TCDD Inhibits Heart Regeneration in Adult Zebrafish

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Normal adult zebrafish can completely regenerate lost myocardium following partial amputation of the ventricle apex. We report that 2,3,7,8-tetrachlorodibeno-p-dioxin (TCDD) significantly impairs this regeneration. Adult male zebrafish were injected with vehicle (control) or TCDD (70 ng/g, ip) 1 day prior to partial amputation of the ventricle apex. Gross observation and histological analysis of the amputated heart at 21 days postamputation revealed that TCDD-exposed fish had not progressed beyond the initial clot formation stage, whereas the vehicle control fish showed substantial recovery and almost complete resolution of the formed clot. In contrast, hearts that were not surgically wounded showed no signs of TCDD toxicity. Striking features in the TCDD-exposed hearts were the absence of the normal sheath of new tissue enveloping the wound and the absence of intense cell proliferation at the site of the wound. In addition, the patterns of collagen deposition at the wound site were different between the TCDD and vehicle groups. Because the receptor for TCDD is the aryl hydrocarbon receptor ligand-activated transcriptional regulator, we examined the effects of TCDD exposure on gene expression in the ventricle using DNA microarrays. Samples were collected just prior to amputation and at 6 h and 7 days postamputation. TCDD-pretreated hearts had dysregulated expression of genes involved in heart function, tissue regeneration, cell growth, and extracellular matrix. Because embryonic, but not adult, hearts are major targets for TCDD-induced cardiotoxicity, we speculate that the need for embryonic-like cells in regeneration is connected with the effects of TCDD in inhibiting the response to wounding.

Key Words: cardiotoxicity; dioxin; regeneration; cardiovascular system.

The heart is a target for 2,3,7,8-tetrachlorodibeno-p-dioxin (TCDD) toxicity in a number of vertebrate species, including primates (Allen et al., 1977), rodents (Thackaberry et al., 2005), birds (Ivnitski et al., 2001), and fish (Carney et al., 2006a; Peterson et al., 1993). In zebrafish (Danio rerio), TCDD exposure during the first few days of development causes pericardial edema, decreased cardiomyocyte number, cardiac valve malformation, reduced cardiac output, altered looping, and ventricular standstill (Antkiewicz et al., 2005; Carney et al., 2006a; Henry et al., 1997; Mehta et al., 2008). Although the newly formed zebrafish heart is exquisitely sensitive to TCDD toxicity, this sensitivity disappears with development, until at the end of the larval stage the heart is no longer sensitive to a lethal TCDD exposure (Lanham et al., 2012).

In addition to being a model system for studying cardiotoxicity, the zebrafish heart has been the subject of intense interest because of its ability to regenerate myocardium after surgical removal of part of the ventricle (Poss et al., 2002; Wang et al., 2011; Chablais et al., 2011). Damage of human ventricular myocardium is common in response to myocardial infarction; however, unlike adult zebrafish, humans are unable to regenerate the damaged cardiac muscle (Chablais et al., 2011). Following partial surgical amputation of a portion of the adult zebrafish ventricle, a fibrin-rich blood clot is formed. Shortly thereafter, cells proliferate and migrate toward the wound site to form a cluster of cardiac progenitor cells (Jopling et al., 2010, 2011; Lepilina et al., 2006; Poss et al., 2002; Schnabel et al., 2011). The regenerating tissue goes through a process of sealing the wound, recruitment of cells, differentiation, and organization to eventually form new contractile myocardium that can hardly be distinguished from the original.

During zebrafish heart regeneration, the regenerating heart is sometimes said to become “embryonic-like” because genes normally expressed during heart development are found expressed in cells along the outer most layer of the heart, the epicardium (Lepilina et al., 2006; Poss et al., 2002). Among these genes is raldh2, encoding a key enzyme in the synthesis of retinoic acid (RA). The expression of raldh2 is thought to

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provide a source of RA that assists cardiomyocyte proliferation (Kikuchi et al., 2011; Lepilina et al., 2006).

Knowing that TCDD is toxic to embryonic but not adult states and that the response to wounding in the adult heart involves the dedifferentiation of cells to an embryonic-like state, we wondered if TCDD exposure would have an impact on the ventricular response to surgical resection. This idea is strengthened by the knowledge that TCDD exposure blocks regeneration of the rat liver (Bauman et al., 1995; Mitchell et al., 2006) and the zebrafish fin (Zodrow and Tanguay, 2003). Here, we report that pre-exposure to TCDD does indeed inhibit regeneration of the heart muscle. TCDD prevents the intense proliferation of cells around the wound site and also alters the pattern of gene expression induced by wounding at the ventricle. We speculate that TCDD exposure inhibits a process that is important for progenitor cells needed for both early heart development and regeneration.

MATERIALS AND METHODS

Zebrafish. Adult male zebrafish (AB strain) were housed in 38 l glass aquaria with recirculating reverse osmosis water, supplemented with Instant Ocean salts (60 mg/l; Aquarium Systems, Mentor, OH). Density of fish in the aquaria did not exceed 20 fish/tank. Water was filtered through biofilter media, activated charcoal, sterilized by ultraviolet light, and maintained at 25°C–27°C with a 14 h/10 h light/dark cycle. Fish were fed TetraMin Tropical flakes (Tetra Holding, Blacksburg, VA) and powdered brine shrimp flakes (Aquatic Eco-Systems Inc., Apopka, FL) twice daily. Fish were anesthetized by waterborne Holding, Blacksburg, VA) and powdered brine shrimp flakes (Aquatic Eco-Systems Inc., Apopka, FL) twice daily. Fish were anesthetized by waterborne exposure to tricaine (160 mg/l; MS 222, Sigma) or euthanized by tricaine overdose.

TCDD treatment. TCDD dosing solutions were prepared as previously described (Walker and Peterson, 1991; Zodrow and Tanguay, 2003). Briefly, TCDD (> 99% purity, Chemsys, Lenexa, KS) dissolved in 1.4-dioxane was added to chicken egg yolk phosphatidylcholine (PC; > 99% purity, Avanti Polar Lipids, Alabaster, AL). The mixture was evaporated using Argon gas to dryness, resuspended in 0.9% NaCl, and sonicated to form liposomes. The mole fraction of TCDD to PC did not exceed 0.6 mmol TCDD/0.1 mmol PC. The same procedure was followed for formulating the PC liposomes without TCDD as the vehicle control. Liposomes were stored under argon at 4°C and used within 1 week. Adult male zebrafish were anesthetized, weighed, and placed in a wet sponge. TCDD (70 ng/g) or vehicle (3.5 µl/g) was administered via ip injection with a 30-G needle and a gas tight Luer tip 10 µl Hamilton syringe (Hamilton Company, Reno, NV) (Zodrow et al., 2004). The amount of TCDD emulsion injected was matched with the fish weight to produce the same procedure was followed for the parallel measurement of β-actin mRNA to produce a final measurement on a standard curve analysis. The qRT-PCR was performed on a LightCycler (Roche Molecular Biochemicals) with a FastStart SYBR Green I kit (Roche Applied Science, Indianapolis, IN) with BM purple (Roche). Hybridization was carried out at 60°C. Images were photographed with an Optronics MicroFire camera mounted onto a Leica MZ16 stereomicroscope.

Quantitative RT-PCR. RNA was isolated from ventricular tissue (RNeasy, QIAGEN, Valencia, CA), and cDNA was synthesized from RNA using oligo(dT) primers (SuperScript II RT cDNA synthesis kit; Invitrogen). The qRT-PCR was performed on a LightCycler (Roche Molecular Biochemicals) with a FastStart SYBR Green I kit (Roche) and was labeled using digoxigenin-UTP for probing with antidigoxigenin-AP Fab fragments (Roche Applied Science, Indianapolis, IN). Image analysis was conducted as previously described (Thoese et al., 1993).

Heart morphology examination. Hearts were collected at the indicated times by dissection of terminally anesthetized fish. Hearts were placed in PBS (Cellgro) and photographed with an Optronics MicroFire camera mounted onto a Leica MZ16 stereomicroscope. For 7, 14, and 21 days postamputation (dpa), vehicle hearts were collected at 7 dpa, placed in PBS, and fixed overnight in 4% PFA. The following day fixed heart samples were rinsed in PBS containing 0.1% Tween-20 (PBST), embedded, and vibratome sectioned (Vezina et al., 2008). Sections were collagenase digested and stained with a mouse monoclonal anti Brdu IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor secondary antibody (Invitrogr, Carlsbad, CA). Heart sections were counterstained with rhodamine phalloidin (Invitrogen) to outline cardiac cells (Beis et al., 2005). Sections (n = 6 individual fish for each treatment) were visualized using a Nikon C1 Laser Scanning Confocal microscope.

In situ hybridization. Hearts were removed and fixed overnight in 4% PFA. The following day hearts were washed in PBS, dehydrated to methanol, and stored at −20°C until needed. Prior to sectioning, hearts were rehydrated into PBST (0.1%) and infiltrated with 30% sucrose in PBST before embedding in Tissue-Tek O.C.T Compound for Cryostat Sectioning. Heart sections (10 µm) were stored on slides at −80°C until needed. In situ hybridization was conducted as previously described (Thisse et al., 1993).

Riboprobe synthesis and detection. A 572-bp raldh2 fragment was amplified from embryonic zebrafish cDNA and subcloned into pCRII-TOPO (Invitrogen). Primers positions were as + 505 to + 1076 of Reference Sequence ID NM_131850 and were: 5′-tct aca ctc aca cat gag-3′ and 5′-ctt tga cca acc cac tta cca-3′. An antisense RNA probe was made with T7 RNA polymerase using vector linearized using BsmBI and was labeled using digoxigenin-UTP for probing with antidigoxigenin-AP Fab fragments (Roche Applied Science, Indianapolis, IN) with BM purple (Roche). Hybridization was carried out at 60°C. Images were photographed with an Optronics MicroFire camera mounted onto a Leica MZ16 stereomicroscope.
This has the advantage of reproducible delivery of a known choline emulsion injected into the peritoneum of adult fish. Established a dosing regimen using TCDD in a phosphatidyl-...work studying the effects of TCDD on fin regeneration has used waterborne exposures; however, a substantial body of work studying the effects of TCDD on heart regeneration other than the formation of the blood clot that initially seals the wound. During the 3-week period following surgery, this clot did not regress, and the normal encompassing white sheath of tissue did not form (Fig. 1; TCDD).

Microarrays. Ventricle apex samples were collected and stored at −80°C in RNA later (QIAGEN). RNA was isolated using QIAGEN RNeasy Micro Kit (QIAGEN, Valencia, CA), and cRNA amplification and hybridization was conducted using an Affymetrix GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. Two independent biological replicate experiments were run for each condition.

Analysis of microarray data was adapted from the methods of Gould et al. (2006). The raw data (.CEL files) were normalized by RMA algorithm using the ExpressionFileCreator module of GenePattern software. Differences in gene expression produced by partial amputation and TCDD exposure were determined using the comparative selection marker module of GenePattern. Cutoffs for statistical significance were a Benjamini-Hochberg corrected False Discovery Rate (FDR) ≤ 0.1 and a minimum fold change ≥ 2. To compare the change in gene expression induced by partial heart amputation in vehicle control or TCDD treatment groups, the log 2 values were calculated and were compared to their respective unamputated 0 h postamputation (hpa) samples (e.g., vehicle 6 hpa to vehicle 0 hpa and TCDD 6 hpa to TCDD 0 hpa). The raw data are stored in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/geo/; series record no. GSE33981).

RESULTS

TCDD Inhibits Heart Ventricle Regeneration

To test the hypothesis that TCDD inhibits regeneration of the adult zebrafish heart ventricle, we treated fish with TCDD or vehicle 1 day prior to surgery as described in the Materials and Methods section. Most of our experiments with embryos have used waterborne exposures; however, a substantial body of work studying the effects of TCDD on fin regeneration has established a dosing regimen using TCDD in a phosphatidylcholine emulsion injected into the peritoneum of adult fish. This has the advantage of reproducible delivery of a known dose, and we followed this established protocol (Zodrow et al., 2004). For the surgery, a small thoracic incision was made, and a small portion (~20%) of the ventricle at the apex was snipped away with scissors (Poss et al., 2002). After bleeding was controlled, each fish was returned to aquarium water for recovery and sacrificed later for heart collection.

Normal regeneration was characterized by the formation of a blood clot at the amputation plane. The clot was then encased by a pale white sheet of regenerating tissue (Fig. 1; vehicle). This sheet initially includes rapidly dividing epicardial cells. As time progresses, a new heart field is thought to form in which dedifferentiated cardiomyocytes replace the lost muscle cells (Jopling et al., 2010; Kikuchi et al., 2010). By 21 dpa, the vehicle control heart had recovered to the point that it was nearly indistinguishable from a normal unamputated heart. The blood clot formed in response to wounding had been largely resorbed.

In striking contrast, TCDD-treated hearts did not show signs of regeneration other than the formation of the blood clot that initially seals the wound. During the 3-week period following surgery, this clot did not regress, and the normal encompassing white sheath of tissue did not form (Fig. 1; TCDD).

This result indicates that TCDD exposure inhibits myocardial regeneration.

We found no evidence that the failure to regenerate was due to overt cardiotoxicity. On the contrary, we were unable to detect any effect of TCDD on hearts from TCDD-exposed sham controls (Fig. 2). In this case, the TCDD-treated hearts were indistinguishable from vehicle controls. The hearts appeared normal despite the fact that the fish were exposed to a high dose of TCDD. At 3–4 weeks postdosing, these fish showed signs of toxicity such as hyperpigmentation, fin degeneration, and wasting; nonetheless, the hearts in these fish remained normal in appearance. This is consistent with results with juvenile zebrafish in which the hearts remained normal a month after receiving what is ultimately a lethal dose of TCDD (Lanham et al., 2012). Therefore, we conclude that although the adult heart is normally insensitive to TCDD, the regenerative process is TCDD sensitive.
We did notice that for both the amputated and sham-operated hearts, the pericardium was affected by TCDD exposure. Upon dissection, we found that the pericardial layer had become sticky, and compared with the vehicle control fish, there was very little fluid in the pericardium surrounding the heart.

To obtain a closer look at the effects of TCDD on heart regeneration, we sectioned the hearts for H&E staining. Microscopic examination showed that both vehicle and TCDD-exposed hearts had similar trabeculation and structure in the area not affected by the wound (Fig. 3). In contrast, there were distinct differences at the wound sites. As before, hearts that were not wounded did not appear affected by TCDD (Fig. 3).

The vehicle control hearts showed extensive regeneration at the wound site: minimal residual blood clot remained at the amputation plane, and distinct myocardial and epicardial layers had closed together to repair the wound (Fig. 3). The TCDD-treated hearts showed a striking lack of repair. The remaining blood clot was especially evident.

At 21 dpa, only 5% of the control fish had a prominent blood clot, whereas 69% of the TCDD-treated fish showed a prominent blood clot with no visible signs of resolution or tissue repair ($p < 0.02$). None of the TCDD-treated hearts had undergone the degree of clot resolution typical in the control hearts.

**TCDD Reduces Cell Proliferation**

Zebrafish heart regeneration involves migration and intense proliferation of cells around the wound site (Poss et al., 2002). Because TCDD produces such a distinct halt in heart ventricle regeneration, we expected that this increased proliferation of cells needed to repair the wound might also be blocked. To test this, we used BrdU incorporation to measure cardiomyocyte proliferation in amputated hearts at 7 dpa. As expected, cells in the control hearts displayed extensive BrdU incorporation, concentrated at the margin of the wound, with a few BrdU-positive cells scattered in the unwounded region of the heart (Fig. 4). In contrast, TCDD hearts lacked BrdU-positive cells concentrated at the wound site.

We consistently observed small groups or individual cells proliferating at the surface of the heart away from the site of the wound. These can be seen in Figure 4 as individual BrdU-positive cells along the left edge of the TCDD-exposed heart. These cells did not appear to be affected by TCDD and were observed at roughly equal frequency in TCDD and DMSO control hearts. We also sometimes observed small groups of proliferating cells at the edge of the wound in TCDD-treated hearts; however, this was inconsistent and not unambiguously
linked to the region of the wound. For example, the cluster of BrdU-positive cells at the right of the TCDD panel in Figure 4 appears to be more closely associated with the atrium than the edge of the wound.

**TCDD Alters the Pattern of Collagen Deposition at the Wound**

Collagen deposition is required for normal wound repair. The pattern of collagen can be observed by AFOG staining in which the collagen shows as blue, and fibrin attracts the orange-red dye. We found that the vehicle control and TCDD-exposed hearts showed very different AFOG staining patterns at the wound site at 21 dpa (Fig. 5). The control heart showed a layer of blue, indicative of collagen matrix, across the regenerating wound. Between this layer and the regenerating heart cells were the remains of the original clot, mostly resorbed. The TCDD-exposed hearts consistently showed the massive original clot, apparently unresorbed, with collagen deposition throughout the clot, with concentrated deposition in some regions of the clot. In contrast to the control, this staining did not reveal an organized sheet across the resorbing clot.

**Raldh2 Is Upregulated in TCDD-Treated Amputated Hearts**

During zebrafish heart regeneration, the RA synthesis enzyme, raldh2 is expressed in epicardial and endocardial cells shortly after heart amputation (Kikuchi et al., 2011; Lepilina et al., 2006). Thus, we wanted to determine whether TCDD misregulates raldh2 expression as the heart responds to amputation. To do this, we used in situ hybridization and qRT-PCR to measure raldh2 expression in TCDD- and vehicle control–amputated hearts.

At 7 dpa, raldh2 expression was highest in regions at or near the amputation plane (Fig. 6). In the TCDD samples, raldh2 was expressed more broadly, especially along portion of the epicardium distant from the wound boundary. In the controls, raldh2 was intensely expressed in cells at the wound plane, whereas in the TCDD-exposed hearts the expression was far less restricted, and the wound plane did not stand out as the region of the highest expression.

Our in situ hybridization experiments consistently showed an overall pattern of increased raldh2 expression in the TCDD samples compared with the vehicle controls. We used qRT-PCR to more precisely measure differences in raldh2 abundance between TCDD-exposed and control ventricles. For these experiments, we removed the ventricles and sectioned them with a scalpel along a plane running parallel to the original wound. This yielded two samples: (1) the tissue immediately adjacent to the wound, including the wounded region itself (apex) and
samples comprising the remainder of the ventricle, farther away from the wound site (upper). TCDD increased raldh2 expression in both samples (Fig. 6B; p ≤ 0.05).

**The Timing of TCDD Exposure Is Critical in Halting Heart Ventricle Regeneration**

TCDD must disrupt some process critical to heart regeneration. The persistence of the initial clot with little sign of wound resolution suggests that TCDD might alter events occurring early in the regenerative process. To better understand when TCDD acts, we exposed zebrafish to TCDD at −1, +1, and +4 days relative to the amputation event, with the day of amputation considered day 0. Hearts were then examined at 14 dpa for signs of regeneration (Fig. 7).

As expected, fish exposed to TCDD at day −1 appeared similar to those shown in Figure 1. Control hearts displayed a

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**FIG. 6.** TCDD exposure increases raldh2 expression in amputated ventricles. Adult male zebrafish were exposed to TCDD or vehicle, the ventricle was amputated the following day as described in Figure 1, and the hearts were collected at 7 dpa. (A) Cryostat sections showing in situ hybridization with a raldh2 probe stained as dark purple. Dark arrows point to hybridization signal at the amputation plane. Unfilled arrow shows example of increased hybridization at ventricle surface. (B) Results from qRT-PCR measurements of raldh2 mRNA. After removal, the ventricle was sliced parallel to the amputation plane to yield a ventricle sample containing the original wounded tissue (apex) and the upper region of ventricle not initially wounded in the surgery (upper). Results are presented as the mean ± SEM, with n = 6 independent experiments sampling tissues and three technical replicate qRT-PCR measurements for each sample. The raldh2 signal from each sample was normalized to a parallel measurement of β-actin mRNA to produce a final measurement on an arbitrary scale.

**FIG. 7.** Timing of TCDD exposure is critical for the effect on heart ventricle regeneration. Adult zebrafish were exposed TCDD or vehicle at −1, +1, or +4 dpa as described in the Materials and Methods section, and hearts were collected at 14 dpa for gross examination as in Figure 1. The yellow line denotes the amputation plane. The white arrows indicate normal regeneration of the heart ventricle; the yellow arrow indicates a clot and no regeneration occurring. The scale bar corresponds to 50 µm.
pale white sheet of cells surrounding the resolving blood clot, indicating regeneration. TCDD-treated hearts lacked this white epithelial-like layer and displayed a persistent blood clot.

When fish were exposed to TCDD at day +1 or +4, the hearts showed normal regeneration. These TCDD-exposed hearts displayed normal blood clot regression and presence of an encasing sheath. These results show that TCDD must be present prior to +1 dpa to halt regeneration of the heart ventricle. These results suggest that TCDD does not block the progression of heart ventricle regeneration, but instead inhibits a process needed to set regeneration into motion.

Transcript Changes in TCDD-Treated Zebrafish Hearts

Because TCDD activates the aryl hydrocarbon receptor (AHR)/ARNT transcription factor, we suspect that TCDD acts by altering the abundance of specific transcripts. We used microarray experiments to identify transcripts altered by amputation in control and TCDD-treated heart tissue. For these experiments, we collected mRNA from ventricles dissected to capture tissue toward the apex, representing the site of the wound. For our comparisons, we collected samples from ventricles at several different time points. In each case, the fish were injected with either TCDD or the vehicle as a control. Using the time of amputation as time = 0, the injections were at day −1.

The first set of samples collected were the ventricle apexes from TCDD- and vehicle-treated fish at 0 hpa. These fish were injected the day before and were not amputated, but instead were sham operated immediately before heart tissue collection. We collected these 0 hpa samples to understand how TCDD injection altered the state of the tissue before it was wounded.

The second set of samples collected were from fish treated as described above with either TCDD or vehicle at −1 dpa, amputated at time 0, and collected at 6 hpa. These were collected to assess mRNA changes soon after amputation.

The final set was at 7 dpa. These fish were treated as above, and at 7 dpa, hearts were removed and ventricle tissue was collected for RNA isolation. These samples were used to assess transcriptome differences between cells in a regenerating heart and those in one prevented from healing by TCDD.

Our general approach in making comparisons was to use filters described in the Materials and Methods section to identify altered transcripts. Our arbitrary cutoffs were a Benjamini-Hochberg–corrected False Discovery Rate of ≤ 0.1 and a minimum fold change ≥ 2. Several types of comparisons were made. One of these was comparing the transcripts between TCDD-treated and vehicle control samples collected at time 0. This shows the effect of TCDD on the state of the ventricle at the time of amputation. Data from this comparison are shown in Supplementary table S1. Comparison of the 0 hpa transcripts from the vehicle- and TCDD-treated hearts shows how TCDD affected the transcriptional state of the cells prior to amputation. We found that exposure to TCDD induced 22 genes by ≥ 2-fold. Interestingly, no genes were significantly repressed following TCDD treatment. As would be expected, genes normally induced by TCDD including cyp1a, cyp1c1, tiparp, and ahr2 were induced. The remaining genes were generally regulators of transcription, but did not share any noticeable common function.

We also compared how TCDD altered the transcriptional response to amputation. Transcripts from control- and TCDD-amputated hearts were compared with their respective 0 hpa baseline samples. At 6 hpa, amputation altered 652 transcripts in the control ventricles and 718 in the TCDD-treated ventricles. Taken together, this produced a total of 817 transcripts in two sets overlapping by 553 transcripts. These transcripts and their responses to amputation are presented in Supplementary table S2.

Figure 8A provides a graphical view of the 6 hpa results. This figure plots the total of 817 transcripts changed twofold or more by amputation in either the control or TCDD samples. In this figure, each of the transcripts is represented by a small square. The position on the x-axis represents the fold change produced by amputation in the vehicle control samples. The position on the y-axis represents how the transcript was affected by amputation in the TCDD-treated hearts. Fold change is plotted on a log 2 scale, and in both cases the 6 hpa samples were compared with their respective 0 hpa baselines. Because each point represents a transcript that was altered by at least twofold in at least one of the conditions, there is an empty square in the middle of the plot, delineating absolute value 1. This represents the log 2 value of a twofold change.

Most of the transcripts fall close to a diagonal line passing through the origin with a slope of 1. For these transcripts, the fold change caused by amputation in the control was roughly equal to the fold change in the TCDD-treated samples. The two diagonal lines on the plot have slopes of 0.5 and 2. Points falling between these lines have x and y values that are within twofold of each other: their responses under the two conditions might be considered similar. Points above or below these lines have x and y values that differ from each other by more than twofold. We considered these to be altered by TCDD. Of the 817 transcripts on the plot, 26 were expressed at least twofold more in the presence of TCDD than in the control, and 80 were expressed at least twofold less in the TCDD group than in the control. These transcripts are listed in Supplementary table S3. The genes induced more in amputation when TCDDs are associated with a range of biological processes, with signal transduction being perhaps the most represented. Among the genes whose response to amputation was dampened by TCDD, we found that cell adhesion, basement membrane production, and metalloproteinases were well represented. In addition, both gata1 and gata5 were downregulated. These latter genes are involved in cell fate determination in heart and blood.

By 7 dpa, the pattern of TCDD effects on transcript abundance was more complex, perhaps reflecting responses in
TCDD-exposed tissue failing to undergo normal wound regeneration. A total of 731 transcripts were altered by at least twofold by amputation in the control hearts, whereas 753 transcripts were altered in the TCDD group, for a total of 914 transcripts changed by amputation with 570 transcripts overlapping between the groups. These genes are listed with their fold responses in Supplementary table S4.

Figure 8B shows a plot of the 7 dpa data following the methods used for the 6 hpa data in Figure 8A. This compares the responses for all 914 transcripts altered by amputation in either the TCDD or control samples at 7 dpa. We found that 106 of the transcripts were expressed at least twofold more in the presence of TCDD than in the control samples, and 69 transcripts were expressed at least twofold less in the TCDD group than in the controls. Among the induced genes were a wide variety of genes, but those involved in cell adhesion and production of connective fibers are well represented, with keratins and epcam near the top of the list. The list of genes repressed more in the TCDD samples contains genes associated with immune function and blood clotting (Supplementary table S5).

DISCUSSION

TCDD did not prevent the initial clot formation, but appeared to prevent any further steps in myocardial regeneration. In some cases, we scored TCDD-treated hearts as partially regenerated; however, in these cases, the observed signs of repair were not robust, were associated with smaller initial wounds, and did not reflect the normal healing rate. It was not likely that hearts would ever completely regenerate. Indeed, although the control fish recovered from amputation, the TCDD-treated fish did not and began to die at around 1 month after surgery. In contrast, the dose of TCDD was not lethal and produced no observable cardiotoxicity in fish without amputation.

A proliferative response is required for heart regeneration (Porrello et al., 2011; Poss et al., 2002). We found that in a normal heart the wound became surrounded by rapidly dividing cells, TCDD did not appear to prevent division of cells away from the wound site as evidenced by the few BrdU-positive cells found in both control and TCDD-exposed hearts at sites away from the wound. However, we saw no dramatic increase in proliferation at the wound site in the TCDD-treated hearts. It is noteworthy that in the embryonic heart, TCDD exposure produces the downregulation of a large cluster of genes termed the “cell cycle gene cluster,” while at the same time halting cell division in the developing heart (Carney et al., 2006b). It is clear that the normal division of cells needed to replace those lost in the amputated heart is blocked by TCDD.

The TCDD dose required to produce this effect is quite high compared with the short 1 h waterborne exposure at 1 ng/ml that is lethal to developing zebrafish embryos. This is also true in
experiments with fin regeneration. We speculate that because of the well-known hydrophobic nature of TCDD, injected TCDD rapidly associates with macromolecules and lipids throughout the tissues of the fish, especially near the injection site. The levels of circulating TCDD reaching the nuclei of cells within the heart may be significantly lower. We note that this dose of TCDD has been used to block fin regeneration, which is AHR dependent, suggesting that despite the high doses the effect is mediated by normal AHR binding (Mathew et al., 2006). Although we followed a standard dosing protocol used to study fin regeneration, lower concentrations of TCDD will inhibit fin regeneration, while in our hands, lower doses fail to inhibit heart regeneration. We do not know whether this represents an intrinsic difference between the tissues or perhaps some difference in distribution of TCDD.

The Consequences of TCDD Exposure on the Heart Vary with the Circumstances

Ordinarily, TCDD exposure in juveniles or adults has no apparent effect on the zebrafish heart (Lanham et al., 2012). On the other hand, exposure of the developing embryonic heart to TCDD produces catastrophic failure (Antkiewicz et al., 2005; Carney et al., 2006b). Our results show that if the heart is wounded, it returns to a TCDD-sensitive state. This is especially interesting in light of the idea that the response to wounding depends on the mobilization of cells that have embryonic properties (Choi and Poss, 2012; Leplina et al., 2006).

One possible explanation for these results is that there is a single process that is being disrupted by TCDD exposure in both the embryonic and wounded adult hearts. If this is the case, it could provide clues about both the cardiotoxicity of TCDD exposure and mechanisms needed for heart development and regeneration.

Window of TCDD Sensitivity

The embryonic heart is most sensitive to TCDD exposure between ~2 and 5 days postfertilization. This coincides with the period during which the epicardial layer is forming around the new heart. The epicardium also plays a supportive role in recovery from wounding in the adult heart (Kikuchi et al., 2011; Leplina et al., 2006). We found that exposure at 24 h following amputation was too late to produce an effect on heart regeneration, whereas exposure prior to surgery produced a profound effect. One possibility is that by 24 hpa, the process affected by TCDD has reached a stage at which it is completed or committed and is no longer a target. For example, it may be that TCDD affects a process needed for cellular dedifferentiation in response to wounding. This process would be most critical in the hours after wounding, and once completed might no longer be affected. An alternative idea is that the molecular responses to TCDD activation of AHR in the target cell change after wounding. In this example, the initial response to TCDD-activated AHR changes. Yet, another idea is that TCDD alters the starting condition of the heart so that it already has an intrinsically altered repertoire of possible responses to wounding. Although we observed little if any overt toxicity in the unwounded heart, TCDD causes gene expression changes in the ventricle that occur prior to wounding. One of these changes may disable the wound repair response.

Because the unwounded heart shows no response to TCDD, we propose that our results are not due to some nonspecific general cardiotoxicity induced by the high dose of TCDD. TCDD may be acting indirectly to prevent wound repair; however, we do not think that TCDD is producing an environment in which the unwounded heart cannot function normally.

AHR and Gene Expression

AHR agonists such as TCDD are thought to transform the inactive AHR into a heterodimeric transcriptional regulator that binds DNA at specific sites called AHR response elements. Although this suggests that TCDD acts by altering gene expression, we do not know what genes are crucial in producing toxicity. This led us to microarray experiments.

In these experiments, we compared the transcriptional response with amputation in TCDD-exposed and control hearts. Previous work has examined the gene expression changes induced in the heart by amputation, giving us the opportunity to compare our results with the control-amputated samples and the results published by Sleep et al. (2010). After normalization, regression analysis showed no significant difference ($p \leq 0.05$, root square = 0.86) between our data sets for comparable samples (data not shown). This provides a degree of validation for both sets of array results.

TCDD exposure altered the transcriptome of the ventricle even prior to amputation. However, the list of 22 genes induced at least twofold did not provide obvious paths toward explaining the effect on heart regeneration. Most of the affected genes are common to the well-characterized TCDD-inducible battery induced in many tissues. We did not observe a common trend in function between the remaining genes in this list except that many are thought to regulate transcription.

Out of the set of 817 transcripts altered by amputation in either the control or treated hearts at 6 hpa, 553 were common to both. Later after amputation (7 dpa), the transcriptome in TCDD-exposed ventricles diverged farther from that of the control ventricle. By this point, a large number of genes were altered in expression by amputation far more in the TCDD group than in the control. Within this group were genes involved in ECM formation, cell-cell adhesion, and epithelial to mesenchymal transition. This may reflect the hyperexpression of genes required for wound repair in response to the blockade itself. Although we were able to detect changes in raldh2 expression using in situ hybridization and qRT-PCR, the data for this gene did not pass the quality control and fold change filters in the microarray experiments and was not counted as changed by TCDD. We speculate that TCDD does indeed alter raldh2 expression, but this is not captured due to a poor signal for this particular transcript on the arrays.
Rather than producing a Venn diagram of overlapping sets, we chose to plot each transcript directly. This provided several advantages. First, the Venn diagram approach can separate transcripts that are not very different. For example, a transcript altered by 2.1-fold in both control and TCDD falls into the common circle, whereas a transcript induced 2.1-fold by amputation in TCDD and 1.9-fold in the control would fall into a TCDD-specific group. Furthermore, transcripts meeting the criteria to be in the common group might be very different in actual response. For example, at 7 dpa, the keratin gene krt5 was induced 3.4-fold in the control; yet, in the TCDD-amputated sample, the transcript was increased by 168-fold; using simple grouping with a twofold cutoff, these would have been grouped together as not TCDD altered.

**TCDD and Wound Healing in Fin and Heart**

The discovery that TCDD inhibits the regeneration of fish fins produced a great deal of excitement. The effect of TCDD on wound healing in the zebrafish fin is relatively well characterized; it is known that TCDD arrests the expansion of the initial blastema, and work has been done characterizing the effects of TCDD on gene expression in the regenerating fin. In a superficial manner, our work shows similarities between the two systems. In both cases, the presence of TCDD blocks cell proliferation and tissue regeneration. Beyond that, the responses appear to diverge. Although TCDD can stop the advancing blastema at the site of fin regeneration several days after the initial damage, in the heart, TCDD is ineffective in blocking regeneration if administered a day following the surgery. Furthermore, although there are regenerating progenitor cells in the regenerating heart, it is not clear that a blastema exists.

Finally, when we compared our results with those from Andreasen et al. (2006), we found little overlap between the genes affected by TCDD in the heart and those affected in the regenerating fin. The lack of concordance can in part be attributed to differences in the methods of data comparison, but only to a limited extent. We chose to compare the amputated tissue to 0 hpa samples of the same treatment, i.e., TCDD 6 hpa to TCDD 0 hpa and 6 hpa PC control to 0 hpa PC control. We might also have compared between TCDD and PC samples at equivalent time points or chosen a single sample such as 0 hpa PC control as the baseline for all comparisons. We have tried all of these types of comparisons with our data, and the major change in outcome is the appearance of genes from the AHR battery such as cyp1a. Our decision to compare genes affected by amputation to unamputated controls in the same treatment group removes genes such as cyp1a that are well-known targets for TCDD induction. This battery of genes is already induced in the 0 hpa TCDD-treated baseline samples and is therefore filtered out with our approach. These genes did not change appreciably in response to amputation. We compared our lists of genes affected by TCDD at 6 hpa with the 36 genes affected in the fin at 1 dpa and found no overlapping genes. Comparison of genes affected in the heart at 7 dpa with the 142 genes affected in the fin at 5 dpa produced an overlap of eight genes: induced, temp2, ccl1, ass1, and btbd6a; repressed, cxcl12a, si:ch1073-459j12.1, f13a1, and si:ch211-23714.5.

However, whenever we compare any heart tissue treated with TCDD with any PC control heart sample, we find that genes in the AHR battery appear; this is amputation independent. In order to better compare our results with those of Andreasen et al. (2006), we have reanalyzed our data using the 0 hpa PC control as the baseline for comparisons. This is as close as we can come to their approach, because our time points differ. The lists from Andreasen et al. (2006) and from our work are shown compared in Supplementary table S6. As indicated above, there were few overlapping transcripts, especially at the earliest time points, 6 hpa (heart) and 24 hpa (fin). At later time points, 7 dpa (heart) and 5 dpa (fin), the most interesting similarities were enzymes such as metalloproteinases needed for basement membrane remodeling and formation. In particular, the transcripts for sox9b and R-spondin 1 affected by TCDD in the amputated fin were not affected by TCDD in our experiments. We speculate that these results reflect fundamental differences between the two regeneration processes.

**CONCLUSIONS**

The ability of the zebrafish heart to regenerate tissue after wounding is of great interest. It is remarkable that the zebrafish heart, although so sensitive to TCDD during a short period of development, should become almost immune to TCDD after the end of larval development and then again become sensitive when it is wounded. Understanding these phenomena will provide helpful clues to unraveling both the mysteries—the regeneration of the heart ventricle and the cardiotoxicity of AHR agonists.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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