

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Inhibits Zebrafish Caudal Fin Regeneration

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Adult zebrafish completely regenerate their caudal fins following partial amputation. Fin regrowth can easily be monitored *in vivo* and regenerating tissues can be used to study this dynamic developmental process. In this study we determined that fin regeneration is significantly affected by exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Zebrafish caudal fins were partially amputated, and the fish received intraperitoneal (ip) injection of 2.8, 14, or 70 ng/g weight TCDD or vehicle control. By 7 days postamputation, fish exposed to the highest concentration of TCDD regenerated 15% of their fin compared to 65% regrowth in control fish. To determine if this effect was stage specific, zebrafish were exposed to 70 ng/g TCDD on 1, 2, 3, or 4 days postamputation. Fin regeneration was significantly inhibited at all time points following TCDD exposure. TCDD exposure also induced hyperpigmentation in *de novo* tissue. Zebrafish were dosed with BrdU, following fin amputation and TCDD exposure, to study changes in cell proliferation. By 4 days postamputation, cell proliferation rates were significantly lower in TCDD-exposed fish. TCDD toxicity is mediated through the aryl hydrocarbon receptor (AHR), and RT-PCR experiments confirmed AHR2, ARNT2b, and TCDD-dependent CYP1A expression in the regenerating tissue. These results demonstrate that zebrafish caudal fin regeneration is a unique model to investigate molecular mechanism(s) of TCDD toxicity.

Key Words: zebrafish; fin regeneration; aryl hydrocarbon receptor; AHR; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD.

Adult zebrafish regenerate their caudal fins within fourteen days after amputation (Geraudie *et al.*, 1995; Poleo *et al.*, 2001). During the first stage of regeneration, up to 12 h postamputation (hpa), the fin forms an epidermal wound covering or cap. From 12 to 24 hpa, mesenchymal cells dedifferentiate and move toward the epidermis. During 24–48 hpa there is proliferation of mesenchymal cells forming a blastema. Finally, from 48 hpa forward, the blastema differentiates and

develops structures required for fin regeneration, including blood vessels, bony rays, and connective tissue (Becerra *et al.*, 1996; Johnson and Weston, 1995; Mari-Beffa *et al.*, 1996; Poss *et al.*, 2000b; Santamaria and Becerra, 1991). Each fin is composed of lepidotrichia, a pair of facing cartilaginous hemirays surrounding connective tissue, nerves, and blood vessels. The lepidotrichia are lined inside by actinotrichia, which extend distally beyond the lepidotrichia at the end of each ray (Laforest *et al.*, 1998). The tissue between the rays is mesenchymal derived inter-ray tissue. During fin regeneration and the formation of the blastema, cells adjacent to actinotrichia differentiate into scleroblasts, important in the synthesis and formation of the lepidotrichia, while those not in contact with actinotrichia differentiate into fibroblasts that form the inter-ray connective tissue (Santamaria and Becerra, 1991).

Fish are among the most sensitive vertebrates to the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), with larval fish several times more sensitive than adults (Peterson *et al.*, 1993; Tanguay *et al.*, in press; Walker and Peterson, 1994). Zebrafish larvae exposed to TCDD exhibit characteristic signs of early-life-stage toxicity, including pericardial edema, yolk sac edema, craniofacial malformations, reduced blood flow, anemia, decreased growth, and mortality (Belair *et al.*, 2001; Henry *et al.*, 1997; Teraoka *et al.*, 2002; Wannemacher *et al.*, 1992). Adult zebrafish exposed to TCDD exhibit hypertrophy of hepatocytes, glycogen depletion, and lipidosis of the liver, as well as hypertrophy and fusion of gill lamellae (Zodrow *et al.*, in press). TCDD also results in fin necrosis in rainbow trout and yellow perch (Spitsbergen *et al.*, 1988a,b). Interestingly, mirror carp (*Cyprinus carpio*) caudal fin wound healing is also impaired by a single TCDD dose (van der Weiden *et al.*, 1994).

TCDD toxicity is mediated by activation of the aryl hydrocarbon receptor (AHR) pathway. Unliganded AHR is located in the cytoplasm bound to two 90-kDa heat shock proteins (hsp90) and the AHR interacting protein (AIP). Ligand-bound AHR translocates to the nucleus, where it binds to the AHR nuclear translocator (ARNT). This heterodimeric complex interacts with AHR-responsive elements (AHREs), altering the transcription of a large battery of genes, including cytochrome P450 1A (CYP1A) (reviewed in Schmidt and Bradfield, 1996;

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Swanson and Bradfield, 1993). Two zebrafish AHRs, AHR1 and AHR2, have been characterized (Andreasen *et al.*, 2002a; Tanguay *et al.*, 1999). The zebrafish ARH2, not AHR1, mediates TCDD dependent toxicity in zebrafish (Andreasen *et al.*, 2002a). Four splice variants of zebrafish ARNT2 have also been characterized and have been named ARNT 2a, 2b, 2c, and 2x (Hsu *et al.*, 2001; Tanguay *et al.*, 2000; Wang, 2000). Biochemical data indicates that ARNT2b can dimerize with AHR2 and transactivate AHRE-containing promoters in the presence of TCDD (Tanguay *et al.*, 2000).

CYP1A induction is a common biomarker for TCDD exposure, and the temporal and spatial CYP1A expression often correlates with toxicity (Andreasen *et al.*, 2002b; Guiney *et al.*, 1997; Henry *et al.*, 1997; Tanguay *et al.*, 1999). Since zebrafish AHR2 and a functional ARNT2b are required for CYP1A expression, AHR2 and ARNT2b should be expressed in tissues expressing TCDD-inducible CYP1A. In developing zebrafish exposed to TCDD, AHR2, ARNT2b, and TCDD-induced CYP1A mRNAs are largely coexpressed in tissues affected by TCDD (Andreasen *et al.*, 2002b). Adult expression of AHR2, ARNT2b, and CYP1A are similar to the expression patterns observed in larval zebrafish (Andreasen *et al.*, 2002a; Tanguay *et al.*, 2000). Exposure to TCDD in adult fish results in induction of CYP1A mRNA in the brain, heart, muscle, swim bladder, and liver and a lower fold induction in the gill, kidney, and fin. Immunohistochemistry reveals that TCDD exposure in adult fish significantly increases CYP1A protein expression in the kidney, liver, and intestine with lower levels of induction in the gill, caudal fin, and cardiac muscle (Zodrow *et al.*, in press).

While TCDD leads to a variety of toxic endpoints in developing and adult fish, the purpose of this study is to determine if TCDD affects zebrafish caudal fin regeneration and to use this as a model to help elucidate mechanisms of TCDD toxicity. Here we report that several stages of adult caudal fin regeneration are significantly impaired by TCDD exposure. TCDD exposure also leads to hyperpigmentation of regenerating tissue. Additionally, AHR2, ARNT2b, and CYP1A are expressed during regeneration. These results indicate that caudal fin regeneration can be used as a model to understand mechanisms of altered dynamic cellular processes due to TCDD exposure.

MATERIALS AND METHODS

Chemicals. TCDD (> 99% pure) was purchased from Chemsyn (Lenexa, KS). Chicken egg yolk phosphatidylcholine (PC, > 99% purity) in chloroform was obtained from Avanti Polar Lipids (Alabaster, AL).

Oligonucleotides for RT-PCR. zfAHR1F, TAGACGATATACAGCAG; zfAHR1R, TCTCTCCAACACCATTTCATG; zfAHR2F2, ACGGTGAA-GCTCTCCATA; zfAHR2R2, AGTAGGTTTCTCTGGCCAC; zfARNT2F2, GACTGAATTCCITTCGCGCCAC; zfARNT2b/cR, CTGGAGCTGCTTGAC-GTTG; zfARNT2aR, CACAGTGAATATTCCTTGATC; zfCYP1F, TGCCGATTCATCCCTTTCC; zfCYP1R, AGAGCCGTGCTGATAGTGTC; zfb-actinF, AAGCAGGAGTACGATGAGTC; and zfb-actinR, TGGAGTCTCAGATG-CATTG.

Maintenance of zebrafish. Zebrafish (*Danio rerio*), AB strain, were maintained in recirculating tanks containing oxygenated reverse osmosis water supplemented with 0.3 g/l Instant Ocean Sea Salt (Marine Biotech, Beverly, MA). The water was filtered through 0.45 μm mesh, denitrified by bacterial filtration, and finally disinfected by ultraviolet light exposure. Fish were fed twice daily, once with dry flake food (Tetra-min, Tetra, Melle, Germany) and once with live artemia (Great Salt Lake Artemia cysts, INVE, Grantsville, UT).

TCDD dosing of adult zebrafish. A stock concentration of TCDD in 1,4-dioxane was added to PC in chloroform. The chloroform was evaporated to dryness, and the residual film was rehydrated with 0.9% NaCl and sonicated, forming microsomes as previously described (Walker and Peterson, 1991). Each zebrafish was weighed to calculate required dose, and injection volumes ranged between 1 and 2.5 μl . For dose-response studies, each group consisted of six adult male zebrafish. Fish were anesthetized with 0.16 g/l Tricaine (MS-222) before intraperitoneal injection with either PC as vehicle (control) or 2.8, 14, or 70 ng/g TCDD in PC liposomes. The caudal fin was partially amputated using a razor blade at the first branch point of the lepidotrichia. The fish were allowed to recover in one-liter tanks before transfer to ten-gallon tanks separated by dividers. Each fish was tracked individually to calculate regeneration progress over time. Zebrafish fins were imaged before amputation and again on day 7 postamputation. Percent fin regeneration was determined based on the area of regrowth divided by the original fin area \pm standard deviation; $n = 6$.

For the partial fin regeneration study, adult zebrafish were anesthetized, injected with vehicle or 70 ng/g TCDD in vehicle, and one-fourth of the caudal fin was amputated. The fish were allowed to recover as stated earlier and housed in ten-gallon tanks for the duration of the experiment. Zebrafish fins were imaged before amputation and at days 1, 3, 5, and 11 postamputation. For the multiple stages of regeneration study, adult zebrafish were anesthetized, and their caudal fins were partially amputated. Zebrafish were injected with 70 ng/g TCDD on the same day as amputation or 1, 2, 3, or 4 days postamputation. The study was conducted for 21 days, and fins were imaged before amputation and at 2, 4, 6, 11, 14, and 21 days postamputation. Percentage fin regeneration was determined for each fish based on the area of regrowth divided by the original fin area \pm standard deviation; $n = 6$.

BrdU incorporation in zebrafish caudal fins. For studies involving bromodeoxyuridine (BrdU), a stock concentration of 50 mg/mL BrdU was prepared in sterile Hanks solution. The fish were anesthetized with tricaine and injected with vehicle (control) or TCDD, and their fins were partially amputated on day 0, as described previously. The fish were then reanesthetized and injected with 250 $\mu\text{g/g}$ weight BrdU at 18, 42, 66, or 90 hpa and allowed to recover. Six hours post-BrdU injection, the fish were euthanized, and their caudal fins were amputated and fixed in 4% paraformaldehyde. The fins were then cut in half and used in whole-mount immunohistochemistry with primary mouse anti-BrdU antibody (Sigma, St. Louis, MO) at a dilution of 1:500 and secondary goat anti-mouse AlexaFlour™ 546 antibody (Molecular Probes, Eugene, OR) at a dilution of 1:500. Fluorescence microscopy was used to visualize and photograph BrdU incorporation. BrdU-positive cells were counted, and area of regenerated tissue were measured using Image Pro Plus software (Media Cybernetics, Silver Spring, MD), mean \pm standard error of the mean; $n = 3$. Old tissue counts were determined by selecting an area of non-amputated tissue adjacent to the plane of amputation and using the same area for all tissue counts in each fin, mean \pm standard error of the mean; $n = 3$.

Reverse transcription-polymerase chain reaction. Adult zebrafish were anesthetized and injected in the abdominal cavity with either vehicle (control) or 70 ng/g TCDD in vehicle. The caudal fins were partially amputated and allowed to regenerate. On days 2, 4, or 6 postamputation, the regenerating fin was surgically amputated, and total RNA was isolated from the regenerating tissue. Total RNA was isolated with TRI reagent (Molecular Research Laboratories, Cincinnati, OH) according to the manufacturer's instructions and as previously described (Tanguay *et al.*, 1999). The reverse transcription (RT) reactions were carried out using 1 μg of total RNA isolated from each sample. Each 20- μl RT reaction contained 1 \times AMV reverse transcriptase buffer (50

mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, and 10 mM DTT), 1mM dNTPs, 250 ng Oligo dT primer, and 4.5 units of AMV reverse transcriptase (Promega, Madison, WI). This reaction was incubated at 42°C for 1 h, followed by incubation at 95°C for 15 min. A 2 μ l aliquot of the cDNA was used as a template for each 50 μ l PCR reaction, which contained 1 \times AmpliTaq buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin), 0.200 μ M forward and reverse primers for AHR1, AHR2, ARNT2a, ARNT2b/c, CYP1A, or β -actin, 0.2 mM each dNTPs, and 1.5 units AmpliTaq. The reactions were cycled in a GeneAmp 9700 Perkin Elmer (Norwalk, CT) thermal cycler using the following conditions: 95°C for 30 s; 58°C for 30 s, 72°C for 90 s, for a total of 35 cycles. PCR products were resolved with agarose gel electrophoresis and ethidium bromide staining.

Statistical analysis. All statistical analyses were performed using SigmaStat software (Chicago, IL). Graphs were created using SigmaPlot software (Chicago, IL).

RESULTS

TCDD Dose Responsive Inhibition of Fin Regeneration

In order to determine the effects of TCDD on zebrafish caudal fin regeneration, adult zebrafish were anesthetized with Tricaine (MS-222) and injected with vehicle (control) or TCDD at 2.8, 14, or 70 ng per gram fish (parts per billion [ppb]). The caudal fins were partially amputated and allowed to regenerate for 7 days. The control fish regenerated 65% of their caudal fins 7 days after amputation, while regeneration was significantly inhibited by TCDD exposure at all three doses. Fish exposed to 2.8, 14, or 70 ng/g TCDD only regenerated 50, 35, or 15% of their caudal fins, respectively, 7 days postamputation (Fig. 1). It is important to note that the fish remained otherwise healthy during the duration of this study. The fish

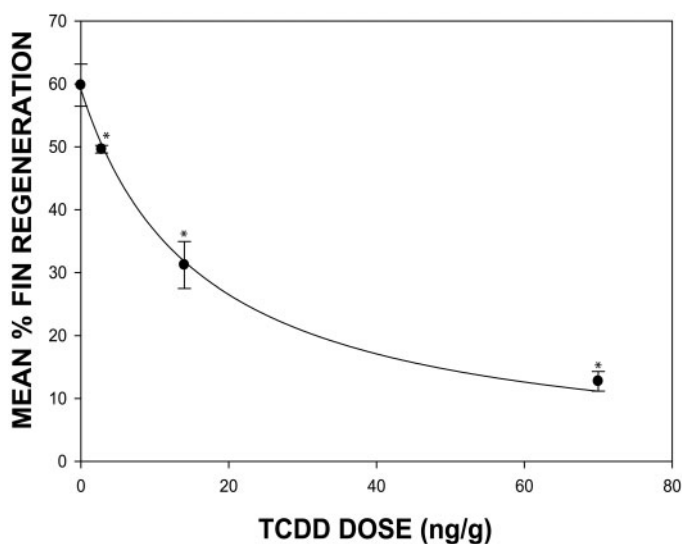


FIG. 1. TCDD inhibits zebrafish fin regeneration in a dose-response manner. Adult zebrafish were injected ip with 0, 2.8, 14, or 70 ng/g TCDD, and their caudal fins were partially amputated. The percentage fin regrowth was calculated after 7 days of regeneration. Mean, SD, $n = 6$. Asterisks indicate significant differences compared to control group (one way ANOVA, $p < 0.001$).

exhibited no change in feeding habits, body weight, or behavior at any of these TCDD doses. This is the first report of a sublethal TCDD dose leading to inhibition of fin regeneration. For the remaining of the described studies, the 70 ng/g dose of TCDD was used, since it had the most pronounced effect on regeneration.

TCDD Completely Abrogates Fin Regrowth

To determine if the block in regeneration is persistent, zebrafish were injected with either vehicle or TCDD in vehicle, and their fins were partially amputated and allowed to regenerate for 21 days. Images of control and TCDD-exposed fish were taken at 2, 4, and 11 days postamputation (Fig. 2A). Two days postamputation control fish regenerated 5% of their caudal fins. By day 4 and 11 postamputation, their fins regenerated 17% and 80%, respectively (Fig. 2A,B). Regeneration was significantly inhibited by TCDD exposure, with only 8% regrowth at day 4 and only 7% 11 days postamputation (Figs. 2A,B). TCDD-mediated inhibition of regeneration persisted up to 21 days postamputation. These results demonstrate that TCDD can severely impair the regeneration process.

It was essential to determine if the TCDD doses used in these studies induced general fin necrosis or if the regenerating tissue was particularly sensitive to TCDD. Following TCDD injection, only one-fourth of each caudal fin was amputated, leaving the intact part of the fin to serve as an internal control. Images from zebrafish with partial fin amputations were taken on 1, 3, 5, and 7 days postamputation (Fig. 3). The partially amputated fin in the TCDD-exposed zebrafish was significantly inhibited from regenerating while the amputated fin in the control zebrafish regenerated normally. Importantly, the intact fin of the TCDD-exposed zebrafish displayed no signs of fin necrosis and was indistinguishable from the uncut section of the fin of the control fish. We have not observed signs of fin necrosis in zebrafish with doses up to 70 ng/g, suggesting that zebrafish are significantly less sensitive to TCDD compared to other species (Kleeman *et al.*, 1988; Spitsbergen *et al.*, 1988a,b). Overall, these results suggest that TCDD inhibits fin regeneration and this effect is specific to regenerating tissue and not due to generalized fin necrosis.

TCDD Affects Multiple Regeneration Stages

TCDD is a stable, lipophilic molecule known to have a long half-life in biological tissues. Therefore, when zebrafish are exposed to TCDD, the chemical persists in tissues for extended periods of time. This is significant for these studies, because if TCDD is delivered at day zero (day of amputation), then TCDD will be present in the fish throughout the duration of study, and all regeneration stages would be potential TCDD targets. To begin to determine which stage(s) are most susceptible to the actions of TCDD, adult zebrafish caudal fins were partially amputated, and fish were dosed with a single injection of vehicle (control) or TCDD on either 1, 2, 3, or 4 days

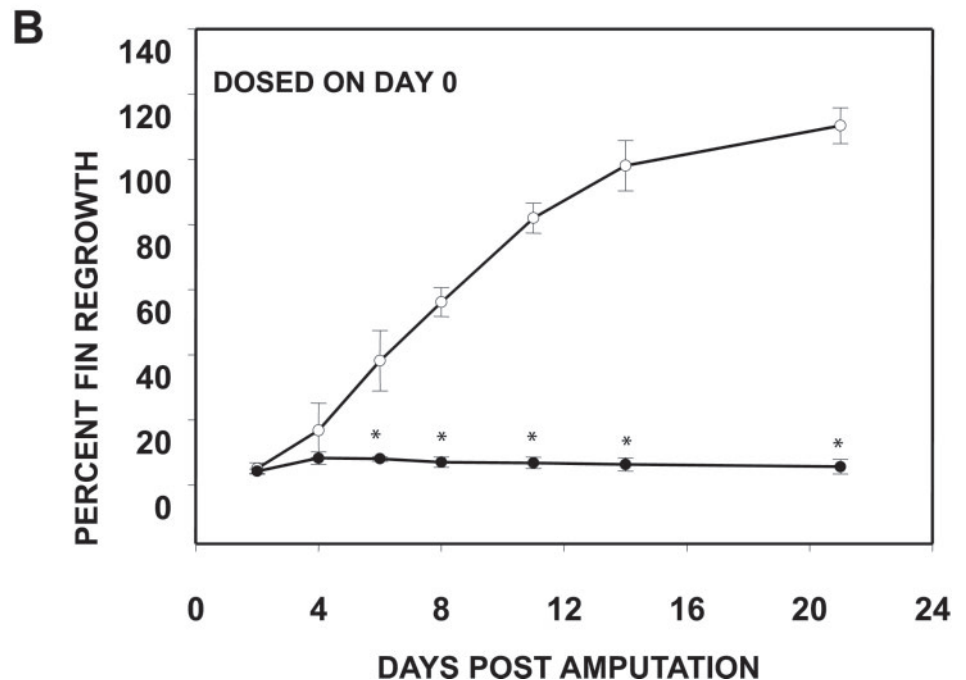
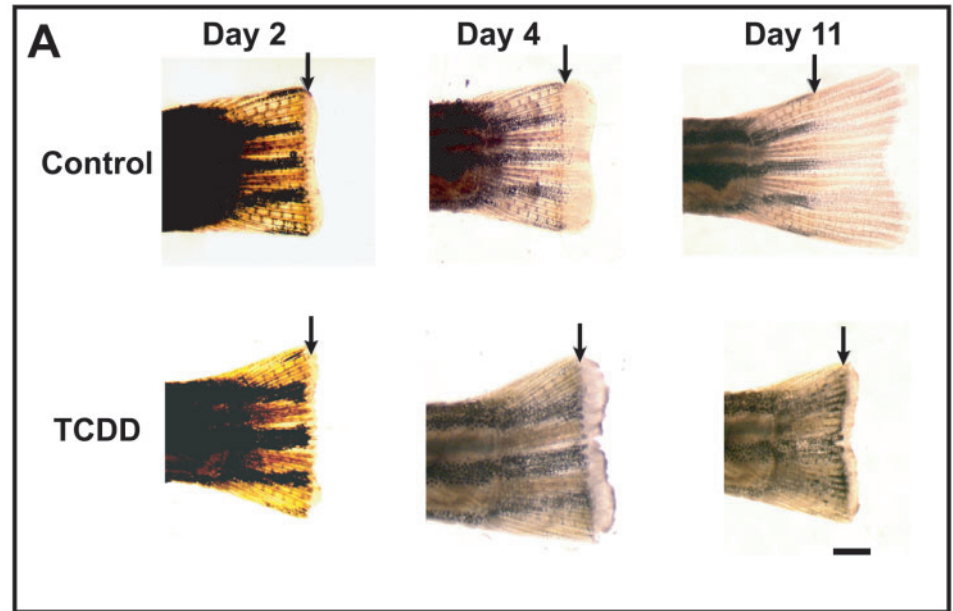


FIG. 2. TCDD exposure results in inhibition of fin regeneration. Adult zebrafish were injected ip with vehicle (control) or 70 ng/g TCDD in vehicle at day 0, followed by partial fin amputation. (A) Fin regeneration images from 2, 4, and 11 days postamputation. Arrows indicate day 0 plane of amputation. (B) Images were acquired on 2, 4, 6, 8, 11, 14, and 21 days postamputation, and the mean percent fin regeneration was determined in TCDD-exposed (closed circle) and vehicle control (open circle) fish. Mean, SD, $n = 6$ per group. Asterisks indicate significant differences compared to the control group at each regeneration stage (t -test, $p < 0.001$). Scale bar = 1.0 mm.

postamputation, and fin regeneration was frequently measured for 21 days. The goal of these studies was to determine if there was a point during regeneration where TCDD exposure no longer inhibited the regeneration process. Fish exposed to TCDD on day 1 regenerated 3% of their fins by day 2, 10% by day 4, and 6% by day 14, while matched control fish regenerated 6% of their fins by day 2, 19% by day 4, and 79% regrowth by day 14 (Fig. 4). The failure of the fin to regenerate in the presence of TCDD occurred in each experimental group.

For example, fish exposed to TCDD on day 3 regenerated 15% by day 4 and 14% by day 14, while control fish regenerated 16% of their fins by day 4 and 70% by day 14. It is apparent from these experiments that after TCDD was delivered to the fish, further regeneration was rapidly and significantly inhibited. Importantly, the inhibition of regeneration is persistent. In all experimental groups, regeneration does not recover if measured as late as 21 days postamputation. In fact, the percent fin regrowth in TCDD-exposed fish appears to decrease between

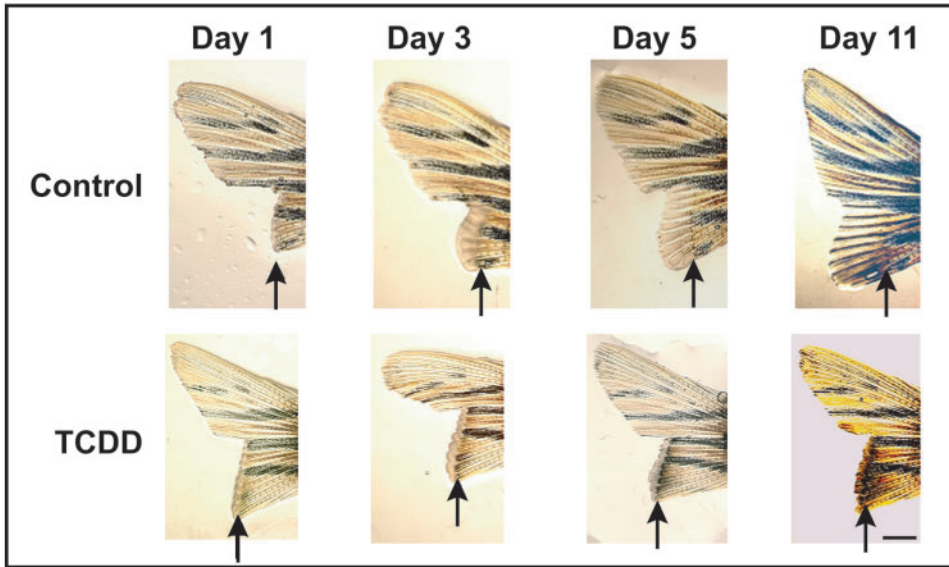


FIG. 3. TCDD preferentially affects regenerating tissue. Adult zebrafish were injected ip with vehicle or 70 ng/g TCDD in vehicle; fins were partially amputated at day 0 and allowed to regenerate. Images were acquired on 1, 3, 5, and 7 days postamputation. Arrows indicate the day 0 amputation plane. Scale bar = 1.0 mm.

14 to 21 days postamputation (Fig. 4). This late stage affect of TCDD on the fin is likely due to shear stress damage to the caudal fin from the lack of peripheral support structure. The overall results of these studies suggest that TCDD interferes with multiple stages of the regeneration process.

TCDD Alters Cell Proliferation in Regenerating Caudal Fins

We have demonstrated that fin regeneration is significantly inhibited by TCDD exposure, which led us to determine what effect TCDD has on cell proliferation in the early stages of fin

regeneration. BrdU is a thymidine analog that incorporates into DNA during the S-phase of cell replication and can be used to measure cell proliferation in tissues. Adult zebrafish were anesthetized and injected with vehicle (control) or TCDD, and their caudal fins were partially amputated. The fish were allowed to recover, and at 18, 42, 66, or 90 hpa, the fish were reanesthetized and injected with 250 μ g/g BrdU. The fish were allowed to recover for 6 h, allowing for BrdU incorporation before complete fin amputation. BrdU positive cells were monitored by whole-mount immunohistochemistry with a primary

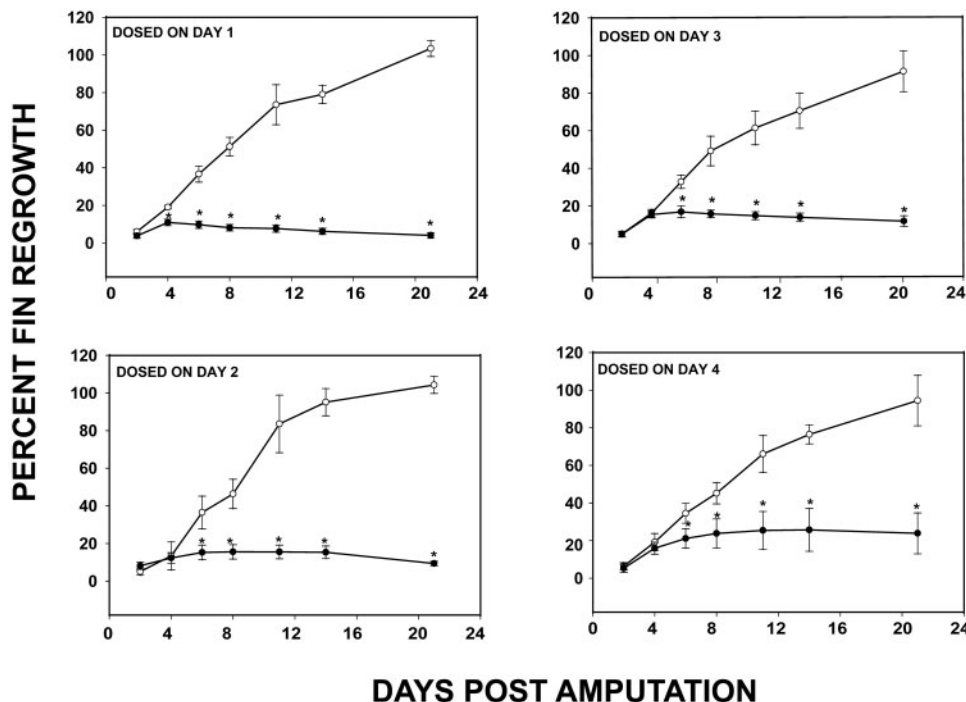


FIG. 4. TCDD inhibits multiple stages of regeneration. Adult zebrafish were anesthetized prior to partial fin amputation on day 0. Vehicle or 70 ng/g TCDD in vehicle was ip injected 1, 2, 3, or 4 days postamputation to determine the effects of TCDD on various stages of regeneration. Images were acquired on 2, 4, 6, 8, 11, 14, and 21 days postamputation, and the mean percentage fin regeneration was determined in TCDD-exposed (●) and vehicle control (○) fish. Mean, SD, $n = 6$ per group. Asterisks indicate significant differences compared to control group at the same regeneration stage (t -test, $p < 0.001$).

anti-BrdU antibody and a fluorescent secondary antibody. Images of BrdU incorporation in the fin were captured using fluorescent microscopy, and BrdU-positive cells were quantified. The number of BrdU positive cells in fins of both control and TCDD-exposed fish were similar for both days 1 and 2 postamputation (Table 1). By day 3, the number of BrdU-positive cells in the regenerating fins of the control fish was 1.25-fold higher than in the TCDD-exposed fish (Table 1). On day 4 postamputation, the number of BrdU-positive cells was 2.4-fold higher in control fish than in TCDD-exposed fish (Table 1 and right side of arrows in Fig. 5A versus Fig. 5B). By day 3 and 4 postamputation, the epidermal cap has formed (asterisk, Fig. 5), and the proliferating cells are concentrated in the blastema of the regenerating fin. It is also important to note that, by 3 and 4 days postamputation, the area of regenerated tissue in the TCDD-exposed fish was significantly smaller than in control fish. Analysis of the nonregenerating adjacent tissue revealed that TCDD did not globally impact cell proliferation in the caudal fin, since BrdU-positive cell numbers in the nonamputated tissue were consistent in vehicle control and TCDD-exposed fins at all time points analyzed (Left of arrows, Fig. 5A versus Fig. 5B).

TCDD Induces Hyperpigmentation of de Novo Tissue

In addition to inhibition of fin regeneration, TCDD exposure also results in hyperpigmentation of new fin tissue. Adult zebrafish caudal fins were partially amputated on day 0 and injected on day 4 with TCDD or control and imaged on day 6 (Fig. 6). The control fins display normal regeneration and pigmentation. However, the TCDD-exposed fish display a darkening of the tissue, or hyperpigmentation, at the end of the fin, specific to the recently regenerated tissue. This hyperpig-

mentation response was a reproducible phenomenon in this experimental system and was evident in the regenerating fins of TCDD exposed-fish in previous experiments (Figs. 2 and 3).

AHR Pathway Is Expressed and Functional during Regeneration

It is widely accepted that TCDD toxicity is mediated by activation of the AHR pathway, and to determine if the AHR pathway was present and active in regenerating fin, we partially amputated the caudal fins of adult zebrafish and injected the fish with vehicle or TCDD in vehicle. The fins were allowed to regenerate for 2, 4, or 6 days, and the recently regenerated tissue was surgically removed for total RNA isolation. Nonquantitative RT-PCR was performed using gene-specific primers for members of the AHR pathway, including AHR1, AHR2, ARNT2a, ARNT2b/c, and CYP1A (Fig. 7). AHR2, ARNT2b/c, and CYP1A were expressed at all three postamputation time points in regenerating fins from both TCDD-exposed and control fish. The precise levels of these transcripts and, more importantly, their protein products remain to be determined. These results do, however indicate that the AHR pathway is indeed present and functional during the stages of regeneration affected by TCDD.

DISCUSSION

Zebrafish fin regeneration consists of several specific stages including epithelial cap formation, dedifferentiation of mesenchymal cells, differentiation of cells forming a blastema (which leads to formation of epithelium, nerves, and vessels), and finally, outgrowth of the blastema and regeneration of the fin (Johnson and Weston, 1995; Poss *et al.*, 2000b; Santamaria and Becerra, 1991). Our results indicate that TCDD interferes with several stages of the regeneration process.

There are a number of potential underlying mechanisms that may explain how TCDD impacts this dynamic developmental process. TCDD-induced activation of the AHR pathway leads to increased CYP1A expression in a variety of tissues in adult zebrafish, including kidney, liver, gastrointestinal tract, gill, heart, and caudal fin (Zodrow *et al.*, in press). In addition, CYP1A mRNA tissue expression was significantly increased in heart, muscle, gill, eye, kidney, and fin following exposure to TCDD (Andreasen *et al.*, 2002a). Cytochrome P4501A1 induction by TCDD has been associated with oxidative stress and DNA damage in mammals (Shertzer *et al.*, 1998; Tritscher *et al.*, 1996). TCDD-induced oxidative stress leads to TCDD-induced embryotoxicity in medaka (Cantrell *et al.*, 1996). Further support for the potential role for AHR2 and CYP1A in TCDD-dependent toxicity in fish was revealed in recent morpholino gene repression studies. Repressing AHR2 protein expression in larvae zebrafish repressed CYP1A expression and blocked the signs of TCDD developmental toxicity including pericardial edema and circulation deficiencies (Prasch *et*

TABLE 1
BrdU Incorporation in Regenerating Caudal Fins

Days post amputation	Total BrdU + cells in new tissue	New tissue area (mm ²)	Cells per area (mm ²)	Cells per area adjacent tissue (mm ²)
Control				
1	98 ± 24	26 ± 8	3.8 ± 2.9	1.63 ± 0.15
2	202 ± 38	37 ± 6	5.5 ± 6.0	1.37 ± 0.09
3	847 ± 64	140 ± 1	6.1 ± 2.9	1.02 ± 0.10
4	1332 ± 48	243 ± 11	5.5 ± 4.8	1.21 ± 0.11
TCDD exposed				
1	110 ± 28	28 ± 2	3.9 ± 3.2	1.31 ± 0.17
2	251 ± 75	51 ± 19	4.9 ± 3.9	1.29 ± 0.06
3	677 ± 47*	59 ± 5	11.4 ± 9.3	1.04 ± 0.07
4	550 ± 84*	108 ± 35	5.1 ± 2.4	1.14 ± 0.08

Note. Asterisks indicate statistical significance compared to controls at same time point; data presented as mean ± SEM for each group; n = 3 (p < 0.05).

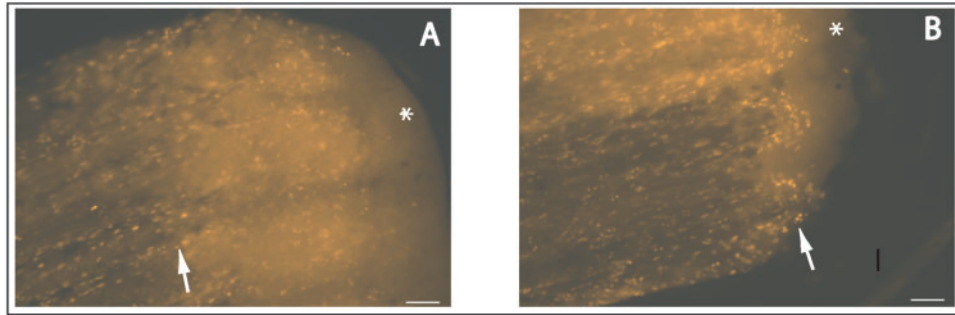


FIG. 5. TCDD exposure results in reduced BrdU incorporation in the regenerating fin. Adult zebrafish were anesthetized and injected with vehicle or 70 ng/g TCDD in vehicle and their fins partially amputated and allowed to regenerate. Fish were anesthetized and injected with BrdU 18, 42, 66, or 90 hpa. Six hours following BrdU injection, the fish were euthanized, and the fins were completely amputated, fixed, and used in whole-mount immunohistochemistry with a primary anti-BrdU antibody and fluorescent secondary antibody. Images display BrdU incorporation on day 4 postamputation in caudal fin of (A) zebrafish injected with vehicle control and (B) zebrafish injected with 70 ng/g TCDD. Arrows indicate day 0 plane of amputation. Asterisk denotes area of epidermal cap. Scale bar = 50 μ m.

al., 2003; Teraoka *et al.*, 2003). Therefore, TCDD-induced abundance of CYP1A in the fin tissue could lead to increased oxidative stress and tissue damage, resulting in impaired fin regeneration.

Other AHR-dependent mechanisms have recently been proposed. It is known from mammalian studies that the AHR interacts with the retinoblastoma protein (RB), which regulates S-phase entry during cell cycle progression (Wang, 1997). When RB is hypophosphorylated, it interacts with the transcription factor E2F and represses transcription of S-phase specific genes containing promoters with E2F sites (Wang, 1997). Phosphorylation of RB by cyclin D-activated kinase 4

(Cdk4) allows separation of RB and E2F, thereby allowing S-phase entry. It has been proposed that AHR binding to RB represses E2F-dependent transcription, allowing induction of cell cycle arrest (Puga *et al.*, 2000). Therefore, TCDD-induced upregulation of the AHR pathway in the fin may lead to increased cell cycle arrest and inhibition of regeneration in the caudal fin.

It remains a possibility that TCDD-mediated block in fin regeneration may be the consequence of pathology in another organ. For example, glycogen depletion has been observed in livers of adult zebrafish exposed to similar levels of TCDD used in this study (Zodrow *et al.*, in press). This could result

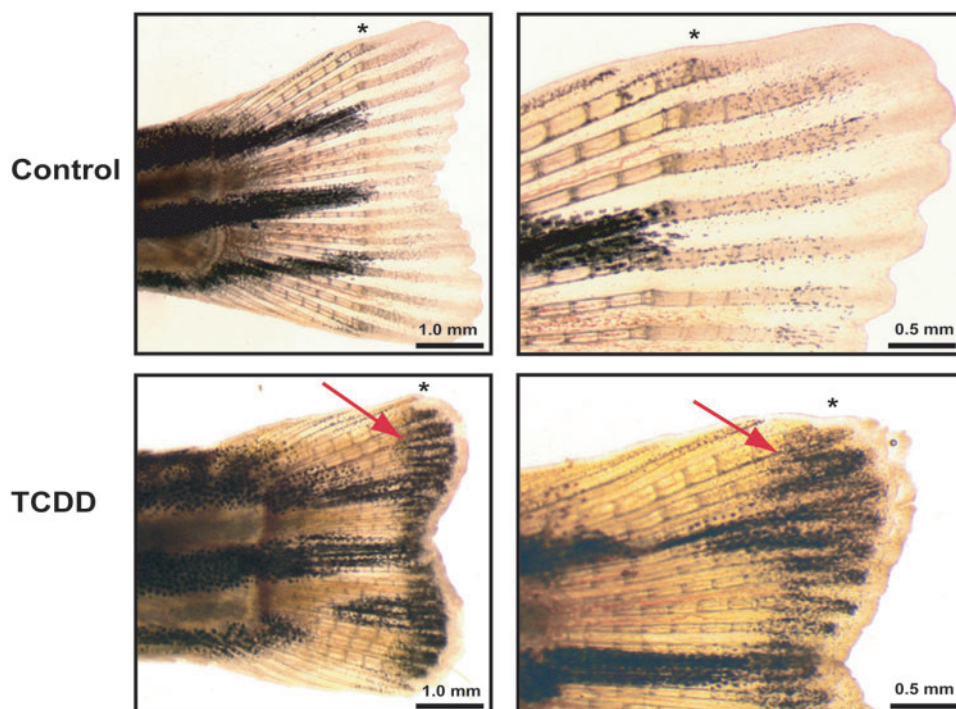


FIG. 6. TCDD exposure results in hyperpigmentation of the fin. Adult zebrafish were injected ip with vehicle or 70 ng/g TCDD in vehicle, and their fins were partially amputated and allowed to regenerate. Full caudal fin and close-up images were acquired 5 days postamputation for both groups. Asterisks represent plane of amputation. Arrows denote area of hyperpigmentation of regenerating fin in the TCDD-exposed fish.

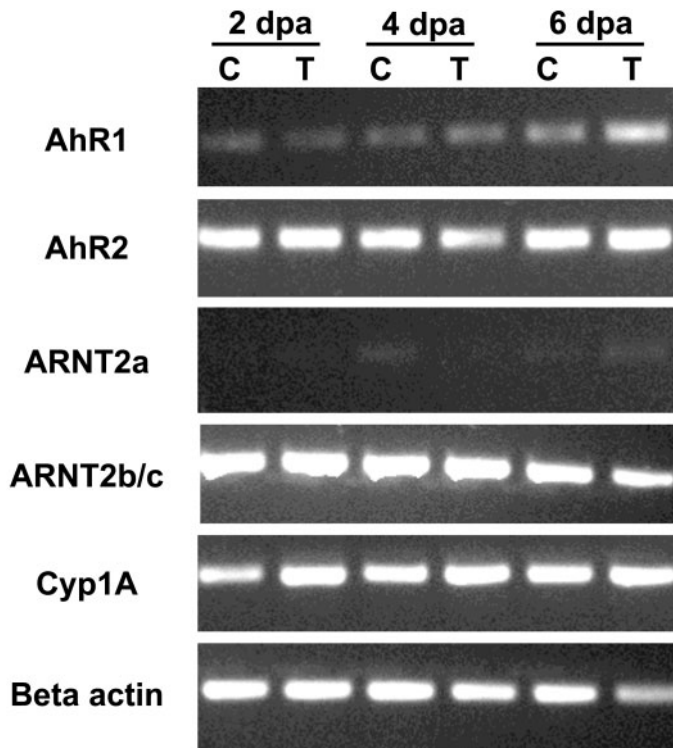


FIG. 7. Non-quantitative RT-PCR to detect members of the AHR signaling pathway during fin regeneration. Adult zebrafish were injected ip with 70 ng/g TCDD (T) or vehicle (C), followed by partial caudal fin amputation. Regenerating fin tissue was removed 2, 4, and 6 days postamputation for total RNA isolation. Total RNA was reverse transcribed and used as templates for PCR using AHR1, AHR2, ARNT2a, ARNT2b/c, CYP1A and β -actin specific primer pairs.

from an alteration in the glycogen synthesis or storage pathways. Alterations in these pathways could lead to alterations in blood glucose levels, decreasing metabolism or energy production and thereby reducing the ability of zebrafish to regenerate their fins. In addition, mammalian studies have demonstrated that TCDD can affect immune system function (Kerkvliet, 2002). The affect of TCDD on the fish immune system is less well studied, a 5- μ g/kg or higher TCDD dose in trout lead to lymphoid depletion following a single TCDD dose (Spitsbergen *et al.*, 1988b). A single 2.93- μ g/kg TCDD exposure in the mirror carp results in lymphocyte depletion and erythrocyte congestion six weeks after TCDD exposure (van der Weiden *et al.*, 1994). The importance of the immune response in orchestrating the regeneration process is not understood, and the effect of TCDD on fish immunity is largely unknown.

In the current study, TCDD exposure produced hyperpigmentation of the regenerating tissue. Pigment in the zebrafish caudal fin consists of melanocytes (black pigmented cells) and xanthophores (yellow pigment cells). The reestablishment of the stripe pattern in the caudal fin begins with the appearance of melanocytes near the preexisting melanocyte stripes at 3 dpa (Goodrich and Nichols, 1931; Goodrich *et al.*, 1954; Rawls and

Johnson, 2000). These melanocytes differentiate *de novo* from unpigmented precursors (Rawls and Johnson, 2000). Skin and fin hyperpigmentation has been observed in carp (*Cyprinus carpio*) and largemouth bass (*Micropterus salmoides*) exposed to TCDD, with increased pigmentation observed with increasing dose (Kleeman *et al.*, 1988). TCDD exposure results in enhanced terminal differentiation and a decrease in epidermal growth factor (EGF) binding in normal human epidermal cells (Osborne and Greenlee, 1985). The differentiation pattern of human keratinocytes is also altered by TCDD exposure (Loertscher *et al.*, 2001), suggesting that the mechanism of hyperpigmentation is tightly tied to altered differentiation. Whether the inability of the fin to regenerate in the presence of TCDD is a consequence of altered differentiation remains to be determined. It is also important to note that hyperpigmentation is a hallmark sign of accidental human TCDD exposures (Caramaschi *et al.*, 1981; Cook, 1981; Crow, 1978; Pocchiari *et al.*, 1979; Reggiani, 1980), and it is intriguing to speculate that the mechanisms underlying these pigmentation responses are similar in these species.

Signaling events between epithelial cells and adjacent mesenchyme play an important role in cell proliferation and patterning during fin regeneration as well as embryonic development (Akimenko *et al.*, 1995; Laforest *et al.*, 1998; Poss *et al.*, 2000a). While a number of the signaling pathways have been studied during fin regeneration, the exact epithelial-mesenchymal signaling interactions have yet to be determined. Retinoic acid receptor γ (White *et al.*, 1994), zebrafish homeobox genes *msxB* and *msxC* (Akimenko *et al.*, 1995), fibroblast growth factor receptor 1 (*fgfr1*) (Poss *et al.*, 2000b), as well as the cell cycle regulator *Mps1* (Poss *et al.*, 2002) are all expressed in the blastemal tissue during fin regeneration. The members of the sonic hedgehog signaling pathway, sonic hedgehog (*shh*), patched 1 (*ptc1*), and bone morphogenetic protein (*bmp2*) are all expressed in the basal layer of the epithelium during fin regeneration (Laforest *et al.*, 1998) in addition to the transcription factor *Lef1* (Poss *et al.*, 2000a). *Ptc1* and *bmp2* are also expressed in scleroblasts and may have a role in formation of the lepidotrichia. Studies are just beginning to understand the important role these signaling molecules play in fin regeneration. For example, exogenous retinoic acid treatment results in decreased expression of *shh*, *ptc1*, and *bmp2*, as well as inhibition of regeneration upon immediate exposure following amputation of the fin, and narrowing of the rays and fusion of the rays when administered at later time points (Ferretti and Geraudie, 1995; White *et al.*, 1994). Results from AHR-null mice have demonstrated the functional importance of AHR for normal retinoid homeostasis (Andreola *et al.*, 1997). When a specific inhibitor of *Fgfr1* (SU5402) is administered immediately following fin amputation in zebrafish, blastema formation is inhibited, while administration during ongoing fin regeneration prevents further outgrowth. SU5402 also results in the downregulation of *msx* and *shh* genes. The potential interaction

between the AHR signaling and other cellular signaling pathways awaits further evaluation.

In the current study, we have demonstrated that fin regeneration is inhibited following TCDD exposure and is independent of the stage of regeneration at the time of exposure (Figs. 2 and 4). In addition, BrdU cell proliferation assays reveal that TCDD exposure results in formation of a blastema at 3 and 4 days postamputation, but blastema outgrowth never occurs as in control fish (Fig. 5). This result is similar to the response seen in zebrafish fin regeneration following administration of the *fgfr1* inhibitor SU5402. No direct link between TCDD and Fgf has been demonstrated; however, Fgf administration leads to an increase of AHR in fibroblast cells (Vaziri *et al.*, 1996).

TCDD-mediated inhibition of fin regeneration may be a consequence of AHR-dependent cross-talk between other signal transduction pathways required for fin regeneration. Direct cross-talk between signaling pathways is possible when transcription factors require a common protein partner to function. When the availability of the common partner is limited, competition could result in reduced transcription of downstream targets termed squelching (Gill and Ptashne, 1988). For example, in addition to AHR binding, ARNT is a dimerization partner for the hypoxia inducible factor 1 α (HIF-1 α). HIF-1 α is a member of a family of proteins that are activated by reduced cellular O₂ concentrations, allowing increased transcription of a number of hypoxia-regulated genes including vascular endothelial growth factor (VEGF), erythropoietin (EPO), and glycolytic enzymes (Bunn and Poyton, 1996; Goldberg and Schneider, 1994; Jiang *et al.*, 1996; Semenza *et al.*, 1994; Wang *et al.*, 1995). These genes are involved in glycolysis, wound healing, and neovascularization, processes that are critical for fin regeneration. Activation of AHR by TCDD could lead to competition for ARNT by HIF-1 α , culminating in the downregulation of genes important in neovascularization. Cell culture experiments clearly demonstrate interactions between the AHR and HIF-1 α pathways (Chan *et al.*, 1999; Gassmann *et al.*, 1997; Gradin *et al.*, 1996). The possibility also exists that currently uncharacterized proteins may also form functionally important dimers with AHR. In addition, activation of AHR by TCDD or other agonists could result in altered protein-protein interactions affecting non-AHR transduction pathways. Therefore, there exist several potential points of cross-talk between the AHR and other signal transduction pathways.

We have observed that TCDD inhibits zebrafish caudal fin regeneration at multiple regeneration stages and induces hyperpigmentation of *de novo* tissue. Since it is well accepted that the initial stages of TCDD toxicity are mediated by the AHR, these results indicate that inappropriate activation of the AHR pathway is detrimental to the complex process of tissue regeneration. Currently, the precise mechanism of TCDD toxicity remains unclear, but this caudal fin model provides unique advantages to further our understanding of TCDD toxicity and tissue regeneration.

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