

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Exposure Prevents Cardiac Valve Formation in Developing Zebrafish

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Cardiovascular malformations are one of the most common congenital birth defects observed in humans. Defects in cardiac valves disrupt normal blood flow. Zebrafish are an outstanding experimental model for studying the effects that environmental contaminants have on developmental processes. Previous research has shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes blood regurgitation in the heart and reduces peripheral blood flow in embryonic zebrafish, suggesting some form of valve failure. To test this we used video microscopy to examine valve function and structure in developing zebrafish exposed to TCDD. TCDD exposure produced blood regurgitation at both the atrioventricular (AV) and bulboventricular (BV) junctions. In marked contrast to control embryos exposed to the vehicle dimethyl sulfoxide, embryos exposed to TCDD failed to form valve leaflets as the heart matured. In addition, whereas TCDD did not block initial formation of the bulbus arteriosus, we found that TCDD exposure prevented the normal growth and development of this portion of the outflow tract. TCDD altered the localization of endothelial cells at the AV and BV junctions and altered the localized expression of mRNAs *bmp4* and *notch1b* normally associated with the nascent valves. Taken together, our results demonstrate that although TCDD does not prevent the initial specification of the presumptive valve locations, TCDD exposure produces severe alterations in valve development, leading to blood regurgitation and failing circulation in the developing zebrafish.

Key Words: zebrafish; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; heart; blood regurgitation; atrioventricular valve; bulboventricular valve.

The cardiac valves play a critical role in heart function by preventing retrograde blood flow, thereby maintaining vascular hemodynamics. It is estimated that 25–30% of all cases of human cardiovascular malformation involve defects in cardiac valves (Armstrong and Bischoff, 2004). Atrioventricular (AV) canal defects are seen in approximately 5% of congenital heart disease, establishing this as the fourth most common congenital heart malformation (Emmanouilides *et al.*, 1998). Valve defects

are also observed in 16% of Down and Noonan syndrome patients as well as infants born to gestational diabetic mothers (Enciso *et al.*, 2003; Stainier *et al.*, 2002). The American Heart Association estimates 95,000 in patient valve procedures were performed in the United States alone in 2003, and about 300,000 worldwide (Thom *et al.*, 2006; Vesely, 2005).

In humans, valve malformations include valvular stenosis where there is a narrowing of the valve opening and valvular insufficiency in which the valves do not close completely, allowing blood regurgitation (Flanagan and Pandit, 2003). Epidemiological studies suggest that people living in areas of water contaminated with halogenated hydrocarbons have an elevated risk for valvular stenosis and other cardiac malformations (Goldberg *et al.*, 1990). Another study identified a region of Baltimore, MD, where an increased frequency of hypoplastic left heart syndrome correlated with the industrial release of solvents, dioxins and polychlorinated biphenyls (Kuehl and Loffredo, 2006). Understanding how genetic predisposition and environmental factors play a role in the development and functioning of the cardiac valves is a critical step in preventing these diseases.

Environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are bioaccumulative, lipophilic halogenated aromatic hydrocarbons that persist in the environment and cause cardiovascular malformations in mammalian, avian and aquatic species (Heid *et al.*, 2001; Henry *et al.*, 1997; Thackaberry *et al.*, 2005b). Fetuses from pregnant mice exposed to TCDD exhibit a reduced heart to body weight, reduced fetal cardiocyte proliferation, and altered expression of cardiac genes involved in extracellular matrix (ECM) deposition, remodeling and cardiac homeostasis (Thackaberry *et al.*, 2005a, b). In chickens, TCDD causes an increase in apoptosis in cells of the outflow tract and the AV canal endocardial cushions (ECs), whereas decreasing cardiomyocyte proliferation (Ivnitski *et al.*, 2001). TCDD also reduced the growth of cells in cardiac ventricle explants isolated from embryonic chickens (Ivnitski-Steele and Walker, 2003).

Embryonic zebrafish exposed to TCDD exhibit a reduction in common cardinal vein area and reduced number of cardiomyocytes by 48 hpf (hours postfertilization). This is

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followed by reduced blood flow, decreased heart size, pericardial edema, impaired looping, reduced cardiac output, anemia, ventricular standstill, and ultimately death (Antkiewicz *et al.*, 2005; Belair *et al.*, 2001; Bello *et al.*, 2004).

The developing zebrafish heart begins to beat at around 22 hpf and circulation is initiated shortly thereafter (Stainier *et al.*, 1993). By 48 hpf, constrictions have clearly partitioned the heart into the sinus venosus, atrium, ventricle, and bulbous arteriosus (Hu *et al.*, 2000). The heart consists of an outer myocardial layer that is contractile and an inner endocardial layer that is continuous with the vascular endothelium. In between these two layers lies the ECM or cardiac jelly (Yelon, 2001). The valves are formed from the endocardial cells at three sites: the sinus venosus valve at the junction between the sinus venosus and the atrium; the AV valve at the junction between the atrium and the ventricle; and the bulboventricular (BV) valve between the ventricle and the bulbus arteriosus (BA) of the outflow tract (Hu *et al.*, 2000).

Two components of the Wnt signaling pathway, bone morphogenetic protein 4 (*bmp4*) and *notch1b*, are initially expressed throughout the heart, but become restricted to myocardial and endocardial cells marking the constrictions at the presumptive valve sites by 37 and 45 hpf, respectively (Walsh and Stainier, 2001).

There is some disagreement between reports describing the processes involved in early valve formation. For several years it has been thought that a subset of the AV endocardial cells differentiate in response to myocardial cell signals, and undergo an epithelial to mesenchymal transformation (EMT) at the site of the nascent valve. In this process, the endothelial cells delaminate and migrate into the cardiac jelly by 60–72 hpf. The delaminated cells form transient structures called ECs that help to prevent reverse blood flow (Walsh and Stainier, 2001). The ECs undergo further remodeling to form the actual valve leaflets at the AV boundary. Many aspects of this process have been observed directly and recently reported (Bartman *et al.*, 2004; Beis *et al.*, 2005; Hu *et al.*, 2000; Hurlstone *et al.*, 2003; Stainier *et al.*, 2002; Wang *et al.*, 2006)

However, a very recent report from Scherz *et al.* (2008) has called this model into question. In this paper, the authors described the use of selective plane illumination microscopy to gather evidence that valve leaflets are formed directly through an invagination process rather than through the intermediate step of forming cushions.

Blood flow and proper myocardial contractility are both believed to play a role in valve development in zebrafish. Reducing blood flow across the AV junction by obstructing the sinus venosus results in the absence of the AV valve and the BA (Hove *et al.*, 2003). However, in other experiments, Bartman *et al.* (2004) found that it was possible to pharmacologically block blood flow entirely, without preventing the initial steps of valve formation.

The anecdotal report that TCDD causes blood regurgitation in the developing zebrafish heart provides a possible explana-

tion for at least part of the circulation failure that TCDD produces in developing zebrafish (Antkiewicz *et al.*, 2005). If TCDD altered the morphology or function of valve structures in the heart, then blood would be expected to leak backwards during systole, reducing cardiac output and blood flow. Here we report experiments designed to test this possibility. We used video microscopy to demonstrate that TCDD causes both retrograde blood flow and valve failure. We found that an initial failure of the nascent valve to form a seal was followed by a failure to form ECs or AV valve leaflets. Although markers of AV junction specification appeared normal in vehicle and TCDD-treated embryos at 48 hpf, the clusters of endothelial cells at the AV and BV junctions became progressively delocalized in the TCDD-exposed embryos at later time points. This coincided with a loss of localization and a general increase in expression of the valve specific markers *bmp4* and *notch1b* in the region of the AV junction. These results demonstrate that TCDD exposure severely disrupts heart valve formation in the developing zebrafish, a response that would be expected to contribute to the subsequent lethality.

MATERIALS AND METHODS

Zebrafish lines. All zebrafish embryos were kept at 28.5° in egg water (60 µg/ml Instant Ocean Salts; Aquarium Systems, Mentor, OH) with a 14-h/10-h light/dark cycle. Wild-type AB embryos were used in assessing blood regurgitation and for *in situ* hybridization experiments. Embryos from the vascular endothelial specific *Tg(flk1:GFP)* fish (kindly provided by Dr Beth Roman and Dr Jau-Nian Chen; Cross *et al.*, 2003) were used for assessing the endothelial cell localization and for assessing AV valve leaflet development. *Tg(cmlc2:gfp)* embryos a kind gift from Dr C. Geoffrey Burns (Rottbauer *et al.*, 2006) were used to assess the development of the BA.

TCDD exposure. TCDD of > 99% purity was obtained from Chemsyn (Lenexa, KS) and dissolved in 0.1% dimethyl sulfoxide (DMSO). Embryos were placed in glass vials containing egg water and exposed to either TCDD (1 ng/ml) or 0.1% DMSO (vehicle) for 1 h on a rocker. After the 1-h exposure, the embryos were rinsed with fresh egg water and transferred to Petri dishes and raised at 28.5° in a 14-h light/10-h dark cycle. The maximum number of eggs/ml of egg water did not exceed 10 embryos. For our experiments, each group of embryos exposed in a single vial constitutes an $n = 1$.

Assessing blood regurgitation and endothelial cell localization. Live embryos were mounted on a slide containing 3% methylcellulose to reduce movement and blood regurgitation across the valves was assessed by following video recordings made with a Nikon TE300 inverted microscope with a high-speed Motion Scope camera. Statistical significance of blood regurgitation incidence at the AV junction was determined via Fisher's p -test. For endothelial cell localization, *Tg(flk1:GFP)* embryos were dosed and collected as above but visualized using a Princeton instruments Micromax charge coupled device camera and MetaMorph imaging software (Molecular Devices, CA).

Assessing development of the BA chamber. *Tg(cmlc2:gfp)* embryos were exposed to TCDD or vehicle as described above. In this strain, *gfp* is seen in the atrium and ventricle, but is absent in the BA. At 24 hpf embryos were transferred to fish water containing 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigment formation. At 36 hpf live embryos were transferred to fish water containing 10µM diamiorhodamine-4M AM (DAR-4M AM) (Calbiochem, San Diego, CA) in fish water adjusted to pH 7.0 and kept overnight in the dark for detection of the bulbus (Grimes *et al.* 2006). DAR-4M AM is an *in vivo* cell permeable, photo-stable nitric oxide reactive dye that marks the

developing BA. New fish water containing DAR-4M AM was replaced every 24 h, and the presence of the bulbous was assessed at 48, 60, 72, 76, 96, and 120 hpf using a Nikon TE300 inverted microscope connected to a Princeton instruments Micromax CCD camera.

Immunohistochemistry. *Tg(flk1:GFP)* embryos were exposed to TCDD or vehicle as described above. At 24 hpf embryos were transferred to fish water containing 0.003% PTU to prevent pigment formation. At 120 hpf they were euthanized in 1.67 mg/ml Tricaine (MS 222, Sigma, St Louis, MO), rinsed in phosphate-buffered saline (PBS, Cellgro), and fixed overnight in 4% Paraformaldehyde in PBS. Whole embryos were then mounted on a glass slide and observed with a Nikon C1 laser scanning confocal microscope.

In situ hybridization. Vehicle and TCDD-treated AB embryos were collected at 48, 60, and 72 hpf. The embryos were euthanized in 1.67 mg/ml Tricaine (MS 222, Sigma), rinsed in PBS and fixed overnight in 4% Paraformaldehyde in PBS. The next day they were washed in PBS, and bleached in a mix of 3% Hydrogen peroxide and 1% potassium hydroxide. They were then washed in PBS, and dehydrated in methanol and stored at -20°C until needed. TCDD-treated fish were tail clipped for identification after dehydration so that both treatment groups could be processed together for the *in situ* hybridization steps. This ensured identical staining. BMP4 and Notch1b plasmids were kindly provided by Dr Randall Peterson. Riboprobes were labeled with digoxigenin-UTP and visualized using anti-digoxigenin-AP Fab fragments (Roche Applied Science, Indianapolis, IN) with 4-nitro blue tetrazolium chloride solution and 5-bromo-4-chloro-3-indolyl-phosphate as substrates. Hybridization was carried out at 65°C . Overall for the 60 hpf samples $n = 16$ from two separate experiments; for the 48 and 72 hpf samples $n = 26$ from three separate experiments. A single representative embryo was selected for each treatment group at each time point and photographed with an Optronics MicroFire camera (Leica) mounted onto a Leica MZ16 stereomicroscope (Leica).

RESULTS

TCDD Exposure Causes Blood Regurgitation at the AV Valve

Anecdotal evidence suggested that a part of the reduction in blood flow produced by TCDD might be due to regurgitation of blood at the sites of the nascent valves in the developing heart. We used video microscopy to examine the incidence of blood regurgitation in TCDD-exposed embryos and in control embryos exposed to DMSO from 48 to 120 hpf. We found evidence that TCDD produced an overall greater incidence of blood regurgitation at the AV boundary compared with vehicle (Fig. 1).

We saw significant blood regurgitation at the AV boundary beginning at approximately 60 hpf in the TCDD-exposed embryos, but not in the controls. This became progressively more severe as development progressed. By 72 hpf, TCDD-exposed embryos demonstrate clear signs of valvular insufficiency. We observed that the ring of tissue at the presumptive AV valve site failed to shut completely during ventricular systole. This left a small opening that allowed blood cells to trickle back into the atrium in TCDD-exposed fish examined at 72 hpf (Fig. 2) (Supplemental Movie: 72hpfAB.mov). As the atrium contracted, blood was expelled from the atrium into the ventricle; however, as the ventricle contracted a small fraction of cells could be seen pushed in a retrograde motion from within the ventricle back into the atrium. By 96 hpf virtually all of the blood moving into the ventricle was then moved back-

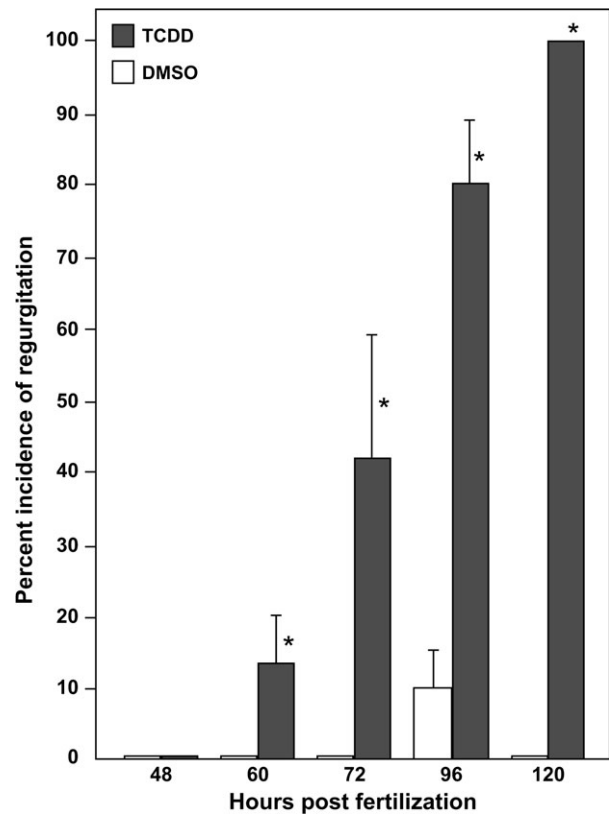


FIG. 1. TCDD causes blood regurgitation in the developing zebrafish heart. Newly fertilized zebrafish eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” Videomicrographs were recorded at the indicated times during development and individuals were scored for retrograde blood flow across the AV junction. Asterisks indicate a statistical difference between TCDD and control hearts $p \leq (0.05)$ determined via one-tailed Fisher’s p -test. Error bars indicate standard error.

wards into the atrium during systole, virtually abolishing blood flow (Supplementary Data, supplemental movie: 96hpfAB.mov).

TCDD Exposure Prevents Formation of Valve Cushions and Leaflets at the AV Junction

We used *Tg(flk1:GFP)* to determine whether TCDD prevents the distinct thickening of endothelial cells at the AV junction that has been reported to be indicative of cardiac cushion formation (Bartman *et al.*, 2004; Stainier *et al.*, 2002). Figure 3 shows successive images from high-speed video recordings in which the endothelial cells are marked with GFP fluorescence. In the control zebrafish, endothelial cells were observed in distinct clusters that appeared to form actual valve leaflets as early as 72 hpf, consistent with the results recently reported by Scherz *et al.* (2008). These leaflets were even more distinct and well developed by 96 hpf. This can be even more readily discerned in movies included as supplemental data (supplemental movies: 72hpf_flk.mov; 96hpf_flk.mov). In striking contrast, there was no evidence of either cushion or leaflet formation in the TCDD-exposed zebrafish at either 72 or 96

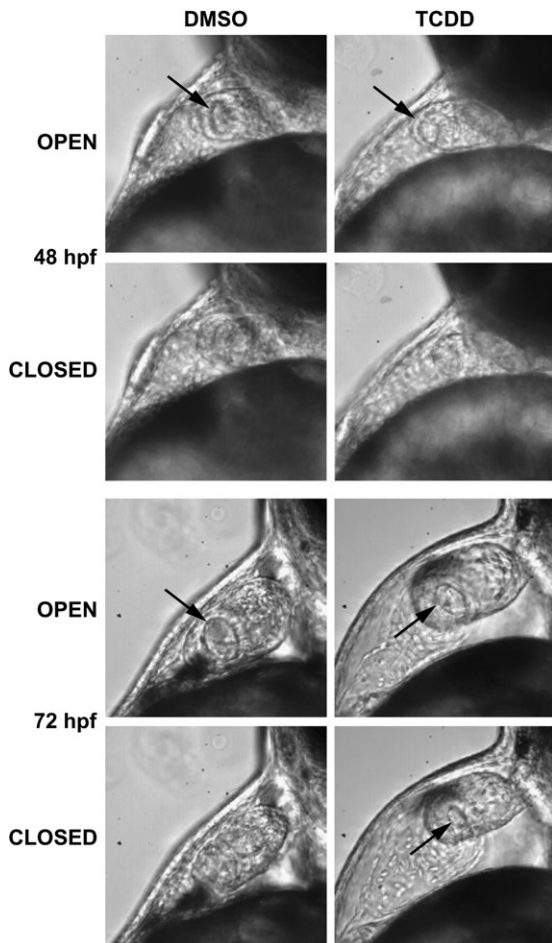


FIG. 2. TCDD prevents closure of the nascent AV valve during systole. Newly fertilized zebrafish eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” Representative lateral view micrographs are shown for hearts at the indicated time in development. The arrows indicate an open passage between the atrium and ventricle. Open: the image was taken during diastole in which the AV junction is relaxed. Closed: the image was taken during systole, during which the AV junction has contracted to its fullest extent. An open passage between the ventricle and atrium was consistently observed associated with retrograde blood movement at 72 hpf (see supplemental movie: 72hpfAB.mov).

hpf. Furthermore, although the endothelial cells in the control hearts were clustered tightly so as to form a mass that decisively occludes the lumen during contraction, the endothelial cells in the TCDD-treated hearts were less concentrated, leaving what appears to be a small gap during contraction. By 96 hpf there was no readily discernible cluster of endothelial cells at the AV junction in the TCDD-treated hearts.

TCDD produced similar effects at the BV junction, in which the control hearts had distinct clusters of endocardial structures forming discernible flaps by 96 hpf, whereas the TCDD-treated hearts had a more diffuse and unorganized arrangement of endothelial cells. These results are consistent with the results shown in Figures 1 and 2, demonstrating that TCDD prevents sealing at the AV junction, allowing retrograde blood flow.

We used confocal microscopy to get a better view of leaflet formation at the AV junction at 120 hpf in treated and vehicle *Tg(flk1:GFP)* embryos (Fig. 4). Because it was possible that TCDD had simply delayed leaflet formation, we chose a time point two days after we had first seen signs of leaflet formation in the control hearts. If TCDD were simply delaying the emergence of leaflets, one might expect to see evidence of leaflet formation in the TCDD-exposed hearts by this time point. At 120 hpf, the nascent valve leaflets were clearly visible at the AV boundary in the control hearts. We saw no signs of leaflets in the TCDD-exposed hearts; aggregation of endothelial cells at the AV boundary was greatly reduced in TCDD-treated fish.

TCDD Exposure Inhibits BA Development

During our confocal microscopy experiments, we began to notice that TCDD produces changes in the BV valve as well as the BA chamber itself. The BA serves as a capacitor as the smooth muscle expands and relaxes (supplemental movie: dar4_120hpf.mov), protecting the gills and providing uniform blood flow (Grimes *et al.*, 2006; Hu *et al.*, 2000). The BA is first noticeable at the outflow side of the ventricle at around 48 hpf, when it can be observed by *in vivo* staining with DAR-4M AM, a membrane permeable compound that detects nitric oxide (Grimes *et al.*, 2006).

We used DAR-4M AM to determine what effects TCDD has on the formation of the BA (Fig. 5). In our hands, we could not clearly detect the BA prior to 76 hpf. By this time, TCDD-exposed embryos already exhibited a dramatically smaller BA than was observed in the vehicle exposed fish. Although the BA in vehicle treated fish steadily increased in size as seen at the 96 and 120 hpf points, the BA in the TCDD-exposed embryos exhibited little if any growth. This indicates that although TCDD did not block the initial formation of a group of DAR-4M AM staining cells at the location of the BA, it prevented any further growth of the outflow chamber.

TCDD Exposure Alters Endothelial Cell Localization at the AV Junction

Among the early steps of valve formation is the accumulation and clustering of endothelial cells lining the heart at the AV and BV junctions. Using *Tg(flk1:GFP)* we followed the migration and accumulation of endothelial cells at the AV boundary over time. We were able to clearly distinguish a cluster of endothelial cells at the AV and BV junction by 36 hpf demarcating the junction between the atrium and ventricle and between the ventricle and outflow tract (data not shown). This group of GFP-positive cells was even more distinct in lateral view images at 48 hpf, where both vehicle and TCDD-treated fish showed a distinct cluster of endothelial cells at the AV junction, and a distinct cluster of endothelial cells at the BV junction (Fig. 6). We were unable to detect any difference between the localization of endothelial cells at the AV or BV

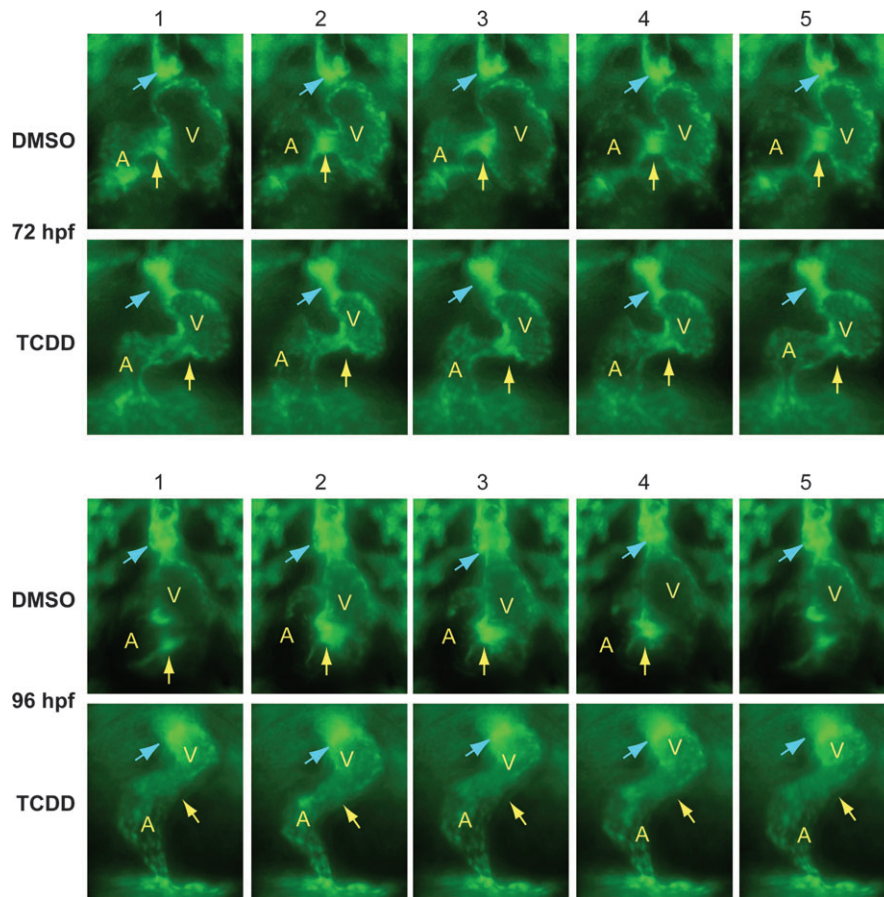


FIG. 3. TCDD alters the arrangement of endothelial cell clusters at valve sites. Newly fertilized *Tg(flk1:GFP)* eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” The figure shows sequential frames taken at six images per second. Representative ventral view fluorescence micrographs showing endocardial cells at the AV and BV junctions are shown for 72 and 96 hpf as indicated. A indicates atrium; V indicates ventricle. Arrows show the location of the AV and BV junctions. The arrows are positioned to indicate forming valve leaflets at the junctions in the DMSO images; leaflets were not observed in the TCDD-exposed hearts.

junction in control and TCDD samples at 48 hpf or before. However, after 48 hpf we observed progressive loss of localization of these GFP-positive endothelial cells in the TCDD-treated samples. This TCDD-induced delocalization of endothelial cells at the AV junction was clearly evident in the ventral view images taken at 72 hpf and 96 hpf (Figs. 3 and 6).

We also found that TCDD caused the GFP-positive endothelial cells at the BV junction to become progressively less restricted at the BV junction over time. At 72 hpf, vehicle exposed embryos clearly exhibited a distinct cluster of endothelial cells at the BV junction. However, in TCDD-exposed fish the endothelial cells at the BV junction were distributed across a larger region of the outflow tract compared with the vehicle control (Fig. 6) (supplemental movie: 72hpf_flk).

TCDD Alters AV Boundary Specification during the EMT Process

During early heart development genes encoding bone morphogenetic protein 4 (*bmp4*) and *notch1b* are expressed

throughout the heart. However, as the heart develops, these markers become restricted to the presumptive AV valve region: *bmp4* is expressed in the myocardial layer, and *notch1b* is found in the endocardial layer (Walsh and Stainier, 2001). We

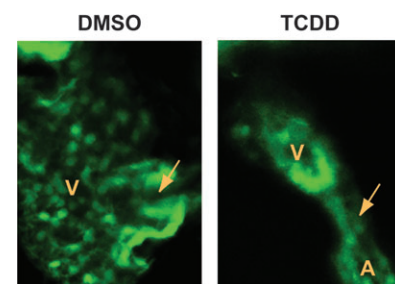


FIG. 4. Valve leaflets fail to form in 120 hpf zebrafish exposed to TCDD. Newly fertilized *Tg(flk1:GFP)* eggs were treated with TCDD or DMSO as a vehicle control and ventral view confocal micrographs were recorded at 120 hpf as described in the Materials and Methods. Representative images show the AV junction, indicated by the arrow. Valve leaflets can be clearly seen flanking the AV passage in the DMSO heart. A indicates atrium; V indicates ventricle.

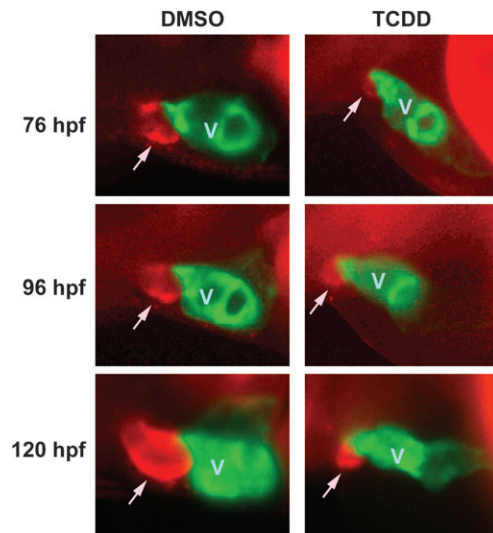


FIG. 5. Development of the BA is inhibited by TCDD. *Tg(cmlc2::gfp)* embryos were exposed to TCDD or DMSO vehicle and treated with DAR-4M AM to stain the BA as described in the Materials and Methods. Embryos were mounted in methylcellulose and visualized using epifluorescence. Lateral view images of representative vehicle and TCDD embryos are shown for 76, 96, and 120 hpf are shown. The head is toward the left of the figure, and the dorsal side is to the top. The ventricle is labeled with GFP, and indicated by a V; the DAR-4 signal identifying the BA is seen as red, and is indicated by an arrow.

used these markers to test the hypothesis that TCDD disrupts AV boundary specification.

At 48 hpf, we consistently observed a small ring or dot of *bmp4* staining, corresponding to the previously reported localized expression at the AV boundary myocardium in both vehicle and TCDD-exposed embryos. This is visible as a small region of staining at the AV boundary and another at the outflow tract junction (Fig. 7). By 60 hpf, a point at which the EMT process has normally started (Beis *et al.*, 2005), we consistently saw *bmp4* expressed in a broader, less restricted region in TCDD-treated embryos. By 72 hpf, the *bmp4* signal was consistently more intense in the TCDD-treated hearts than in the controls, and covered a region that extended completely beyond the heart, making it quite evident that expression was no longer restricted to the AV boundary.

We observed very similar results with *notch1b* staining. As with *bmp4*, we initially observed a distinct spot of *notch1b* staining marking the AV junction endocardium in both control and TCDD-treated fish (Fig. 8). However, in the TCDD-treated fish this pattern of localized expression steadily broadened as the notch signal became progressively delocalized and stronger at 60 and 72 hpf.

These results demonstrate that TCDD alters processes specifying valve development at the AV boundary. The timing of the changes in *bmp4* and *notch1b* localization corresponds to the loss of the localized pattern of endothelial cells at the AV and BV junctions depicted in Figure 6, and takes place at a stage in development during which critical processes affecting valve formation have been reported to occur. Thus, it is not

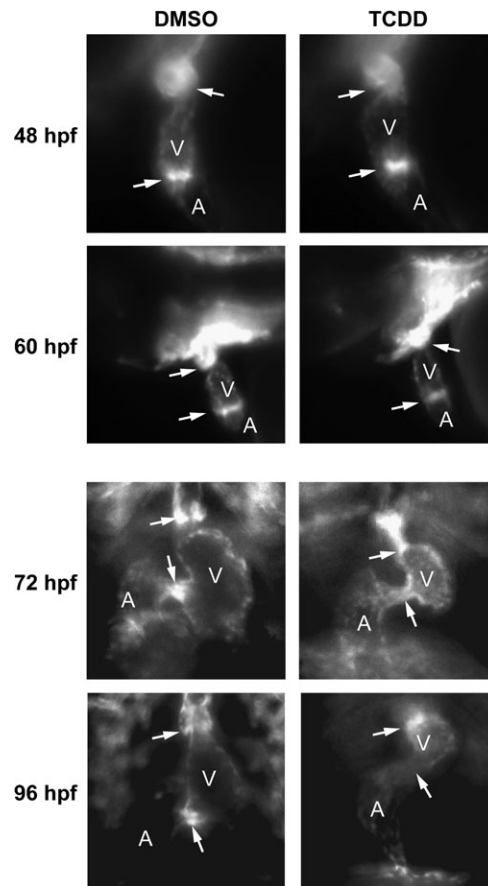


FIG. 6. TCDD alters endothelial cell localization at the AV and BV junctions. Newly fertilized *Tg(flk1:GFP)* eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” Representative fluorescence micrographs showing lateral views (48 and 60 hpf) and ventral views (72 and 96 hpf) of the heart are shown. These orientations were selected to give the best view of the heart at each stage of development. A indicates atrium; V indicates ventricle. Arrows show the locations of the AV and BV junctions.

surprising that TCDD prevented subsequent formation of valve structures, because TCDD leads to a loss of cellular conditions required for valve formation.

DISCUSSION

Our results demonstrate that TCDD exposure produces alterations in valve formation in the developing zebrafish heart. These effects were first observed as a failure of the AV junction to completely seal as the ventricle beats, allowing blood cells to slip backwards during ventricular systole. Although the observed blood regurgitation at the AV and BV valves is not the only defect caused by TCDD, the failure of a fraction of the blood cells to pass through the valves in the proper direction must contribute to the loss of peripheral circulation that TCDD exposure produces.

The changes in heart valve formation and function were associated with disruption of cellular patterns associated with

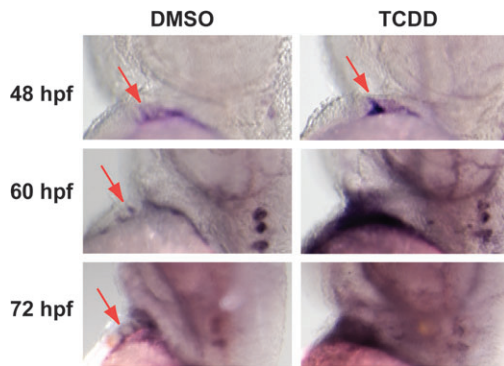


FIG. 7. TCDD alters *bmp4* mRNA localization in the developing heart. Newly fertilized zebrafish eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” Representative lateral view micrographs are shown for hearts stained by *in situ* hybridization with a probe specific for *bmp4* mRNA. Arrows indicate *bmp4* signal at the location of the AV boundary myocardium. The head is toward the top of each panel and the dorsal side is to the right.

normal valve formation. The localized clustering of endothelial cells that is normally observed at the AV and BV junctions as a precursor to valve development was disrupted by TCDD. Initially these localized clusters of endothelial cells formed normally despite the presence of TCDD, and were clearly present at 48 hpf. However, in the TCDD-exposed fish the endothelial cells do not remain localized to these junctions. This loss of endothelial cell localization coincided closely with delocalization of *bmp4* and *notch1b* expression.

It is interesting to note that TCDD did not simply delay or halt the process of valve formation. At early time points the contractile process at the AV junction was normal, producing a functional barrier to retrograde blood flow despite the presence of TCDD. In addition, the localization of endothelial cells and the expression of *notch1b* and *bmp4* appeared normal at early time points, despite the presence of TCDD. However, as time progressed, all of these normal features of early valve formation deteriorated, reversing whatever progress toward valve formation that had been made. Similarly, although TCDD did not prevent the initial formation of the BA, the size of the BA was greatly reduced in TCDD-exposed zebrafish. As with valve formation, TCDD did not appear to block the initial process of tissue specification, but appeared to prevent the development needed to complete the nascent structure.

TCDD and Endothelial Cells in the Heart

This work was initiated in part by casual observations that TCDD alters the distinct clusters of endothelial cells labeled with GFP at the sites of valve formation. Thereafter, we conducted numerous experiments directed at determining whether TCDD affects the different steps in the EMT process described in detail by Beis *et al.* (2005). As we were preparing this manuscript a report from Scherz *et al.* (2008) described work suggesting that there is no EMT process in the

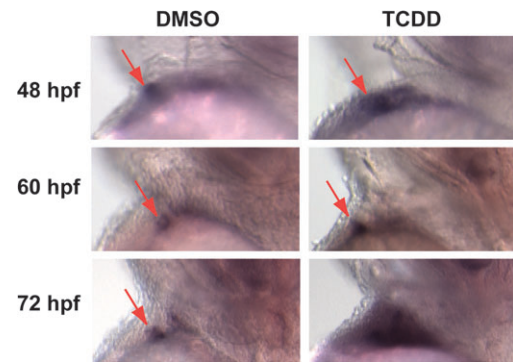


FIG. 8. TCDD alters *notch1b* mRNA localization in the developing heart. Newly fertilized zebrafish eggs were treated with TCDD or DMSO as a vehicle control as described in the “Materials and Methods.” Representative lateral view micrographs are shown for hearts stained by *in situ* hybridization with a probe specific for *notch1b* mRNA. Arrows indicate *notch1b* signal at the location of the AV boundary endocardium. The head is toward the top of each panel and the dorsal side is to the right.

developing heart, and that instead the leaflets form directly from layers of endothelial cells. Some of our work, notably our observation of visible leaflets of GFP-positive endothelial cells in movies of hearts as early as 72 hpf, supports this recently proposed model. Beyond this, our work has little to contribute to the question of whether zebrafish valve formation goes through the classical EMT. Instead, our results demonstrate that the AHR agonist TCDD inhibits valve formation, entirely disrupting endothelial cell positioning at the presumptive valve site.

TCDD Alteration of bmp4 and notch1b Expression

Bmp4 and *Notch1* are Wnt/ β -catenin pathway components needed for normal valve development. Zebrafish mutants such as *jeekyll* lack localization of *bmp4* and *notch1b* at the AV boundary. These mutants fail to form ECs at 96 hpf and have retrograde blood flow (Stainier *et al.*, 1996, 2002). Experiments in mice with a hypomorphic *bmp4* allele indicate that *bmp4* is specifically required for proper AV septation after the cushions have formed (Jiao *et al.*, 2003). Mutation in the *notch1* receptor in mice or the *RBPJk* effector results in abnormal endocardial adhesion complexes and abortive endocardial EMT *in vivo* and *in vitro* (Grego-Bessa *et al.*, 2004). Thus, the effects of TCDD on *bmp4* and *notch1b* could be upstream of the morphological defects that we observed.

However, increased *bmp4* and *notch1b* expression has been associated with promoting valve formation. Expression of mouse *Notch1* (N1IC) in zebrafish embryos produces hypercellular ECs and enlarged AV valves, whereas suppression of N1IC causes atrophic ECs (Timmerman *et al.*, 2004). Activation of the Wnt/ β -catenin pathway leads to excessive development of valve cushions. This is associated with an upregulation of *bmp4* and *notch1b* (Hurlstone *et al.*, 2003). One might therefore expect the increased *bmp4* and *notch1b* expression

produced by TCDD would lead to increased formation of valve structures, rather than what we observed.

It is likely though that EC formation is regulated by a more complex signal than the simple levels of *notch1b* and *bmp4* mRNAs. For example, zebrafish *cloche* mutants fail to develop endocardium and lack *notch1b* mRNA. This failure to express *notch1b* is associated with a loss of *bmp4* localization at the AV junction. Thus, the absence of one layer abolishes processes needed to specify the site of the AV ring (Milan *et al.*, 2006). It is possible that the elevated *bmp4* and *notch1b* signals produced by TCDD result from a blockade of a process downstream in the Wnt signaling pathway needed for endothelial cell localization and valve formation. If this were the case, feedback mechanisms might upregulate *bmp4* and *notch1b* in an attempt to restore normal Wnt signaling.

Blood Regurgitation, Valve Function, and Circulation

Because TCDD affects stroke volume and blood flow (Carney *et al.* 2006), it is possible that the effect on valve formation is secondary to an emerging loss of contractility and blood movement through the heart. The flow of blood through the heart is thought to generate signals that play an important role in valve formation (Hove *et al.*, 2003). Silent heart (*sih*) mutants, with no blood flow, exhibit no EC formation. Similarly, *cardiofunk* mutants with no early blood flow, have defective valve cushion formation (Bartman *et al.*, 2004; Sehnert *et al.*, 2002).

It is noteworthy however that although neither the *silent heart* nor *cardiofunk* mutations alters the restricted *bmp4* expression at the AV boundary, indicating that loss of blood flow does not alter AV boundary specification (Bartman *et al.*, 2004). The effect of TCDD on localization of endothelial cells and molecular markers at the AV boundary is far more similar to the *jeekyll* mutant, which produces delocalized *bmp4* and *notch1b* expression, pericardial edema, a loss of endothelial cell localization at the AV boundary, and blood regurgitation by 48 hpf (Walsh and Stainier, 2001). Thus, although TCDD could produce altered valve development by altering blood flow, it is also possible that TCDD interferes with a developmental process that is independent of blood flow.

Environmental Factors and Valve Development

Over the past decade numerous studies have identified and characterized zebrafish mutants in an attempt to understand the key genes needed for normal heart development and function (Chen *et al.*, 1996; Lee *et al.*, 1994; Stainier *et al.*, 1993, 1996). Although these studies have been useful in identifying genetic factors contributing to pathological conditions involving the cardiovascular system, there is little doubt that environmental causes also lead to heart abnormalities. By using the zebrafish, a well-established developmental model system, to study the effects of TCDD on heart valve development, we can combine our results with a wealth of knowledge obtained using genetic

methods. This is a step toward the long-term goal of understanding how environmental and genetic factors combine to cause heart abnormalities and disease.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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