

Aryl Hydrocarbon Receptor Activation Impairs Extracellular Matrix Remodeling during Zebra Fish fin Regeneration

Eric A. Andreasen,*†‡§ Lijoy K. Mathew,*†‡§ Christiane V. Löhr,†‡§ Rachele Hasson,*†‡§ and Robert L. Tanguay‡§¹

*Department of Environmental and Molecular Toxicology; †Department of Veterinary Medicine; ‡Environmental Health Sciences Center; and §Marine and Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, Oregon 97331

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Adult zebra fish completely regenerate their caudal (tail) fin following partial amputation. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits this regenerative process. Proper regulation of transcription, innervation, vascularization, and extracellular matrix (ECM) composition is essential for complete fin regeneration. Previous microarray studies suggest that genes involved in ECM regulation are misexpressed following activation of the aryl hydrocarbon receptor. To investigate whether TCDD blocks regeneration by impairing ECM remodeling, male zebra fish were i.p. injected with 50 ng/g TCDD or vehicle, and caudal fins were amputated. By 3 days postamputation (dpa), the vascular network in the regenerating fin of TCDD-exposed fish was disorganized compared to vehicle-exposed animals. Furthermore, immunohistochemical staining revealed that axonal outgrowth was impacted by TCDD as early as 3 dpa. Histological analysis demonstrated that TCDD exposure leads to an accumulation of collagen at the end of the fin ray just distal to the amputation site by 3 dpa. Mature lepidotrichial-forming cells (fin ray-forming cells) were not observed in the fins of TCDD-treated fish. The capacity to metabolize ECM was also altered by TCDD exposure. Quantitative real-time PCR studies revealed that the aryl hydrocarbon pathway is active and that matrix-remodeling genes are expressed in the regenerate following TCDD exposure.

Key Words: AHR; TCDD; regeneration; zebra fish; extracellular matrix.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known to cause a wide array of toxicities that are primarily dependent upon activating the aryl hydrocarbon receptor (AHR) (Fernandez-Salguero *et al.*, 1996; Mimura *et al.* 1997). The events downstream of AHR activation that mediate these toxicities remain elusive partially because of the broad diversity of toxicities mediated by TCDD. The use of alternative whole animal models may help to identify unifying mechanisms of TCDD toxicity that cause such diverse toxicities in

mammals as epithelial hyperplasia and metaplasia, lymphoid involution, porphyria, wasting syndrome, tumor promotion, and death (reviewed in [Birnbaum and Tuomisto, 2000; Gu *et al.*, 2000]) in addition to reproductive and developmental toxicities (reviewed in [Birnbaum and Tuomisto, 2000]). The regenerating fin may reveal some common mechanistic responses to TCDD since this regenerative process is halted by TCDD exposure (Zodrow and Tanguay, 2003), and the regenerating fin requires dynamic physiological alterations including cellular differentiation and dedifferentiation, wound healing, revascularization, innervation, and boney ray formation. Both mammals and zebra fish (*Danio rerio*) require the AHR to mediate TCDD toxicity. The AHR signal transduction pathway is similar in mammals and fish except that fish have two or more AHR genes while mammals have only one (reviewed in [Hahn *et al.*, 1997; Tanguay *et al.*, 2003]). TCDD toxicity in zebra fish is AHR2- and ARNT1-dependent (Andreasen *et al.*, 2002a; Mathew *et al.*, 2006; Prashc *et al.*, 2003, 2004, 2006).

Upon amputation, adult zebra fish have the capacity to completely regenerate their caudal (tail) fin within 14 days by an epimorphic process (reviewed in [Poss *et al.*, 2003]). Epimorphic regeneration occurs by the reprogramming and migration of cells that differentiate and restore a tissue to its original form. TCDD impairs this process if exposure occurs within 4 days of amputation (Zodrow and Tanguay, 2003). The caudal fin is comprised of a series of boney rays that are attached to the skeleton by ligaments. Each ray, referred to as a lepidotrichium consists of two longitudinal hemirays that form a tube-like structure. The hemirays are divided into repeating segments joined by ligaments. Fins grow by successive addition of hemiray segments to the most distal (end of fin) segment. Blood vessels, nerves, and mesenchymal cells populate the area between the hemirays of a lepidotrichium. After amputation, the wound site is covered within 12 h by migrating non-proliferating epithelial cells forming an epithelial cap, several cells thick, which contains a basement membrane. Once this apical epithelial cap is formed, dedifferentiated cells proximal to the wound site migrate under the epithelia cap and proliferate forming a structure called the blastema by 2 days

¹ To whom correspondence should be addressed at Department of Environmental and Molecular Toxicology, Oregon State University, 1007 ALS, Corvallis, OR 97331-7301. Fax: (541) 737-7966. E-mail: robert.tanguay@oregonstate.edu.

postamputation (dpa). Most of the blastema cells are derived from cells within two hemiray segments anterior to the amputation site, but can be derived from cells as far as seven segments away (Poleo *et al.*, 2001). These blastemal cells proliferate and differentiate to form fibroblasts and ray-forming cells (lepidotrichial-forming cells [LFCs] or scleroblasts) in the regenerating tissue. Formation of fin rays occurs by direct mineralization of collagen laid down by the LFCs (Geraudie and Landis, 1982). The new tissue is then innervated and repopulated with functional vascular tissue and boney fin rays during the regenerative outgrowth phase that occurs from 2 to 14 dpa.

In addition to regeneration, wound healing also occurs during fin regeneration. Wound repair and regeneration requires interactions between the plasminogen (fibrinolytic system) and matrix metalloproteinase (MMP) enzyme systems to regulate extracellular matrix (ECM) composition, cell migration, and adhesion (reviewed in [Li *et al.*, 2003; Lund *et al.*, 1999]). In addition to wound healing, the plasminogen and MMP systems have been implicated in development, branching morphogenesis, angiogenesis, ECM degradation, bone remodeling, nervous system development, mammary gland development, ovulation, tumorigenesis, and embryo implantation. A brief summary of how these systems interact is described below (Fig. 1) (reviewed in [Binder *et al.*, 2002; Irigoyen *et al.*, 1999; Li *et al.*, 2003; Lijnen, 2002; Visse and Nagase, 2003; Vu and Werb, 2000]). Plasminogen is an inactive proenzyme that is produced primarily in the liver and circulates in the blood. It is proteolytically activated to form plasmin which serves to degrade fibrin into soluble products, activate MMPs

(1,2,3,9,10,13), latent TGF- β , and urokinase plasminogen activator (uPA). It is enzymatically activated to plasmin at sites of action by plasminogen activators, uPA or the tissue-type plasminogen activator (tPA). Plasminogen activation is inhibited by the plasminogen activator inhibitors (PAIs). MMPs are a group of at least 23 extracellular proteases that are secreted into the ECM, are proteolytically activated, and function in wound repair to degrade ECM components including collagen (MMP-1,8,13) and gelatin (MMP-2,9). The activity of MMPs is inhibited and regulated by tissue inhibitors of metalloproteinases (TIMPs). TGF- β plays a key regulatory step in activating MMP and plasminogen activity. Latent TGF- β is activated by plasmin which then stimulates the expression of PAI-1 and TIMP-1, while suppressing expression of uPA. Alterations in this complex regulatory pathway could directly affect the structural architecture of a tissue but it can also affect cell growth, cell migration, cell-cell communication, apoptosis, and angiogenesis since ECM also serves as a source and store of biologically active molecules which can be released and or activated by MMPs (reviewed in [Mott and Werb, 2004]). It is not surprising then that the dynamic and complex event of fin regeneration requires a functional MMP system (Bai *et al.*, 2005).

Global gene expression studies determined that the largest category of genes affected by TCDD during fin regeneration were those involved in the structure and remodeling of the ECM (Andreasen *et al.*, 2006). Thirty-four of the 41 ECM genes misregulated by TCDD were repressed, suggesting that TCDD inhibits the maturation of the ECM. This is consistent with the findings that proper regulation of collagen processing and MMP activity is necessary for fin regeneration (Bai *et al.*, 2005; Bechara *et al.*, 2000). Because of this and the recognition that both wound healing and regeneration are occurring, the effects of TCDD on ECM parameters including collagen and proteoglycan localization, MMP activity, neovascularization, and innervation were evaluated. Three days after amputation and TCDD exposure, collagen and proteoglycan localization were impaired. TCDD exposure also repressed the regeneration of the vascular and neuronal tissues. MMP9 activity remained elevated in the fins of TCDD-exposed fish. These findings support the previous microarray studies in that they suggest that the ECM is disorganized in response to AHR activation.

MATERIALS AND METHODS

TCDD dosing and fin amputation. Adult male zebra fish (AB strain, Eugene, OR) were anesthetized with tricaine methanesulfonate (MS-222), and then their caudal fins were amputated near the bifurcation of the fin rays. Fish were then injected with phosphatidylcholine liposomes (vehicle control) alone or liposomes incorporated with TCDD resulting in a final dose of 50 ng TCDD/g fish (Andreasen *et al.*, 2002b). This dose is equivalent to the EC₉₀ for impaired fin regeneration and does not lead to overt toxicity such as fin necrosis, hemorrhaging, wasting, or alterations in swimming behavior and feeding. At the indicated dpa, fish were euthanized with an over dose of MS-222. Caudal fin tissue was isolated from regenerating (posterior) and nonregenerating (anterior) portion of each fin (Fig. 2). Samples denoted as posterior were comprised of the

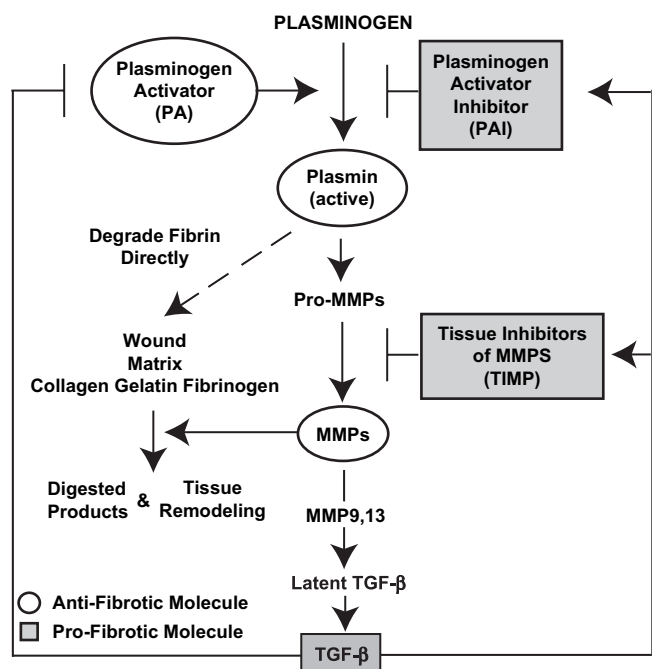


FIG. 1. Plasminogen and MMP pathway in wound repair. Modified from Li *et al.* (2003).

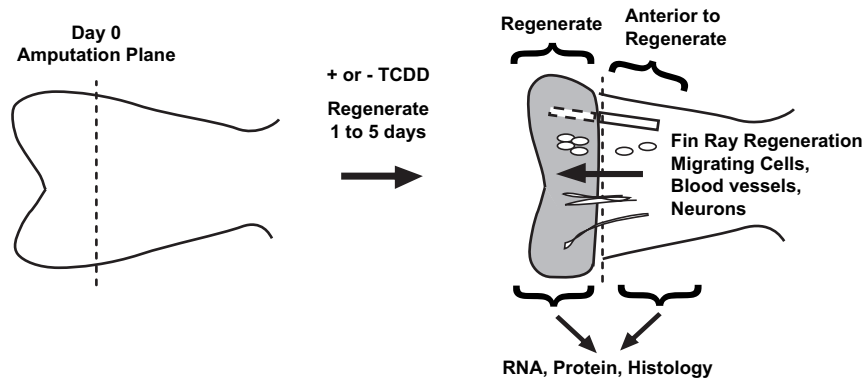


FIG. 2. Overall experimental design. Adult male zebra fish caudal fins were amputated and the fish were injected with vehicle or 50 ng/g TCDD and allowed to regenerate up to 5 days. Regenerating tissue posterior to the amputation plane and also anterior to the amputation site were collected for mRNA, protein and histological analysis at 1, 3, and 5 dpa.

regenerating tissue within 2 hemiray units anterior to the original amputation site. Anterior tissues were comprised of the nonregenerating fraction of the caudal fin specifically 2–7 hemiray units anterior to the original amputation plane. In some cases, nonregenerating fins (0 dpa, never amputated) were used as a control.

Vascular localization and whole mount immunolocalization of neurons and cartilage. The tissue distribution of neurons and a condensation of cells previously identified as the LFCs in zebra fish caudal fins were determined using the zn-12 and zns5 antibodies, respectively (U. Oregon, Eugene, OR) (Johnson and Weston, 1995; Trevarrow *et al.*, 1990). Identification of the AHR responsive gene product cytochrome P4501A (CYP1A) was determined by immunolocalization with a fish-specific anti-CYP1A antibody (Cayman Chemical, Ann Arbor, MI). Fins of TCDD or vehicle control-exposed fish were fixed overnight in 4% paraformaldehyde in PBS and washed 3 × 15 min in PBS + 0.1% Tween-20 (PBST). Fins were washed in dH₂O for 1 h, then permeabilized with – 20°C acetone for 20 min, and rinsed in dH₂O before digestion with collagenase (1 mg/ml) for 90 min. Permeabilized fins were blocked in 10% normal calf serum in PBST, then incubated for 14 h with the primary antibodies (1:500) at 4°C. Following 3 × 10 min washes with PBST, fins were incubated with a secondary antibody for 5 h at 22°C. The zns5 and zn-12 antibodies were detected with Alexa-488-conjugated goat antimouse secondary antibody while the CYP1A antibody was detected with Alexa-546-conjugated goat antirabbit secondary antibody (Molecular Probes, Eugene, OR). Tissues were then washed 3 × 10 min in PBST and visualized by epifluorescence microscopy. The co-localization of CYP1A and the vasculature were determined with the use of a transgenic fish that expresses green fluorescent protein in the vasculature and a fish-specific CYP1A antibody. The green fluorescent protein (GFP) transgene was driven by the vascular-specific fli promoter and was kindly provided by Dr Brant Weinstein of the Unit on Vertebrate Organogenesis at the National Institutes of Health.

Histochemical localization of collagen and histopathology. Fins from control and TCDD-treated fish were fixed in Bouins solution for 20 h followed by decalcification in 5% EDTA for 3 days. Tissues were then dehydrated, blocked in paraffin, and 6 μm and 3 μm sections were prepared. After removal of paraffin and hydration, 6-μm sections were stained in 0.2% Sirius Red in saturated picric acid (Becerra *et al.*, 1996; Bechara *et al.*, 2000) and 3-μm section stained with Hematoxylin and Eosin. Tissues were then dehydrated and mounted. Collagen was detected by visualization under polarized light using a Leica DMRB microscope (Bannockburn, IL) with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Histopathologic examination was performed by a board-certified pathologist using a Nikon Eclipse E400 light microscope.

mRNA abundance. Total RNA was isolated in triplicate from vehicle control and TCDD-treated fish at 0, 1, 3, and 5 dpa ($n = 10$ /replicate). cDNA was prepared from 2 μg of total RNA per group using Superscript II (Life Technologies, Gaithersburg, MD) and oligo dT primers in a 20 μl volume.

Quantitative PCR using gene-specific primers was conducted using the Opticon 2 real-time PCR detection system (MJ Research, Waltham, MA). Specifically, 1 μl of each cDNA pool was used for each PCR reaction in the presence of SYBR Green (Finnzymes, Espoo, Finland), according to the manufacturer's instructions. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were used to confirm the formation of specific products. Negative controls for each experiment consisted of RNA without reverse transcriptase and another without template. Standard curves for each primer pair were generated in duplicate for each experiment from known amounts of DNA template. The abundance of each transcript was normalized to the level of β-actin. Oligonucleotide primers used for these experiments are listed in Table 1 with the sense strand labeled F and the antisense primer labeled R. All oligonucleotides were synthesized by MWG-Biotech (High Point, NC).

Zymography. Fin tissue anterior and posterior to the amputation plane were isolated at 0, 1, 3, and 5 dpa from vehicle- or TCDD-exposed fish as described above. Tissues from six animals were pooled to make a single sample, and the experiments were replicated in triplicate. The tissue was homogenized in extraction buffer (10mM sodium phosphate buffer, pH 7.2, 150mM

TABLE 1
Oligonucleotides Used for qRT-PCR

Target gene	Sequence 5' to 3'
F AHRR1	ACCAGACAGACGTGATGCACC
R AHRR1	ATCAGACTGGAAACAGGCTGC
F AHRR2	TGTGGCTGTTCTCTAGTGC
R AHRR2	GAAACATGCGAGTTGAGAGGC
F AHR2	TGGACTAGATCAGACAACCC
R AHR2	GAAGAGGGAGAGTCATTGTG
F β-actin	AAGCAGGAGTACGATGAGTC
R β-actin	TGGAGTCTCAGATGCATTG
F CYP1A	TGCCGATTTTCATCCCTTTCC
F CYP1A	AGAGCCGTGCTGATAGTGTC
F MMP2	TTGCTGGGAAACAAGTTCTGG
R MMP2	AAGGGCATAGAGCTGAATGG
F MMP9	TCTACTGGAGAATGAATGCC
R MMP9	ACATTTCTGTCTGTCTCTCC
F MMP13	AGGTGAGAAGGTTTGGGCTC
R MMP13	AGTTTAAACCTCTGTACTGG
F TGF-β1	TTCCGAAAGATCTGGGTTGG
R TGF-β1	TTGCTCCACCTTGTGTTGCC
F tPA	TCAGAGAGGATCAAGCGTGG
R tPA	AACCCATCAGAGTCATCCTG

NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% Sodium Azide), and the lysate was purified by centrifugation at 12,000 g. The protein concentration was determined with the BCA assay (Pierce, Rockford, IL). From each pool of tissue, 10 μ g of protein was resolved under nondenaturing conditions in an 8% SDS-PAGE gel containing 0.8 mg/ml gelatin (Calbiochem, San Diego, CA) or 1.0 mg/ml β -casein (ICN Biochemicals, Aurora, OH). Resolved gels were then washed 3 \times 15 min with 2.5% Triton X-100 in 50mM Tris (pH 7.5). The gels were then washed 3 \times with 50mM Tris (pH 7.5) and incubated at 37°C for 16 h in reaction buffer. Gelatinase reaction buffer (50mM Tris-HCl, 5mM Ca²⁺, 1 μ M Zn²⁺, 0.02% NaN₃) contained Zn²⁺ and Ca²⁺ while the β -caseinase did not (50mM Tris-HCl, 0.02% NaN₃). Detection of enzymatic activity was determined as loss of substrate from the gel as detected by Coomassie Brilliant Blue G-250 Blue staining.

Statistical analysis. qRT-PCR data were log₁₀ transformed, and two-way ANOVA was performed to determine if there was a significant effect of TCDD exposure on mRNA abundance. Differences between groups were analyzed by the Tukey method ($p < 0.05$). Statistical analyses were performed using SigmaStat software (Chicago, IL).

RESULTS

Immunohistochemistry

CYP1A was localized in vehicle control- or TCDD-exposed fins at 3 and 5 dpa to identify tissues with an active AHR

pathway (Fig. 3). Knowing that CYP1A is highly expressed in the endothelium, we also documented the expression of a GFP transgene under the control of the fli vascular-specific promoter to determine if neovascularization was impaired by TCDD (Fig. 3). Since fin regeneration is not overtly impaired by TCDD until after blastema formation (2 dpa) and the vasculature is not particularly evident until 2–3 dpa, only the 3 and 5 dpa time points are shown. Neovascularization of the regenerating fin occurs by first healing the severed vessels (joining the arteries and veins) by 1 dpa from which sprout an unstructured plexus consisting of a broad unorganized endothelium (Huang *et al.*, 2003). The plexus is pruned back, forming a more ordered vasculature during the first week of regeneration. Outgrowth and neovascularization of the fins at 3 dpa were substantially greater in the vehicle control-exposed fish as illustrated by the extent of fli-directed GFP expression in the regenerating tissue. By 3 dpa, CYP1A is evident in the vasculature of regenerating and nonregenerating (anterior to the amputation site) tissue of TCDD-exposed fish but not vehicle control-exposed fish (Fig. 3). The vasculature of the vehicle-treated fish at 5 dpa is more mature and extensive than the TCDD-exposed fish as revealed by the fli transgene. This impaired neovascularization

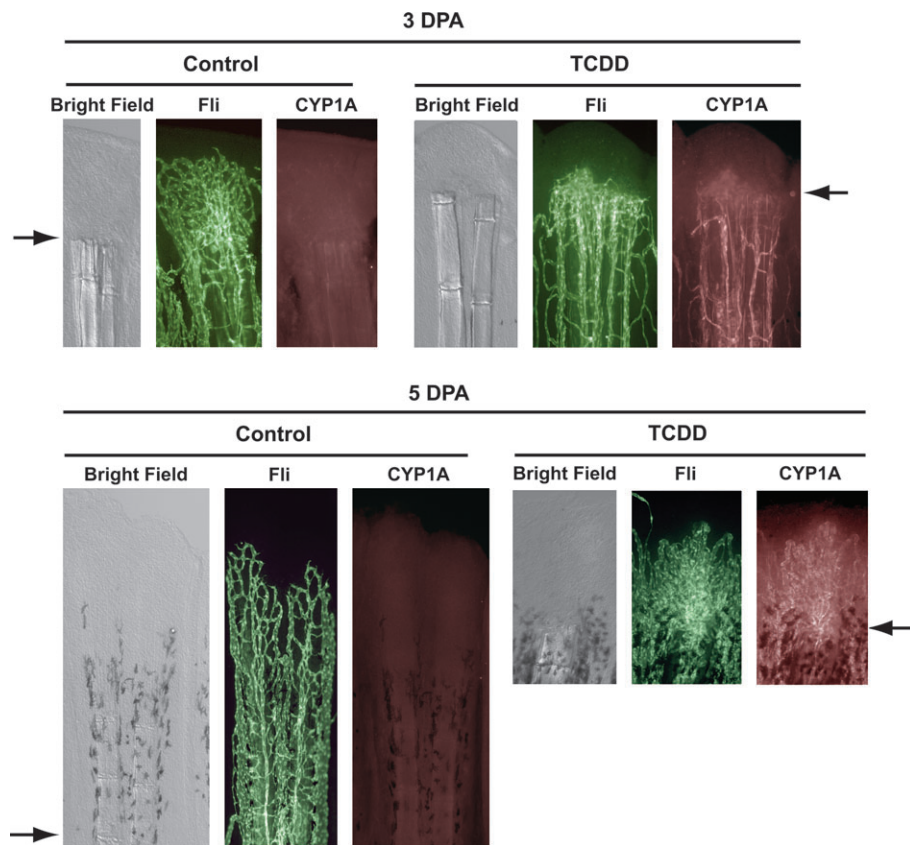


FIG. 3. Whole mount immunolocalization of CYP1A and colocalization with a vascular marker in the regenerating fins of vehicle and TCDD-exposed fish at 3 and 5 dpa captured with at 20 \times magnification objectives. Vascular tissue was identified by the expression of a GFP transgene under the control of the endothelial-specific fli promoter. CYP1A was detected with a fish-specific antibody for CYP1A (Cayman Chemical). Arrows indicate the plane of amputation; representative images from six control and six TCDD-exposed fish.

observed in the TCDD-treated regenerate is further revealed by the abundant TCDD-dependent CYP1A expression at 5 dpa.

Neuronal processes were immunohistochemically identified in the regenerating fins (Fig. 4A). The processes migrate distally in the regenerating tissue in an ordered branching manner at 3 and 5 dpa in the vehicle control–exposed fish. However, this ordered structure is impaired in the TCDD-exposed fish at both 3 and 5 dpa. LFCs are derived from the blastema from where they migrate and condense and help to give rise to the fin rays (Santamaria and Becerra, 1991). The antibody used to identify LFCs detects all stages of LFC subsequent to their migration and condensation and detects LFCs in the regenerating and nonregenerating tissues (Johnson and Weston, 1995). Condensations of the LFCs were identified at 3 dpa in the regenerating and nonregenerating tissues of vehicle-exposed fish (Fig. 4B). The broad localization of LFCs was curtailed to the distal end of the regenerate by 5 dpa. In

TCDD-exposed fish, this pattern of immunoreactivity was less defined in the regenerating tissue at 3 dpa, however, it was localized along the tracts of the developing fin rays at 5 dpa.

Tissue Structure and Collagen and Proteoglycan Localization

In the regenerating fin tissue, collagen is primarily observed in the epithelia distal to the blastema in both control and TCDD-treated fish at 3 dpa and appears to be more abundant in fins of TCDD-treated fish (Fig. 5). By 5 dpa, collagen in control fins aligns with the developing rays (Fig. 5). In TCDD-treated fish, collagen is not accumulating in the location of the developing fin ray but forms an unorganized fibrotic deposit directly distal to the amputation plane that is not observed in the control fins (Fig. 5). Hematoxylin- and Eosin-stained sections of fins at 5 dpa indicate that TCDD impairs blastema formation, ECM localization, LFC development, and localization along the developing fin ray (Fig. 6). Control tissues develop an apical epithelium, blastema, LFCs, and their associated matrix (Figs. 6A–C). The ray is beginning to regenerate as evidenced by deposits of lepidotrichial matrix extending longitudinally from the amputated mineralized bony lepidotrichia (Fig. 6A). However, this deposit is disorganized forming a dense concentration of ECM between the hemirays in the TCDD-treated fin and along the basement membrane zone of the apical cap (Figs. 6D and 6E). Scattered immature LFCs with cytoplasmic secretory granules were identified in the fins of TCDD-treated fish. However, they did exhibit proper orientation and were arranged in rows along the inner surface of the abnormal lepidotrichial matrix (Figs. 6D and 6E).

Unlike bone, fin rays do not have a cartilaginous stage prior to mineralization. Instead, collagenous precursor rays are directly mineralized forming the bony fin ray (Geraudie and Landis, 1982). The maturation of the ECM in the location of the developing ray was assessed by histochemically detecting proteoglycans using alcian blue. This histochemical stain is commonly used to detect cartilage in whole mount tissues. Tracts of proteoglycans extend from the fin rays at the amputation site into the regenerating tissue at 3 and 5 dpa (Fig. 7). These proteoglycan tracts are not evident at 3 dpa in TCDD-exposed fish and are greatly reduced in comparison to control fins at 5 dpa (Fig. 7).

Zymography

Since the alterations in collagen localization due to TCDD exposure could be due to altered metabolism, we conducted zymographic experiments to determine if TCDD altered gelatinase (MMP2 and MMP9) activity. Fin tissue anterior to the amputation site was also analyzed since some of these cells migrate into the regenerating tissue. Two gelatinases were identified in the regenerating fins, a roughly 90-kDa form (pre-MMP9) and a 72-kDa form (pre-MMP2) (Fig. 7). Both putative MMP2 and MMP9 activity formed two bands indicative of the physiologically inactive preform and the proteolytically active

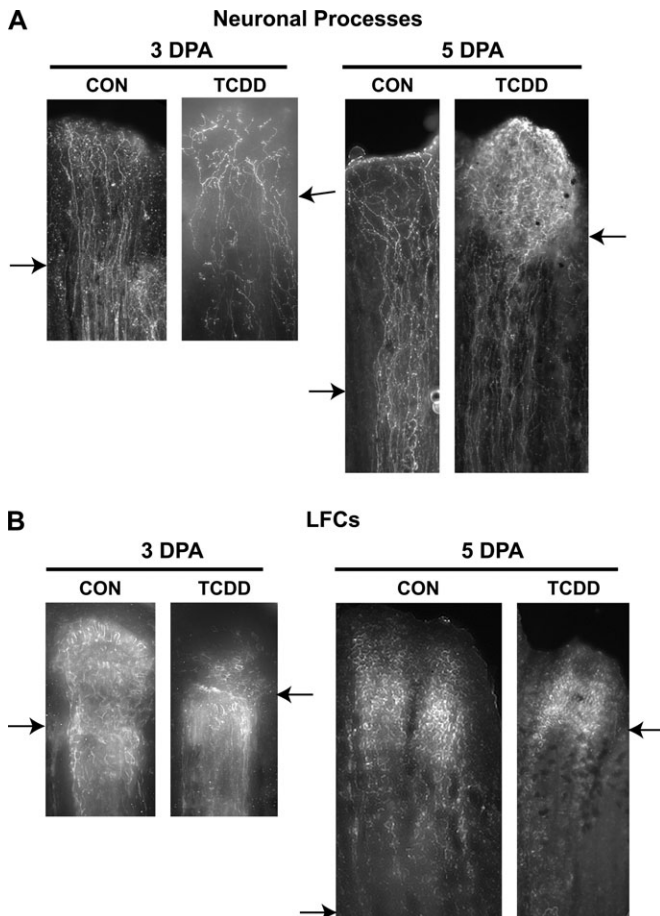


FIG. 4. Whole mount immunolocalization of neuronal and LFCs in regenerating fins of vehicle and TCDD-exposed fish at 3 and 5 dpa. The tissue distribution of neurons and LFCs in zebra fish fins was determined immunohistochemically using the zn-12 and zns5 antibodies (U. Oregon). Arrows indicate the plane of amputation. Images are from representative fins from six control and six TCDD-exposed fish.

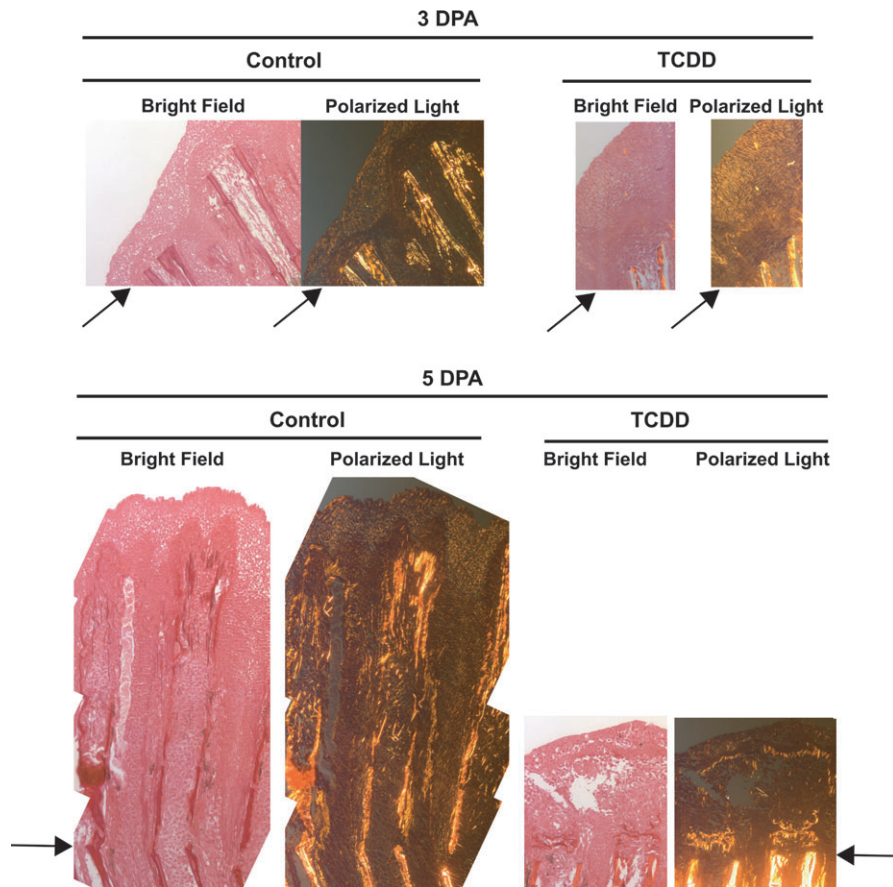


FIG. 5. Histological localization of collagen in regenerating fins of vehicle or TCDD-dosed fish at 3 and 5 dpa captured with at 20 \times magnification objectives. Following outgrowth, 6- μ sections were stained with picosirius red. Tissues were imaged in bright field and the birefringent Sirius Red-stained collagen was detected under polarized light. Arrows indicate the amputation plane. Images are representative sections of fins from six control and six TCDD-exposed fish.

lower molecular weight protein. Based upon molecular weight, pre-MMP2 activity was similar in regenerating and nonregenerating (not amputated, 0 dpa) fins in both the regenerate (Fig. 7B) and fin tissues anterior to the amputation plane (Fig. 7A) at all times and was not altered by TCDD exposure. MMP9 activity was low prior to amputation (0 dpa) and became elevated in regenerating tissue and fin tissue anterior to the amputation site by 1 dpa (Figs. 7A and 7B). The MMP9 activity in vehicle control-exposed fins declined after 1 dpa in the regenerate and fin tissue anterior to the amputation site. MMP9 activity was also elevated by 1 dpa in the TCDD-exposed fin tissues; however, it remained elevated at 3 and 5 dpa (Figs. 7A and 7B). Metabolism of β -casein and fibrinogen (plasmin substrates) was also assessed and did not reveal obvious effects of TCDD exposure (fibrinogen data not shown). The equivalent β -casein activity indicates that equivalent amounts of protein were loaded in each lane. Reverse zymographic techniques using plasminogen- and fibrinogen-containing gels were also studied to assess plasminogen activator activity, but the activity was too low to assess with confidence (data not shown).

qRT-PCR Evaluation of mRNA Abundance

AHR2 and its inhibitors AHRR1 and AHRR2 were quantified to determine if amputation or TCDD exposure altered their expression and CYP1A was measured to evaluate whether the tissues were responsive to TCDD. Additionally, mRNA abundance of ECM-metabolizing enzymes, MMP2, MMP9, and MMP13, and genes that regulate MMP activity (tPA and TGF- β 1) were measured. Regulators of neovascularization VEGF and EPAS1 were not altered by TCDD or amputation (data not shown). Members of the fgf-signaling system (fgf1, FGFR1, fgf8) were also not altered by TCDD exposure (data not shown). Both the regenerating fin tissue and fin tissue anterior to the amputation site responded to TCDD with strong induction of CYP1A message from 1 to 5 dpa (Tables 2 and 3). AHR2 message was detected but unaltered by TCDD in the regenerate and anterior to the regenerate. AHRR1 was detectable only in TCDD-exposed regenerate at 3 and 5 dpa. AHRR2 was more abundant than AHRR1 and responded similarly to TCDD with increased message at 1, 3, and 5 dpa in the regenerate.

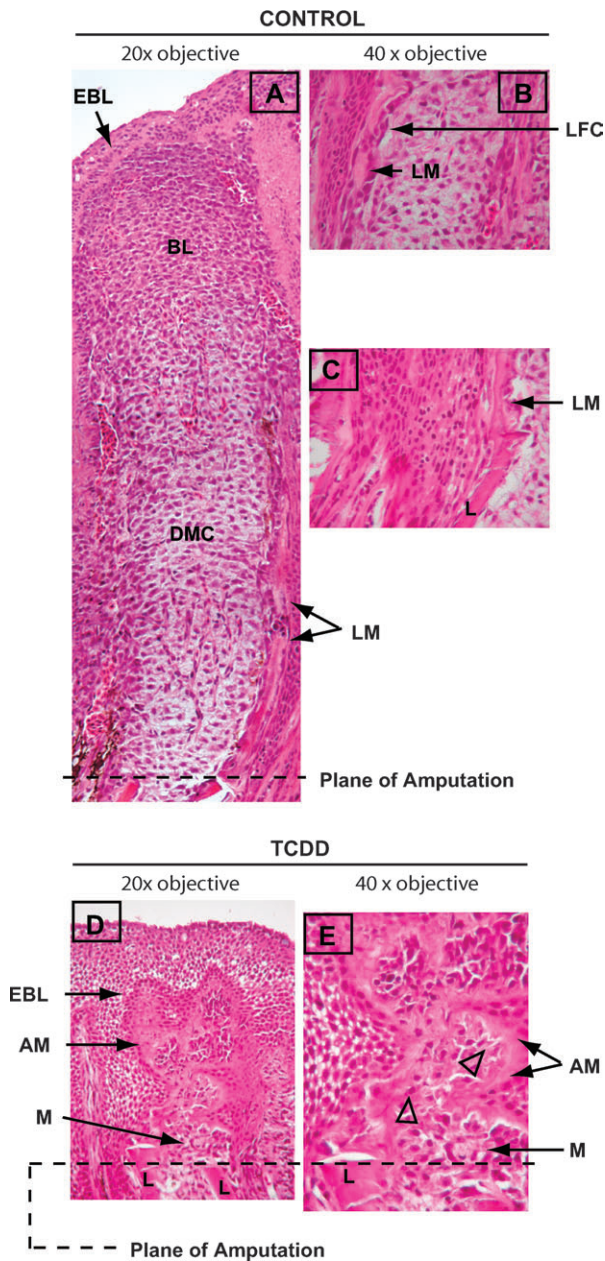


FIG. 6. Structure of the regenerating fin tissues at 5 dpa in vehicle- or TCDD-exposed fish. Hematoxylin- and Eosin-stained tissues at (A) 20 \times vehicle-exposed fin showing the EBL, epidermal basal layer of the apical epidermal cap, the BL, blastema and an ordered column of DMC, differentiating mesenchymal cells. Deposits of lepidotrichial matrix (M) extend longitudinally from the amputated mineralized bony lepidotrichia (L). (B) 40 \times image of vehicle-exposed fin showing a row of LFCs lining the inner surface of the lepidotrichial matrix. (C) Deposition of lepidotrichial matrix is observed to extend from the amputation plane of the lepidotrichia shown here at 40 \times magnification. (D) Fin of a TCDD-treated fish at 5 dpa stained with H&E at 20 \times magnification. Apical epidermal cap, its basal layer (EBL) and lepidotrichia (L) are present; however, the blastema (B) is not evident. Convoluted masses of abnormal ECM (AM) enclose the mesenchymal cells (M) which do not appear to differentiate. (E) The lack of mature LFCs indicated with an open triangle (Δ) is evident along the inner surface of the regenerating ray. Images are representative sections of fins from six control and six TCDD-exposed fish.

MMP2 mRNA expression was only modestly reduced in TCDD-exposed fish at 1 and 3 dpa in both the regenerate and anterior to the regenerate. MMP9 was enhanced by amputation comparing day 0 to the regenerating stages. MMP9 was more that twofold enhanced by TCDD exposure in the regenerate but was not altered by TCDD in tissue anterior to the amputation site. The increased MMP9 activity in response to TCDD is consistent with the increase MMP9 activity (Fig. 8). MMP13, nondetectable at day 0, was greatly elevated by amputation and further elevated by TCDD exposure in the regenerate at 3 and 5 dpa and anterior to the amputation site at 1 and 3 dpa. tPA was not altered by TCDD exposure. Plasmin is the enzyme primarily involved in proteolytic activation of MMPs and is produced primarily in the liver. Plasminogen message was only monitored in the liver and was not altered by TCDD exposure (data not shown).

DISCUSSION

Fin regeneration is a complex process requiring precise regulation of several events including wound healing, ECM production, revascularization, innervation, and bony ray formation. These events require precise regulation of the ECM. Misregulation of the ECM can lead to fibrosis that ultimately inhibits cell migration and distribution of signaling molecules. TCDD impairs fin regeneration after the initiation of blastema formation, a time that is concurrent with the outgrowth, innervation, neovascularization, and bone formation. During this time frame, cell migration and proliferation are prominent. Analysis of microarray data from regenerating larval and adult caudal fins of TCDD-exposed zebra fish indicated that the most prominent category of genes altered in transcript abundance were those involved in ECM composition and function (Andreasen *et al.*, 2006), suggesting that a disruption of the ECM is correlative if not a causative mechanism of toxicity. Although this has not been definitely demonstrated, it is not improbable since pharmaceutical disruption of collagen processing has been shown to impair outgrowth of the regenerating fin (Bai *et al.*, 2005; Bechara *et al.*, 2000). Here, we use zebra fish to investigate whether TCDD alters ECM composition and the plasminogen/MMP pathways that regulate its composition in the regenerating caudal fin. If the ECM is altered, then the impaired fin regeneration due to TCDD exposure may be due to improper ECM remodeling, a primary function of the plasminogen and MMP pathways.

ECM Disruption

TCDD was shown to disrupt the localization of collagen and proteoglycans in the regenerating fin. The cause of this dislocalization of ECM components could be due to transcriptional misregulation. Sox9b, the master regulator of chondrogenesis, was shown to be the most strongly repressed gene by

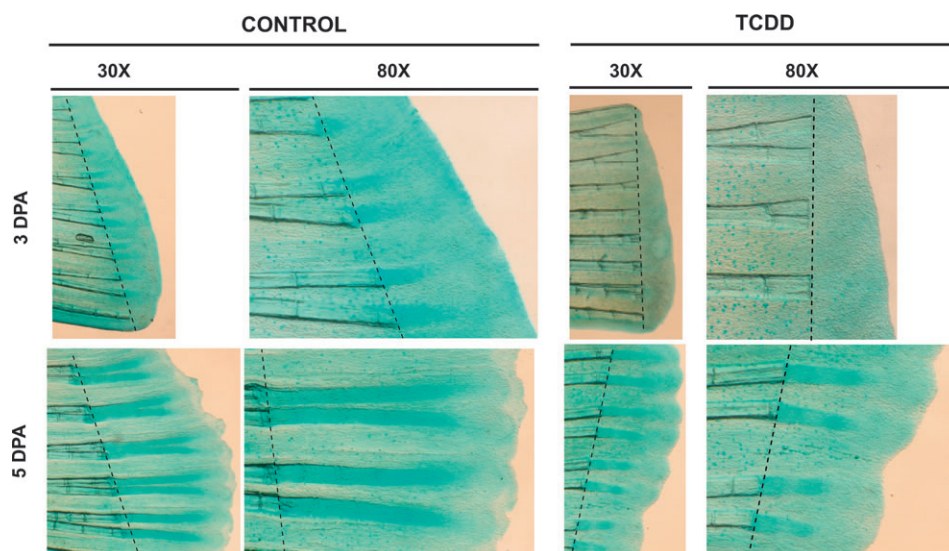


FIG. 7. Histochemical localization of proteoglycans in regenerating fins of vehicle and TCDD-exposed fins at 3 and 5 dpa as detected by whole mount alcian blue staining visualized at 30 \times and 80 \times magnification. Proteoglycans were observed to extend from the lepidotrichia at the amputation site into the regenerating tissue at 3 and 5 dpa in the vehicle-exposed fish but this was severely repressed in the TCDD-treated fish. Images are from representative whole fins from six control and six TCDD-exposed fish. Dashed lines indicate the original amputation plane.

TCDD exposure in the regenerating larval and adult fins (Andreasen *et al.*, 2006; Mathew *et al.*, 2006; #2512). It is conceivable that Sox9b regulates proteoglycan and collagen synthesis since Sox9 is known to regulate the expression of procoll2a1, procoll9a1, procoll11a2, CD Rap, aggrecan, and cartilage link protein (CRTL) in mammals (reviewed in [de Crombrughe *et al.*, 2000]). According to microarray data, the second most strongly repressed gene identified by TCDD is CRTL (Andreasen *et al.*, 2006; Mathew *et al.*, 2006), a protein

that stabilizes proteoglycan aggregates giving cartilage its tensile strength and elasticity. CRTL insufficiency could contribute to the lack of ray growth by dispersing the proteoglycan matrix that forms the provisional matrix needed for collagen to adhere to before calcification, since both collagen and proteoglycan localization were altered by TCDD and both are necessary for mineralization of bone-forming matrix (Landis and Geraudie, 1990). Additionally, repressed CRTL may directly alter ECM constituents since proteolytically activated CRTL stimulates proteoglycan and type II collagen synthesis (Liu *et al.*, 2000). In addition to abundance and localization, the secondary, tertiary, and quaternary

TABLE 2
Abundance of AHR Pathway and ECM Transcripts in the Regenerating Fin following TCDD Dosing

	Posterior to amputation plane							
	0 dpa		1 dpa		3 dpa		5 dpa	
	Control	Control	TCDD	Control	TCDD	Control	TCDD	
AHR pathway								
CYP1A	1.3	0.8	20.6*	0.1	99.1*	0.9	141.1*	
AHRR1	ND	ND	ND	ND	8.2*	ND	13.9*	
AHRR2	1.1	0.2	1.1*	0.1	5.1*	1.0	15.7*	
AHR2	9.0	16.7	18.0	29.9	28.5	16.4	20.4	
ECM								
TGF-B1	5.2	13.0	28.7	6.9	14.8	6.3	16.2*	
MMP2	58.6	22.9	10.0*	195.4	95.0*	168.3	166.0	
MMP9	7.5	38.1	48.3	49.2	112.0*	28.0	59.6*	
MMP13	ND	5.7	15.7	0.9	14.4*	1.1	16.6*	
tPA	9.6	32.9	23.2	16.4	20.7	16.6	20.8	

Note. ND, not detected.

*Significantly different from time-matched vehicle control, $p \leq 0.05$.

TABLE 3

	Anterior to amputation plane					
	1 dpa		3 dpa		5 dpa	
	Control	TCDD	Control	TCDD	Control	TCDD
AHR pathway						
CYP1A	0.2	16.2*	0.1	21.6*	0.3	17.2*
AHR2	4.7	5.7	5.1	6.3	5.1	5.1
ECM						
TGF-B1	5.1	4.0	1.8	2.1	2.0	2.6
MMP2	29.6	10.6*	37.8	13.5*	32.3	16.6
MMP9	15.9	17.9	19.2	24.4	9.8	13.3
MMP13	0.6	2.8*	0.4	1.7*	0.1	0.4
tPA	5.5	4.1	2.8	2.9	2.4	3.6

*Significantly different from time-matched vehicle control, $p \leq 0.05$.

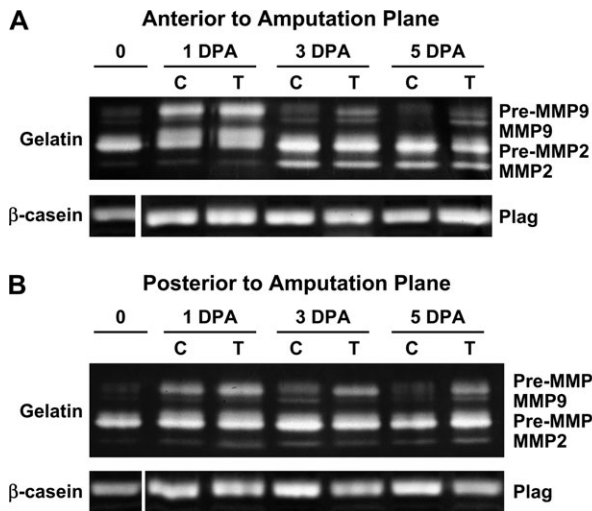


FIG. 8. TCDD alters MMP9 abundance and metabolism capacity but does not alter MMP2 or plasmin activity in the regenerating fin. Gelatin and β -casein digestion activity in extracts of the (A) anterior or (B) posterior fin or 10 μ g of fin extract was resolved in a nondenaturing 8% SDS-PAGE gel copolymerized with 0.8% gelatin or 1% β -casein. The resolved gels were incubated for 16 h in substrate-specific reaction buffer at 37°C and stained with Coomassie Blue. The image is depicted in gray scale with active digestion activity depicted by light bands.

structure of collagen could also be adversely affected by TCDD since TCDD represses the message of several collagen-processing genes from the nucleus to the cytosolic compartments (Andreasen *et al.*, 2006). This alteration in collagen structure could lead to a localized fibrotic tissue resulting in impaired cell migration and repressed mobility of extracellular-signaling molecules. This fibrosis between the apical epithelial cap and the blastema would be disastrous since signaling between these tissues needs to occur for regeneration to occur (reviewed in [Akimenko *et al.*, 2003; Poss *et al.*, 2003]). Fibrosis could also act as a physical barrier to neuronal and vascular outgrowth. Indeed, innervation of the regenerating tissue was strongly repressed in the adult fin by 3 dpa as it was in the larval fin (Mathew *et al.*, 2006). This is important since innervation is required for proper fin regeneration (Geraudie and Singer, 1985). It is intriguing to speculate that the well-studied embryonic cardiac responses to TCDD may also be explained by alterations in the ECM (Antkiewicz *et al.*, 2005, 2006).

LFCs, cells that give rise to the fin ray, are impacted by TCDD exposure. The morphology of the LFC extending from the lepidotrichia is not consistent with mature morphology, and the absence of mature LFC could account for the lack of ray regeneration and outgrowth. It cannot be discerned whether TCDD directly or indirectly impairs fin ray growth by altering the localization, proliferation, or differentiation of LFCs. Blastemal cells differentiate into LFCs when they contact actinotrichia, otherwise they form fibroblasts (Santamaria and

Becerra, 1991). Actinotrichia are long hyperpolymerized elastoidin (collagen-like) fibrils that extend from the distal end of the lepidotrichia into the blastema and are thought to guide the localization of the LFCs, and thus the site of ray formation and outgrowth (Geraudie and Landis, 1982). Elastoidin depolymerization and turnover are thought to be controlled by LFCs (Mari-Beffa *et al.*, 1989). LFCs produce the ECM that becomes mineralized forming the lepidotrichia (Landis and Geraudie, 1990; Mari-Beffa *et al.*, 1996). Mislocalization and or repressed differentiation of LFCs could be due to impaired actinotrichia formation since actinotrachea are much less abundant in the fins of TCDD-treated fish. Loss of actinotrachea and LFCs could lead to extensive local collagen deposition by the fibroblasts and formation of abnormal matrix at the site of amputation. The observed loss of mature LFCs in TCDD-treated fins may be due to reduced differentiation of blastemal cells into LFCs due to reduced interaction with the actinotrachea.

Target Tissues of AHR

The regenerative toxicity of TCDD in the fin is dependent upon AHR2 and ARNT1 signaling, since this toxicity is averted if either of these genes is repressed in larval zebra fish (Mathew *et al.*, 2006). Due to the complex morphological changes during regeneration, it is not surprising that AHR activation alters the abundance of hundreds of genes in the regenerating fin including those relating to the ECM (Andreasen *et al.*, 2006). It is not known, however, whether AHR alters transcript abundance of ECM genes directly or if it acts indirectly by altering cell differentiation or proliferation. The most well-documented AHR-regulated gene, CYP1A, is induced by TCDD prior to outgrowth as early as 1 day following amputation. Induction of CYP1A was used here as a marker of exposure and is not thought to be essential for TCDD toxicity in zebra fish since translational repression of CYP1A in zebra fish embryos does not alter responses to TCDD (Carney *et al.*, 2004). The regenerating vascular tissue has an active AHR pathway since AHR-dependent CYP1A expression was co-localized to this tissue upon its formation at 3 dpa. The regenerating vasculature formed as a disorganized vascular plexus upon AHR activation. Inhibition of vascular regeneration itself would not be expected to halt early outgrowth since VEGF inhibitors have been shown to inhibit angiogenesis and slightly reduce the amount of tissue regrowth but did not inhibit neuronal outgrowth or boney ray formation during adult fin regeneration (Bayliss *et al.*, 2006). However, repressed vascular regeneration in combination with the fibrosis observed with TCDD exposure could significantly alter regeneration. Another interesting possibility is that toxicity could also occur by induction of AHR repressors (AHRs) by TCDD leading to repression of endogenous AHR signaling. AHR was first identified in mice and found to be induced by TCDD and to functionally inhibit AHR by competition for ARNT- and

XRE-binding sites (Mimura *et al.*, 1999). This negative regulatory pathway has been suggested to potentially account for tissue-specific sensitivity to TCDD. Zebrafish have two AHRR genes that can repress TCDD-mediated signaling (Evans *et al.*, 2005). Basal expressions of both AHRR1 and AHRR2 were nearly undetectable in the regenerate. However, they were markedly induced by TCDD. The functional significance of AHRR induction in regenerating tissue remains to be resolved.

AHR and the MMP/Plasminogen Systems

The AHR plays a role in the development and function of several tissues including antral follicle, uterine epithelia, mammary tissues, reproductive tissues, and vascularization of the liver (reviewed in [Abbott *et al.*, 1999; Benedict *et al.*, 2000; Hushka *et al.*, 1998; Lahvis *et al.*, 2000]) in addition to mediating most signs of TCDD toxicity (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997; Peters *et al.*, 1999). These developmental events not only require the AHR but also require proper regulation of the plasminogen and MMP pathways (reviewed in [Parks and Mecham, 1998; Vu and Werb, 2000]). A correlation between pathology of TCDD toxicity and the plasminogen/MMP systems exists. Several of the most sensitive signs of TCDD toxicity are the result of developmental exposure to low doses of TCDD commonly involving processes that require epithelial and mesenchymal interactions. For example, *in utero* and lactational exposure to TCDD impairs branching morphogenesis and differentiation of the breast (Fenton *et al.*, 2002; Lewis *et al.*, 2001) and prostate (Ko *et al.*, 2002), events which are regulated by the plasminogen and MMP pathways (Elfman *et al.*, 2001; Simian *et al.*, 2001; Timme *et al.*, 1994; Wiseman *et al.*, 2003). Likewise, *in utero* and lactational exposure to TCDD also impairs molar eruption (Kattainen *et al.*, 2001; Miettinen *et al.*, 2002), another MMP/plasminogen-regulated event (Bartlett *et al.*, 2003; Beertsen *et al.*, 2002; Okaji *et al.*, 2003; Sahlberg *et al.*, 1999; Tsubota *et al.*, 2002). Despite numerous investigations of TCDD-dependent alterations in gene expression, few studies have directly evaluated whether AHR activation modulates MMP and plasminogen pathways. One study suggests that improper regulation of the MMP system has been associated with TCDD-induced endometriosis in an explant study by disrupting progesterone-mediated regulation of MMPs (Bruner-Tran *et al.*, 1999). Further evidence for interactions between AHR and the plasmin/MMP systems includes the TCDD-dependent alterations in the abundance of members of the MMP and plasminogen systems by regulating message stability (Shimba *et al.*, 2000) or expression in an AHR-dependent fashion (Son and Rozman, 2002). Furthermore, TCDD increases expression of collagenases (MMP1, MMP13) and gelatinases (MMP2, MMP9) in cell lines or the mouse heart (Murphy *et al.*, 2004; Partridge *et al.*, 2000; Thackaberry *et al.*, 2005; Villano *et al.*, 2006; Vogel *et al.*, 2004). The physiological consequences of these alterations in gene expression have not yet been determined.

The regulation of MMPs is complicated and tissue specific. In the regenerating fins, MMP9 was elevated at the message and activity levels. This may seem contradictory since the tissue appears to be fibrotic. This may be a compensatory response to the elevated and mislocalized collagen in the regenerate. This would help to explain why the opposite response was seen in the liver where MMP9 activity was strongly repressed by TCDD (data not shown). MMP2 activity was not perturbed by TCDD in the fin, and the message was slightly repressed. The collagenase MMP13 was strongly induced by TCDD. The elevation in MMP13 message may be due to direct regulation by AHR or it could be a compensatory response to impaired collagen metabolism as seen in mice with impaired collagen metabolism (Beare *et al.*, 2005). The differential activity and expression of MMP2, MMP9, and MMP13 suggest that they are regulated independently or expressed in different cell types. This study strongly supports the importance of whole animal studies to understand molecular responses to TCDD particularly with regard to the plasminogen and MMP systems.

In summary, the data presented here including localized fibrosis, reduction in proteoglycans, and nondetection of LFCs support previously reported microarray studies which revealed that components and regulators of the ECM were misexpressed following AHR activation in the regenerating fin. The localized fibrosis, particularly the accumulation of abnormal matrix along the basement membrane zone, appears to form a blockade to vascular and neuronal outgrowth and likely also form a blockade of the exchange of signaling factors between the blastema and the apical epithelium, which are all necessary for outgrowth.

The precise mechanistic explanation for this cellular response remains unclear, but this model provides a platform to determine the molecular targets of AHR that disrupt the ECM.

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