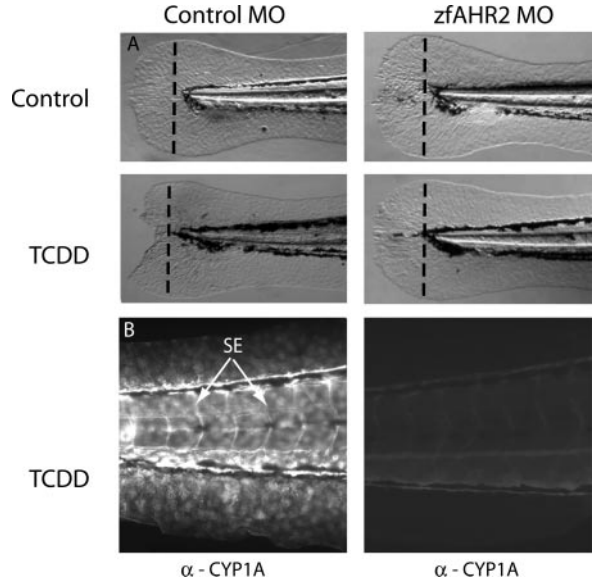

Fig. 7. Fin regeneration occurs in *zfahr2* morpholino-injected embryos in the presence of TCDD. A, the control morpholino (Control MO) and *zfahr2* morpholino (AHR2 MO)-injected embryos were amputated at 48 hpf and exposed to vehicle or TCDD. The fin regeneration images were taken at 3 dpa. Broken line marks the plane of amputation. B, immunolocalization of zfCYP1A on Control and *zfahr2* morphants exposed to TCDD.

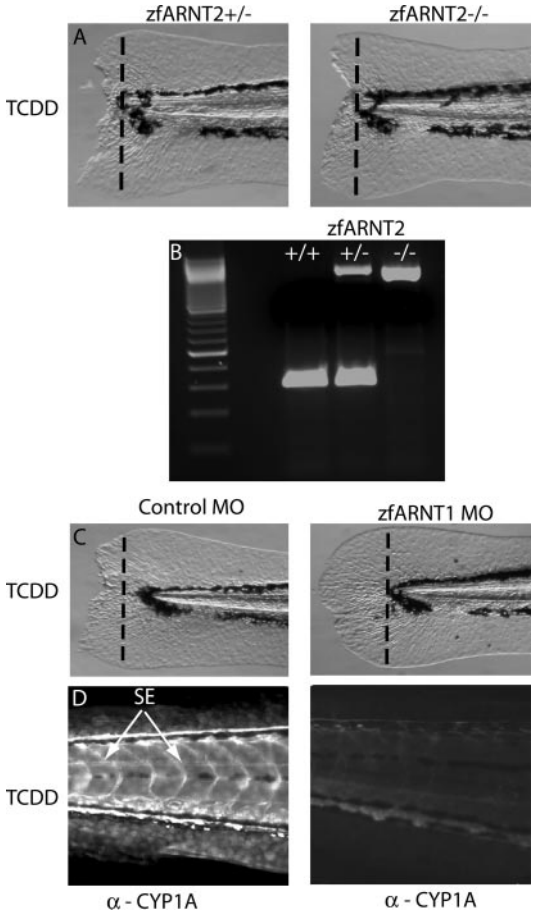

Fig. 8. Role of zfARNT members in the inhibition of fin regeneration by TCDD. A, heterozygous (HET, *zfarnt2*$^{+/-}$) and homozygous *zfarnt2*$^{-/-}$ mutants were exposed to vehicle or TCDD. The images are of *zfarnt2*$^{+/-}$ and *zfarnt2*$^{-/-}$ larvae characterized genotypically by PCR. The images of the mutants exposed to DMSO are not shown. B, genotyping the *zfarnt2* mutants by PCR. C, the control (Control MO) and *zfarnt1* morphants (ARNT1 MO) were exposed to vehicle or TCDD at 0 dpa. The images were captured at 3 dpa. Images of the control and *zfarnt1* morphants exposed to DMSO are not shown. D, whole-mount immunolocalization of zfCYP1A in control and *zfarnt1* morphants exposed to TCDD.

Morphometrically, TCDD- and FGFR1-Mediated Inhibition of Fin Regeneration Are Different. It is clear that AHR activation impairs or interferes with signaling pathways required for tissue regeneration, but these downstream targets remain unknown. To begin to understand the underlying cellular responses, the effects of TCDD were compared with the effects of another chemical known to impact regeneration. Inhibition of fibroblast growth factor receptor 1 (FGFR1) by a lipophilic drug (SU5402) has been shown to inhibit both adult and larval fin regeneration (Poss et al., 2000b; Kawakami et al., 2004). At the gross level, SU5402 inhibited fin regeneration but to a lesser extent than TCDD (Fig. 9, A and B). It is noteworthy that over the course of the regeneration period (3 days), SU5402 had a significant reduction in the overall embryonic and larval growth, whereas TCDD had no measurable effect on overall growth during this developmental window (data not shown). During regeneration, neuronal axons and their peripheral processes grow and extend into the newly formed tissue (R. L. Tanguay, unpublished observation). The growth and pathfinding of these structures can be used to assess tissue reorganization. In situ immunolocalization using an α-acetylated tubulin antibody is a convenient way to label the axons and their peripheral processes. In the control larvae, the peripheral processes branch off the axonal trunks and fan out into the regenerate. After 3 days, a complex but ordered network of branches is formed in the new tissue (Fig. 9C). In the TCDD-exposed animals, the peripheral processes consistently extended toward the amputation plane and then made lateral turns, nearly perpendicular to the plane of amputation. It is noteworthy that the branches continued to extend in the presence of TCDD, but the pathfinding is altered. These observations are consistent with the concept that that TCDD impairs the ability of the neuronal processes to migrate directly into the tissue (Fig. 9, C and D). Further illustration of this point is revealed when only the ventral half of the fin is amputated (Fig. 9E). TCDD consistently leads to an altered neuronal growth pattern in which the new processes in the partially amputated fin continued to grow, but they made lateral turns in the absence of sufficient new tissue to accommodate their length. These results suggest that in TCDD exposed larvae, the peripheral processes in the regenerating fin maybe incapable of growing straight because of a physical barrier or improper growth factor signaling. The neuronal response in SU5402-exposed larvae was noticeably different. The pathfinding of the neuronal branches was not affected. The processes reached the end of the new tissue and had a branching pattern indistinguishable from that in control animals (Fig. 9E). Taken together, these results suggest that the inhibition of regeneration mediated by TCDD and FGFR1 inhibitor is different.

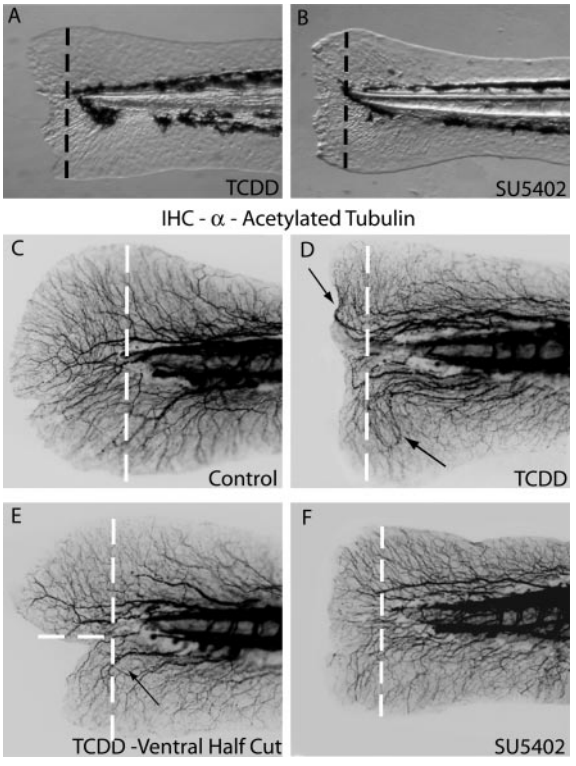

Fig. 9. Inhibitory effect on the process of fin regeneration by TCDD and FGFR1 inhibitor is different. A and B, the 48-hpf larvae were amputated and exposed to vehicle, TCDD, or FGFR1 inhibitor (SU5402). Images of the TCDD- and SU5402-exposed larvae were taken at 3 dpa. C, D, and F, whole-mount immunolocalization of acetylated tubulin that stains axons and the peripheral processes of larvae exposed to vehicle, TCDD, or FGFR1 inhibitor (SU5402). The arrows indicate the path of diverging peripheral processes D, immunohistochemical staining was done on TCDD-exposed larvae with ventral half cuts to delineate the peripheral processes that are diverging at the site of inhibition of fin regeneration. The arrow on the ventral half cut reveals the deviating path of peripheral processes (E). The images are representative of 12 larvae, and the images were inverted.

Discussion

Regeneration of tissue is a well-orchestrated process in which the injured or lost structure is completely replaced. In both adult and larval zebrafish, an epithelial wound covering is formed within 12 hpa, and this response does not involve cell proliferation (Santamaria et al., 1996; Nechiporuk and Keating, 2002). Wound closure is followed by the development of blastema, and the wound epidermis is hypothesized to be a source of growth factors that stimulate and regulate the formation of the blastema. In adult zebrafish, FGFs present in the wound epidermis interacts with the mesenchymal FGFR1. FFGR1 inhibitor studies suggest that active FGF signaling is required for the formation of blastema and regenerative outgrowth (Poss et al., 2000b). The differential expression of *mps1, shh, lef1, wnt3a, wnt5, RAR-γ,* and *msx* homeobox genes illustrates the complexity of signaling pathways during the regeneration process (White et al., 1994; Akimenko et al., 1995; Poss et al., 2000a,b). During regenerative outgrowth, cell proliferation propagates from distal zone to the proximal zone of fin primordia, with intense proliferation occurring in the proximal region during the late phase of repair (Nechiporuk and Keating, 2002; Kawakami et al., 2004). In larvae, fin regeneration is completed in 3 to 5 days by controlled cellular proliferation and migration. It has been established that TCDD impairs the outgrowth phase of regeneration (Zodrow and Tanguay, 2003). Although it had been presumed that the block in regeneration is mediated by TCDD binding and activation of AHR signaling pathway, it had not been demonstrated experimentally.

In mammals, studies have demonstrated that *Ahr* null mice develop hepatic defects and have reduced liver size, implicating a role of AHR in normal liver growth and development (Schmidt and Bradfield, 1996; Lahvis et al., 2000).

The underlying deficit seems to be a congenital vascular defect, failure of ductus venosus closure (Walisser et al., 2004). In addition to this endogenous developmental role, functional AHR is required to mediate TCDD toxicity (Gonzalez and Fernandez-Salguero, 1998; Mimura et al., 1999). Both zfAHR1 and zfAHR2 were present in the regenerating fin of vehicle- and TCDD-exposed larvae (Fig. 4). Previous studies have reported that zfAHR1 does not mediate TCDD toxicity because it does not bind to AHR ligands and has a very limited tissue distribution (Andreasen et al., 2002a). Knockdown of zfAHR2 by antisense morpholinos revealed that zfAHR2 mediates the endpoints of TCDD-dependent developmental toxicity (Prasch et al., 2003). zfAHR2 morphants regenerated the fin tissue completely in the presence of TCDD, confirming that AHR2 is necessary for TCDD to block fin regeneration. Recently, a third AHR gene was identified in zebrafish, which is located adjacent to the AHR2 gene in the zebrafish genome. This duplicated gene was designated AHR1b. In vitro studies indicate that AHR1b has AHR-like properties (Karchner et al., 2005). The AHR2 morpholino studies demonstrate that AHR1b cannot play a role in mediating the well-studied in vivo responses to TCDD. The endogenous role for AHR1b remains to be identified.

In mammals, ARNT1 is the functional dimerization partner for AHR, mediating many endpoints of TCDD toxicity (Schmidt and Bradfield, 1996). *arnt2* knockout mice die within 24 h of birth and have impaired hypothalamic development, confirming its function during normal development (Hosoya et al., 2001). In zebrafish, four splice variants of zfARNT2 have been characterized, and zfARNT2b has been demonstrated to be transcriptionally active with zfAHR2 in vitro (Tanguay et al., 2000). Unexpectedly, antisense repression of ARNT2 did not impact embryonic responses to TCDD. Similar results were obtained in zebrafish *arnt2* mutants. Collectively, these results confirm that zfARNT2 is not the in vivo partner of zfAHR2, mediating the developmental toxicity by TCDD (Prasch et al., 2004). zfARNT1 was recently characterized in zebrafish (Prasch et al., 2006). In regenerating larval fin, zfARNT1 was highly abundant in both vehicle- and TCDD-exposed larvae, whereas the expression of zfARNT2 was low. Fin regeneration in zfARNT2$^{-/-}$ was impaired when exposed to TCDD, whereas the zfARNT1 morphants completely regenerated, confirming that zfANRT1 is the heterodimer of zfAHR2.

Because TCDD inhibits adult zebrafish fin regeneration when dosed on either 0, 1, 2, 3, or 4 dpa, this suggests that TCDD interferes with multiple stages of regeneration (Zodrow and Tanguay, 2003). Similar results were found in larvae. When larvae were exposed to TCDD at 1 dpa, fin regeneration was also impaired (data not shown). It is noteworthy that because wound healing and blastema formation occur in the first day, regenerative outgrowth may be the TCDD target.

Pharmacological inhibition of FGFR1 inhibits blastema formation and outgrowth, indicating that FGF signaling is required during fin regeneration (Poss et al., 2000b; Kawakami et al., 2004). Although FGFR1 inhibitor effectively reduced regenerative outgrowth, overall embryonic growth was also affected. Both FGFR1 inhibitor and TCDD impaired fin regeneration, but the tissue response to these chemicals was different. Using neuronal outgrowth as a marker, TCDD led to a significant disorganization of peripheral processes. Because matrix degradation and turnover are important in wound healing, tissue remodeling, tissue repair, and inflammation, one possibility is that the extracellular matrix (ECM) metabolism is affected by TCDD. Matrix metalloproteinases (MMPs) are considered to be primarily responsible for the turnover of ECM. These proteolytic enzymes play a major role during development and morphogenesis. Improper regulation of these proteinases may result in pathologies such as arthritis, cancer, atherosclerosis, aneurysms, tissue ulcers, and fibrosis (Visse and Nagase, 2003). It has also been reported that after central nervous system injury, the optic nerve astrocytes are stimulated by regenerating axons by secreting active MMP and down-regulating tissue inhibitors of metalloproteinases (TIMPs), resulting in the degradation of scar tissue, creating a permissive growth environment (Ahmed et al., 2005). Tenascin-R, a glycoprotein deposited into the ECM, acts as a repellant guidance molecule in boundaries during normal growth and regeneration of optical axons (Becker et al., 2004), clearly demonstrating that ECM components play critical roles in axonal guidance. We therefore propose that if matrix remodeling is impaired by TCDD, the neuronal growth cone may be unable to traverse through the matrix, resulting in abnormal pathfinding.

ECM is a dynamic environment that plays a crucial role in regulating cellular functions during normal and pathological remodeling processes such as embryonic development, tissue repair, inflammation, tumor invasion, and metastasis. ECM macromolecules are critical for creating a conducive cellular milieu for proper proliferation and migration of different cell types. The MMPs are specifically controlled at the transcriptional level, activation of precursor zymogens, cell-ECM interactions, and inhibition by endogenous TIMPs (Nagase and Woessner, 1999). The expression and functional role of MMPs has been studied in adult zebrafish regenerating fin and have demonstrated that membrane-type *mmp, mmp-2,* and *timp-2* mRNA transcripts were expressed in the regenerating fin tissue. Fin outgrowth was significantly reduced by GM6001, an MMP inhibitor, emphasizing the magnitude of these proteinases during fin regeneration (Bai et al., 2005). Likewise, MMP inhibitor studies demonstrate that these enzymes are required for normal newt limb regeneration, and *mmp3/10a, mmp3/10b,* and *mmp-9* are up-regulated within hours of limb amputation. The temporal expression of MMPs in the regenerating newt limb suggests that these enzymes are involved in blastema formation, maintenance, and growth (Vinarsky et al., 2005). Aryl hydrocarbons such as TCDD and benzo(*a*)pyrene induced the expression of MMP-9 in PC-3 and DU145 human prostrate cancer cells (Haque et al., 2005). TCDD increased the expression and activity of MMP-1, MMP-2, and MMP-9 in transformed melanoma cell (A2058) and increased invasion (Villano et al., 2006). Gene expression in the fetal murine heart after TCDD exposure in utero suggests possible alterations in cell-cycle control and ECM production and remodeling (Thackaberry et al., 2005). Furthermore, TCCD-enhanced expression of MMP-1, -9, and TIMP-3 in lung airway epithelial cells by microarray analysis, implying that MMP expression maybe a common endpoint for AHR pathway activation (Martinez et al., 2002). In normal human keratinocytes, TCDD induces MMP-1 expression, and cotreatment with all *trans*-retinoic acid enhanced the MMP-1 expression additively (Murphy et al., 2004), suggesting the cross-talk of AHR pathway with other signaling

pathways. These studies correlate with the finding that ECM remodeling is impaired in the regenerating fins of TCDD exposed adult zebrafish (E. Andreasen, personal communication).

In summary, our data provide evidence that activation of AHR pathway inhibits larval zebrafish fin regeneration. The inhibition of fin regeneration is mediated by TCDD activation of zfAHR2 and its in vivo dimerization with zfARNT1. Preliminary data and the supporting literature point to an association between AHR pathway activation and impaired extracellular tissue remodeling. The interactions between cells and ECM are tightly controlled by membrane proteins, proteolytic enzymes, cytokines, and growth factors during numerous physiological processes and during regeneration. Inappropriate expression or activity of any of the factors can mediate a variety of pathologies such as tumor metastasis, cardiovascular disease, or toxicity. The larval fin regeneration model presented herein can be exploited to unravel the cross-talk between AHR-activated and other critical signaling pathways.

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