Mol Biol Rep (2014) 41:7973–7983
DOI 10.1007/s11033-014-3692-6

Toxic effects of polychlorinated biphenyls on cardiac development in zebrafish

Mengmeng Li · Xuejie Wang · Jingai Zhu · Shasha Zhu · Xiaoshan Hu · Chun Zhu · Xirong Guo · Zhangbin Yu · Shuping Han

Received: 10 February 2014 / Accepted: 21 August 2014 / Published online: 28 August 2014

Abstract Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants that may pose significant health-risks to various organisms including humans. Although the mixed PCB Aroclor 1254 is widespread in the environment, its potential toxic effect on heart development and the mechanism underlying its developmental toxicity have not been previously studied. Here, we used the zebrafish as a toxicogenomic model to examine the effects of Aroclor 1254 on heart development. We found that PCB exposure during zebrafish development induced heart abnormalities including pericardial edema and cardiac looping defects. Further malformations of the zebrafish embryo were observed and death of the larvae occurred in a time- and dose-dependent manner. Our mechanistic studies revealed that abnormalities in the arylhydrocarbon receptor, Wnt and retinoic acid signaling pathways may underlie the effects of PCBs on zebrafish heart development. Interestingly, co-administration of Aroclor 1254 and diethylaminobenzaldehyde, an inhibitor of retinaldehyde dehydrogenase, partially rescued the toxic effects of PCBs on zebrafish heart development. In conclusion, PCBs can induce developmental defects in the zebrafish heart, which may be mediated by abnormal RA signaling.

M. Li and X. Wang have contributed equally to this study and they should be regarded as joint first authors.

M. Li · X. Wang · J. Zhu · S. Zhu · X. Hu · C. Zhu · X. Guo · Z. Yu · S. Han (✉)
State Key Laboratory of Reproductive Medicine, Department of Pediatrics, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing 210029, China
e-mail: xuhan70@sina.com

Z. Yu
e-mail: zhangbinyu@njmu.edu.cn

Keywords Zebrafish · Polychlorinated biphenyls · Heart · Development · Signaling pathway

Introduction

Congenital heart disease represents for nearly one-third of all major congenital anomalies, representing a major global health problem [1]. It is considered to be a multifactorial complex disease with environmental and genetic factors playing important roles, whose etiology and pathogenesis is not yet fully elucidated [2]. It is widely accepted that environmental insults during fetal development increase the risk of congenital heart disease, including exposure to chemical teratogens [3]. Furthermore, the fetal development stage is a critical period for heart development and normal cardiac function, because fetal sensitivity and the ability to absorb toxins and environmental chemical pollutants are higher than that of adults [4]. As such, environmental chemical pollutants affecting normal development, particularly in the heart, have attracted increasing attention.

Polychlorinated biphenyls (PCBs) are semi-volatile organic pollutants with high toxicity, whose chemical and physical stability prevents their degradation, leading to their ubiquitous presence in the biosphere and bioaccumulation [5]. They can be found in human blood, adipose tissue, and breast milk [6, 7], and have been shown to cross the placenta and reduce gestational length and birth weight, as well as affect fetal development [6, 8–10]. Additionally, embryonic exposure to PCBs can lead to pericardial sac edema, yolk sac edema and growth retardation in zebrafish embryos [11]. We hypothesize that maternal PCB exposure seriously affects the fetal development of multiple organs, including the heart. Since the heart is the first organ to form and function during vertebrate embryogenesis [12],

elucidating the toxic effects and its potential mechanism on cardiac development is essential for prevention of diseases due to PCB exposure, particularly congenital heart defects. Similarly, although there is growing evidence in zebrafish to support the hypothesis that PCBs have adverse effects on zebrafish development, the mechanisms is not well understood.

Zebrafish is an attractive and widely used vertebrate model for studying cardiovascular development. Owing to the conserved developmental program within vertebrates, fish and mammals share many similar developmental processes. For example, it has been shown that zebrafish is responsive to chemicals, such as small molecules, drugs and environmental toxicants in a similar manner as mammals [13–16]. Additionally, severe defects in the heart do not lead to immediate lethality as in many vertebrate models, and even in the total absence of blood circulation, they receive enough oxygen by passive diffusion to survive and continue to develop in a relatively normal fashion for several days, thereby allowing a detailed analysis of animals with severe cardiovascular defects [17].

Cardiac development is a very complicated and elaborate biological process those goes through a series of important morphological events, including cell specification, migration and differentiation [18], and involves a number of signaling pathways, such as the arylhydrocarbon receptor (AhR), Wnt and retinoic acid (RA) signaling pathways [19–21]. Additionally, there is a wide range of crosstalk between these signaling pathways to ensure the coordinated development of the embryonic heart in time and space. This study aimed to use zebrafish as a toxicogenomic model to capture cardiac developmental phenotype and transcriptomic changes in these signaling pathways, and identify the potential molecular mechanism that contributes to the cardiotoxicity of early-life exposure to PCBs.

Materials and methods

Reagents

Aroclor 1254 (lot 48586), a commercial mixture of PCBs, was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Other reagents were of analytical grade.

Animal husbandry

The zebrafish used in this study were housed in the zebrafish facility of the Model Animal Research Center (MARC), Nanjing University, Tübingen (Germany) strain, in accordance with IACUC-approved protocol. Embryos were obtained from natural spawning of wild type adults, and they were grown at 28 ± 1 °C in embryo medium as previously described [22]. Morphological features were used to determine the embryonic developmental stage, as described by Kimmel et al. [23]. Embryos older than 24 h post fertilization (hpf) were incubated in 0.003 % phenylthiourea to inhibit pigment formation.

PCBs exposure and DEAB treatment

Two types of experiments were performed in this study, general toxicity tests and rescue experiments. In the toxicity tests, immediately after fertilization, embryos were randomly exposed to 0, 0.125, 0.25, 0.5 or 1 mg/L Aroclor 1254. In the rescue experiments, embryos were treated with 4-diethylaminobenzaldehyde (DEAB) at nominal concentrations between 0, 5, 10 and 25 nmol/L, and with 1 mg/mL Aroclor 1254. In all studies, Aroclor 1254 was dissolved with 0.01 % methanol into rearing solution and the embryos were kept within a 100 mL glass Petri dish. The rearing solution contained 1 g/L NaCl, 0.3 g/L KCl, 0.4 g/L $CaCl_2$ and 1.6 g/L $MgSO_4$. The solution containing 0.01 % methanol served as the overall control. Dishes were covered to prevent the evaporation of PCBs and solutions were changed and degenerating embryos removed daily.

Counting hatching rate, malformation rates and lethality and observation of zebrafish heart development

After Aroclor 1254 exposure we counted the hatching rate at 72 hpf, malformation and lethality rates at 24, 48, 72, 96 and 120 hpf, observing the heart-specific phenotype. Embryos were photographed with a Olympus DP71 digital camera (Olympus, Tokyo, Japan) to observe the heart-specific phenotype and the digital images were processed using Adobe Photoshop software CS6.

Measurement of heart rate

Embryos were anesthetized in ethyl aminobenzoate (Tricaine, Sigma-Aldrich Co.). The Tricaine solution stock was made as follows: 400 mg Tricaine powder, 97.9 mL DD water and 2.1 mL 1 mol/L Tris (pH 9). The solution was adjusted to pH 7 and stored in the freezer. To anesthetize fish, 4.2 mL Tricaine solution stock was diluted in 100 mL clean tank water. The anesthetized embryos were then transferred to a recording chamber perfused with modified Tyrode's solution (136 mmol/L NaCl, 5.4 mmol/L KCl, 0.3 mmol/L NaH_2PO_4, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 5 mmol/L glucose, pH 7.3) at different time points. The heart rate was calculated by counting the number of sequential contractions, beginning and ending at the end diastole. This was conducted under a dissect microscope in 30 s intervals.

Histology

Paraformaldehyde fixed embryos were processed routinely, embedded in paraffin, sectioned at 2 μm using a RM 2135 microtome (Leica, German) and stained with hemotoxylin and eosin for light microscopic examination.

Preparation of RNA probes for in situ hybridization

RNA probes for in situ hybridization were generated for: atrial myosin heavy chain (amhc), ventricular myosin heavy chain (vmhc), cardiac myosin light chain-2 (cmlc2), retinaldehyde dehydrogenase gene (Raldh2) and Cytochrome P450 26A1 (Cyp26a1). The probes were amplified from cDNA generated from embryos at 24 hpf and subcloned into pMD-18T vectors (Takara, Japan). These plasmids were linearized and transcribed as follows: Amhc, Raldh2 and Cyp26a1, NcoI (Takara)/SP6; vmhc and cmlc2, SalI/T7; RNA probe was transcripted by using of MAXIscript In Vitro Transcription Kit (Ambion, USA). Digoxigenin (DIG)-labeled single strand RNA probes were prepared using a DIG RNA labeling kit (Roche Diagnostics, USA) according to the manufacturer's instructions, and stored at −80 °C for future use.

Whole-mount in situ hybridization

In situ hybridization was performed as previously described [24], using riboprobes specific for vmhc, amhc, cmlc2, Raldh2 and Cyp26a1 [25, 26]. All embryos were fixed in 4 % paraformaldehyde (PFA) at 4 °C for 12–16 h, before undergoing serial dehydration through graded methanol solutions (25, 50, 75, 100) and stored at −20 °C for future use. Hybridization was performed at 65 °C, in 50 % formamide buffer with digoxigenin-labeled RNA anti-sense probes over-night. After a series of washes, embryos were treated in blocking solution for 1–4 h. Hybridization was detected by Anti-Digoxigenin-AP Fab fragment and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The vmhc, amhc, cmlc2, Raldh2 and Cyp26a1 expression domains were visualized using NBT/BCIP (Roche, USA) staining. The control and experimental embryos were processed in parallel. In situ images were captured using Olympus DP71 digital camera (Olympus, Tokyo, Japan).

Quantification of gene expression by real-time quantitative reverse transcriptase PCR

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the relative expression levels of Ahr2, Arnt, cyp1a1, Wnt1, Wnt5a, Wnt11, Cyp26a1, Raldh2, and β-actin (for control) using an ABI 7300 detection system (Applied Biosystems, Foster City, CA). Total RNA was extracted from 30 embryos using Trizol reagent (Invitrogen, USA) for each assay. RNA were reverse-transcribed and PCR were performed using the SYBR Green method following the manufacturer's protocol. Real-time PCR reaction conditions were: 95 °C for 10 s followed by 40 cycles of 94 °C for 5 s, and 72 °C for 30 s with a final extension of 7 min at 72 °C. Primers used for qRT-PCR are listed in Table 1. Relative quantification of each gene was performed by the comparative CT method. The transcript level of target genes was normalized to the mRNA level of β-actin according to standard procedures.

Table 1 Sequences of primers used in qRT-PCR

Gene name	Sequences
Ahr2	Sense: 5′-acggtgaagctctcccata-3′
	Antisense: 5′-agtaggtttctctggccac-3′
Arnt	Sense: 5′-caccttggatcacatctcattg-3′
	Antisense: 5′-tcaccctccttagacggacc-3′
Cyp1a1	Sense: 5′- gctccgaataggtaattgavgat-3′
	Antisense: 5′-agagccgtgctgatagtgtc-3′
Wnt1	Sense: 5′-gctgagagggaggtgacaaa-3′
	Antisense: 5′-tacaaaggcaggagggaatg-3′
Wnt5a	Sense: 5′-cccagacatcagggagtgat-3′
	Antisense: 5′-ctcgtggataccgcaagatt-3′
Wnt11	Sense: 5′-gtaagtgccatggggtgtct-3′
	Antisense: 5′-gcttccaagtgaaggcaaag-3′
Cyp26a1	Sense: 5′-cccagacatcagggagtgat-3′
	Antisense: 5′-ctcgtggataccgcaagatt-3′
Raldh2	Sense: 5′-cccagacatcagggagtgat-3′
	Antisense: 5′-ctcgtggataccgcaagatt-3′
β-action	Sense: 5′-caacagagagaagatgacacagatc a-3′
	Antisense: 5′-gtcacaccatcaccagagtccatcac-3′

Immunofluorescence staining for cyp1a1

Immunofluorescent staining on zebrafish was essentially performed as described previously [27]. In brief, fish were fixed in 4 % PFA made in 0.1 M phosphate buffer. Blocking, primary, and secondary antibody incubations were performed in 10 % goat serum containing 0.5 % Triton X-100 in PBS at either room temperature (blocking step, 2–5 h) or 4 °C (antibody incubations, 16–48 h). Finally, the embryos were detected by fluorescence microscope examination. The primary antibody used was monoclonal 1-12-3 against fish cyp1a1 [28].

Statistical analysis

Statistical analyses were performed using the χ^2-test and Fisher's exact test for the categorical data, or a one-way

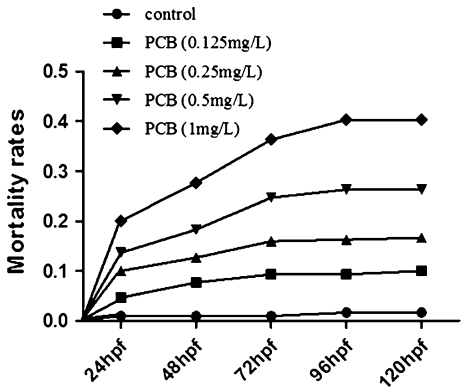

Fig. 1 The acute toxicity (mortality rate) test of Aroclor 1254 on zebrafish in a time- and dose-dependent manner. The rates of lethality ranged from 7.7 to 40.3 % in Aroclor 1254 exposed embryos. With increasing Aroclor 1254 concentration and time, the mortality rates of zebrafish were increased, indicating specific time- and dose-dependent effects

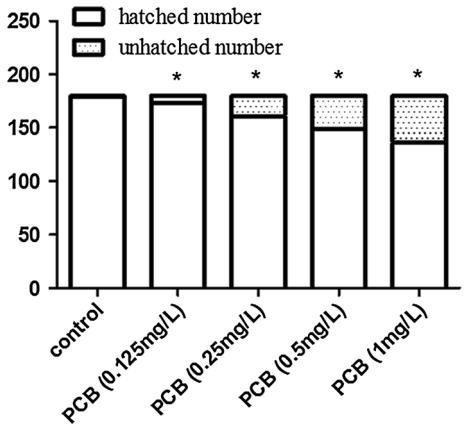

Fig. 2 The acute toxicity (hatchability at 72 hpf) test of Aroclor 1254 on zebrafish in a dose-dependent manner. The rates of hatched embryos ranged from 75.56 to 96.48 % in Aroclor 1254 exposed embryos. With increasing Aroclor 1254 concentration, the mortality rates of zebrafish were decreased, indicating specific dose-dependent effects. $*P < 0.05$ when compared with the normal control group, $P < 0.05$

analysis of variance (ANOVA, SPSS 16.0, SPSS, USA) for the measurement data. The dose–response relationship was analyzed by the Mantel–Haenszel test. A P value <0.05 was considered statistically significant. Every experiment was independently performed at least three times.

Results

Lethality and hatchability of PCB-exposed zebrafish embryos

To characterize Aroclor 1254 toxicity during zebrafish development we evaluated the effects of increasing concentrations of Aroclor 1254 on mortality rate and hatchability at different development stages (24, 48, 72, 96, and 120 hpf). Our results show that the rates of lethality ranged from 4.7 to 40.3 % in Aroclor 1254 exposed embryos. At a concentration of 0.125 mg/L Aroclor 1254, the mortality rate at 24 hpf was not different than the 1.0 % rate observed in the control group. Meanwhile, the hatching rates were from 96.48 to 75.56 % in Aroclor 1254-exposed groups. With increasing Aroclor 1254 concentration and time, the mortality rates of zebrafish were increased and the hatching rates declined, indicating specific time- and dose-dependent effects (Figs. 1, 2). These findings demonstrate that PCBs can retard the development of zebrafish embryos and lead to the death of zebrafish embryos and larvae.

The effect of PCBs on gross morphology

To select a suitable concentration for future work, the effects of increasing concentrations of Aroclor 1254 on malformation rate at different stages (24, 48, 72, 96 and 120 hpf) were evaluated. The malformation rate is identified as the ratio of embryos and larvae with gross morphological deformities including smaller size, pericardial edema and abnormal curly tail (Fig. 3) The results suggest that the malformation rate of zebrafish embryos and larvae increased in a time- and dose-dependent manner. The malformation rates ranged from 10 to 50 % in Aroclor 1254-exposed groups when compared with control groups (Fig. 4). Since exposure to 1 mg/L Aroclor 1254, the phenotypes were the most visible, therefore we chose a concentration of 1 mg/L Aroclor 1254 to investigate its effect on cardiac development.

The effect of PCBs on heart morphology in zebrafish

In normal cardiac development, the linear heart tube has formed at 24 hpf and the tube lies along the anterioposterior axis, with the atrial end to the left of the midline. Subsequently, visible distinct ventricular and atrial chambers have formed by 30 hpf, and the heart undergoes looping morphogenesis by 36 hpf, with functional valves formed by 48 hpf [17]. At 48 hpf, the early cardiac development and posterior circulation function has completed, thus, we next chose 72 and 96 hpf to assess cardiac development. As shown in Fig. 5, obvious abnormalities in heart morphology was observed in Aroclor 1254-exposed embryos, including severe pericardial edema and cardiac looping defects, which are consistent with the phenotypes described previously [29]. Instead of the normal looped and S-shaped hearts that we observed in control embryos, the heart chambers from Aroclor 1254-treated embryos were

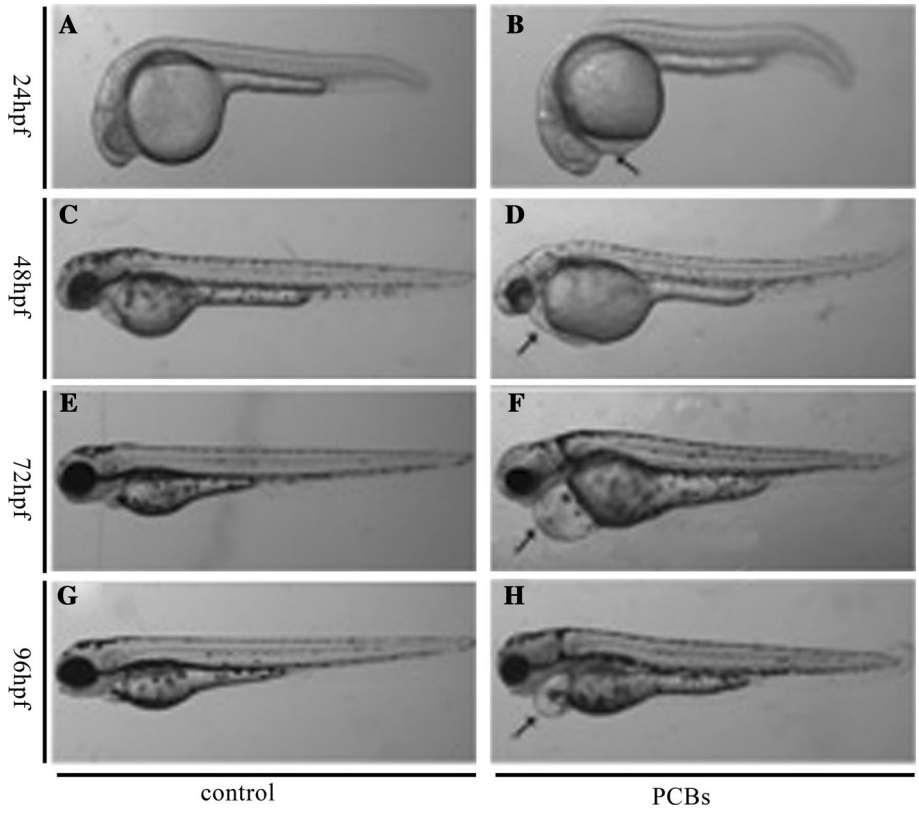

Fig. 3 Effect of PCB exposure on the gross morphology of zebrafish embryos and larvae. All embryos are positioned with their anterior side to the left and their lateral side to the front. Embryos were treated with vehicle as previously described (**a**, **c**, **e**, **g**) or with Aroclor 1254 at concentration of 1 mg/L (**b**, **d**, **f**, **h**), respectively and observed at a magnification of 3.2 times. Compared with the control group, embryos treated with Aroclor 1254 at 24 hpf displayed slight developmental arrest and pericardial edema (**b** vs. **a**). At 48 hpf, Aroclor 1254-exposed embryos displayed slight pericardial edema and bent tails (**d** vs. **c**). At 72 hpf (**f** vs. **e**) and 96 hpf (**h** vs. **g**), Aroclor 1254-exposed embryos displayed similar phenotypes to embryos at 24 and 48 hpf with more severe pericardial edema. *Black arrows* represent the pericardial edema

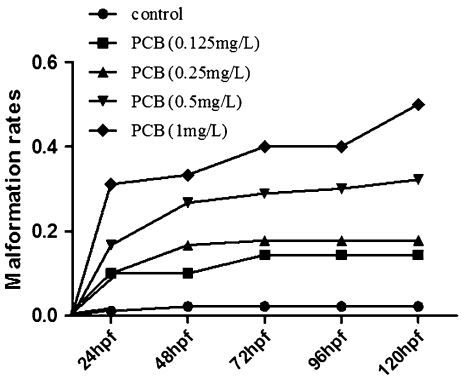

Fig. 4 The acute toxicity (malformation rate) test of Aroclor 1254 on zebrafish in a time- and dose-dependent manner. The rates of malformation ranged from 10 to 50 % in Aroclor 1254 exposed embryos. With increasing Aroclor 1254 concentration and time, the malformation rates of zebrafish were increased, indicating specific time- and dose-dependent effects

string-like and elongated. In the control group, the ventricle and atrium overlapped and were not distinguishable in the lateral view. In contrast, ventricles of the treated embryos were positioned anterior to the atrium, so the chambers could be easily distinguished with little overlap. These results indicated that PCBs caused abnormal heart tube development in zebrafish.

The effect of PCBs on cardiac function

The cardiac circulation function in zebrafish is completed by 48 hpf, so the heart rate was examined at 48, 72 and 96 hpf to evaluate the effects of PCBs on cardiac contraction. As shown in Fig. 6, the mean heart rate at 48 hpf were 163.5 ± 7.6 beats per minute (bpm), 133.3 ± 6.3, and 123.5 ± 4.9 bpm for the control group, 0.5 mg/L Aroclor 1254 treated group and 1 mg/L Aroclor 1254 treated group, respectively ($n = 50$). Moreover, the average heart rates at 72 hpf were 180 ± 9.4, 152.8 ± 7.9 and 138 ± 12.3 bpm for the control group, 0.5 mg/L Aroclor 1254 treated group and 1 mg/L Aroclor 1254 treated group, respectively. Moreover, the average heart rates at 96 hpf were 187.1 ± 12.9, 148.7 ± 9.5 and 130.8 ± 13.4 bpm for the control group, 0.5 mg/L Aroclor 1254 treated group and 1 mg/L Aroclor 1254 treated group, respectively. The results displayed that the heart rate was significantly decreased by PCB exposure in a dose-dependent manner. Conversely, the ventricles and atria of control embryos exhibited vigorous, rhythmic contractions, ensuring circulation throughout the body. In the PCB-treated groups, irregular and weak contractions were observed readily. Taken together, these data indicate that PCB exposure lead to a remarkable change in the contractile function of the heart.

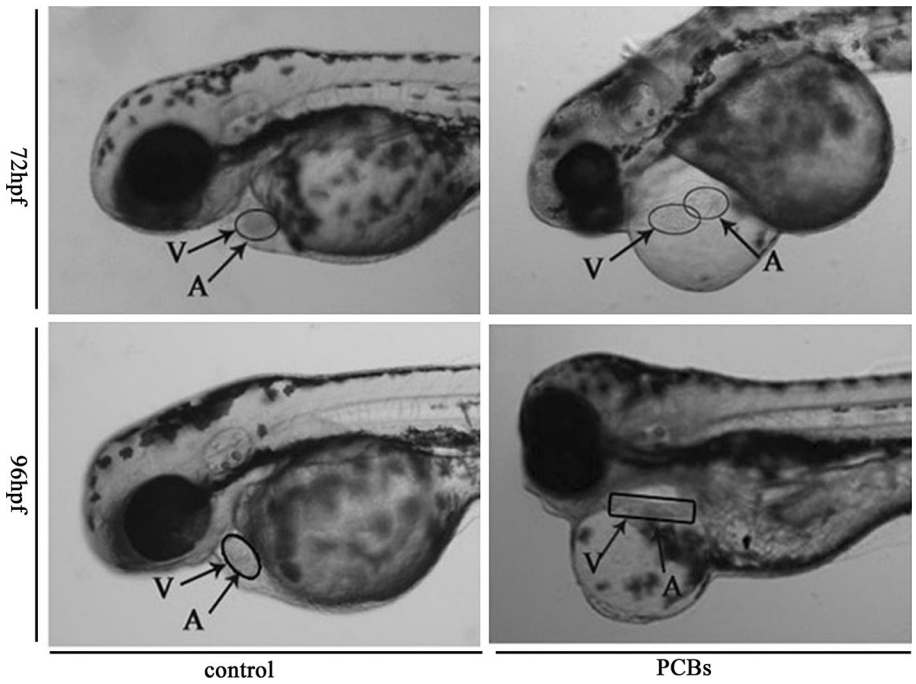

Fig. 5 The effect of PCBs on cardiac morphology in zebrafish embryos and larvae. All embryos are positioned with their anterior side to the left and their lateral side to the front. Embryos were treated with vehicle as previously described (**a**, **c**) or with Aroclor 1254 at a concentration of 1 mg/L (**b**, **d**) respectively. Compared with the control group, Aroclor 1254-exposed embryos at 72 hpf displayed severe pericardial edema at a magnification of 3.2 times (**a**, **b**). At a magnification of 6.3 times, the ventricle and atrium largely overlapped with each other in the lateral view in the control group; however, the ventricles of the treated embryos were positioned anterior to the atrium with little overlap at 96 hpf. Instead of the normal looped and S-shaped hearts observed in the control group, the heart chambers from Aroclor 1254 treated embryos were string-like and elongated (**c**, **d**). *V* ventricle, *A* atrium

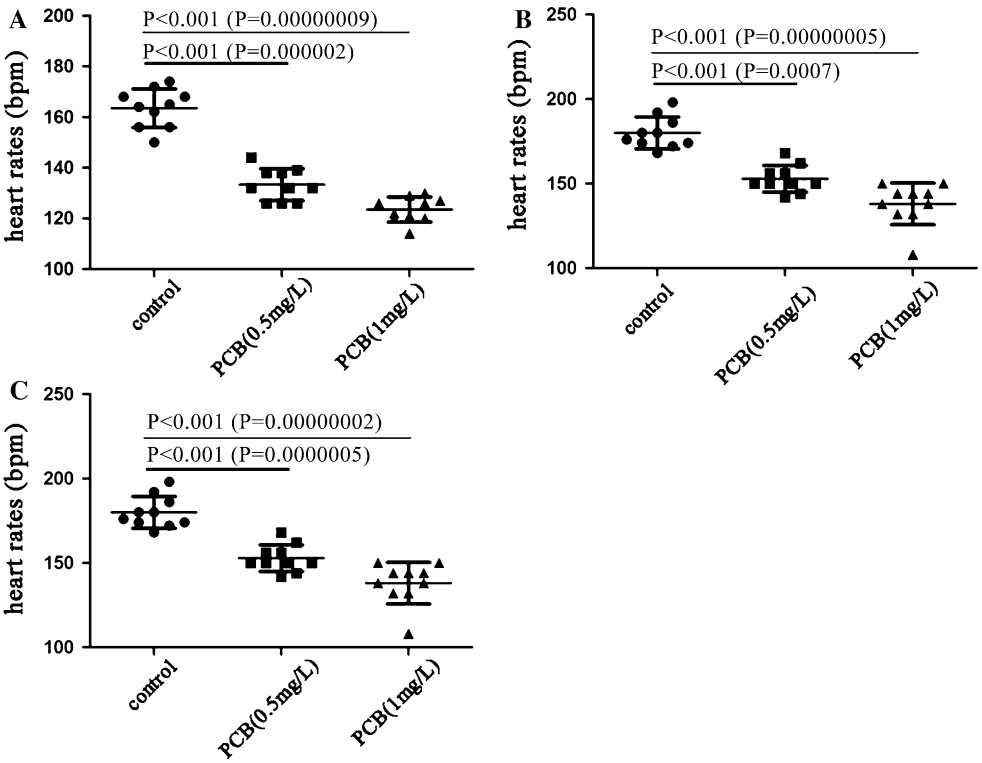

Fig. 6 PCB exposure decreased heart rates in zebrafish embryos and larvae. The heartbeats (bpm) were counted in embryos and larvae treated with vehicle, 0.5 mg/L Aroclor 1254 and 1 mg/L Aroclor 1254 at 48 hpf (**a**), 72 hpf (**b**) and 96 hpf (**c**). The heart rate gradually increased with development; however, it was reduced in PCB-exposed embryos and larvae when compared with controls at 24, 48 and 72 hpf

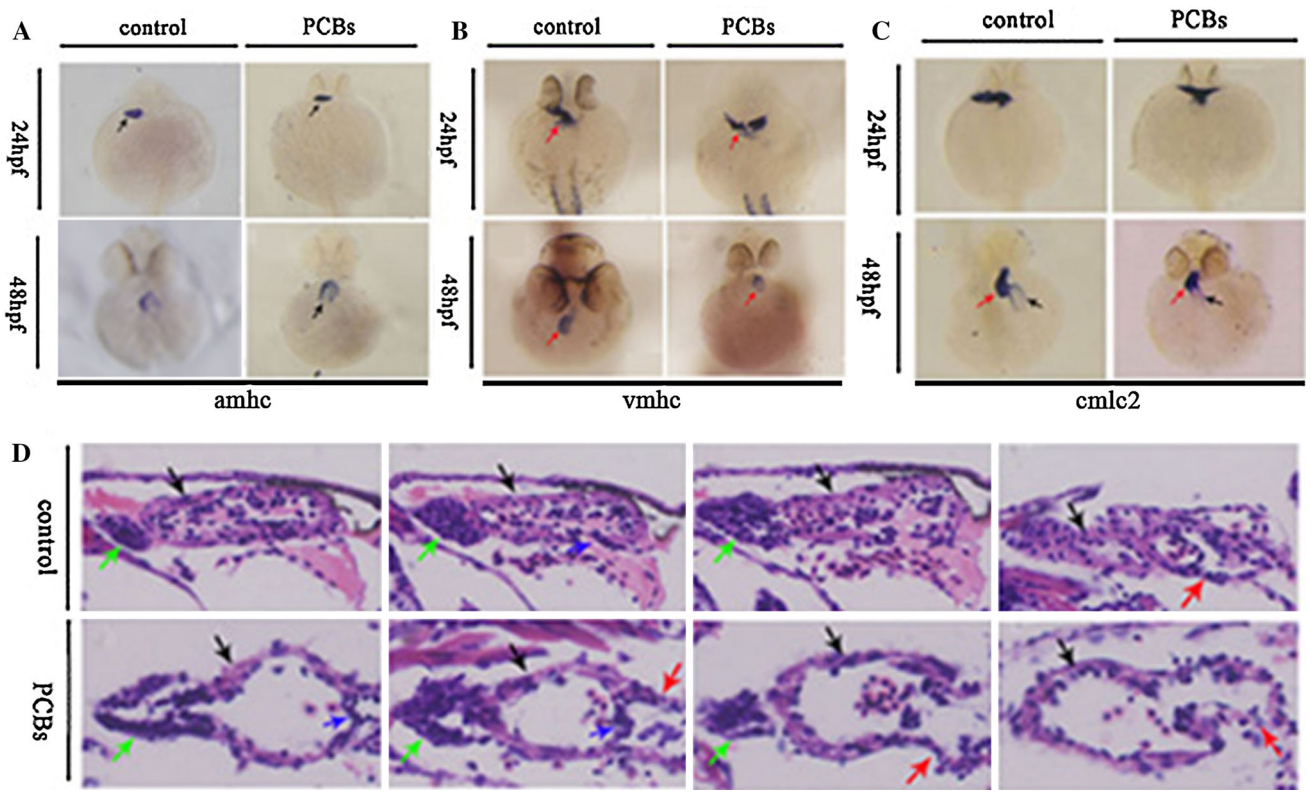

Fig. 7 The effect of PCBs on cardiac chamber development in zebrafish. **a** In situ mRNA hybridization for amhc at 24 and 48 hpf. **b** In situ mRNA hybridization for vmhc at 24 and 48 hpf. **c** In situ mRNA hybridization for cmlc2 at 24 and 48 hpf. **d** Cardiac histology in zebrafish larvae at 96 hpf. The cardiac sections at 96 hpf displayed obvious cardiac phenotypes, including wall thinning due to reduced myocardium and chamber dilation in the Aroclor 1254 treated group when compared with the control group, while, the valves seemed indistinguishable in the exposure group (*black arrowhead* atrium, *red arrowhead* ventricle, *green arrowhead* venous sinus, *blue arrowhead* atrioventricular valve). (Color figure online)

The effect of PCBs on cardiac chamber development in zebrafish

To further investigate the effect of PCBs on cardiac development at the molecular level, the expression of three cardiac chamber marker genes was examined at 24 and 48 hpf by whole-mount in situ hybridization, including amhc, vmhc and cmcl2, expressed in atrium, ventricle and throughout the heart, respectively [26, 30, 31]. Compared with control embryos, embryos exposed to Aroclor 1254 showed an abnormal expression position of amhc at 24 and 48 hpf, resulting in an abnormal atrial development (Fig. 7a). Similarly, as compared with control embryos, vmhc expression was abnormal at 24, and by 48 hpf the expression level was significantly reduced and the extent of expression area was visibly more narrow (Fig. 7b). We also determined cmlc2 expression, to observe the position of the ventricle and atrium directly. We found that the expression pattern of cmlc2 was altered in Aroclor 1254-treated embryos, compared with the control embryos at 24 hpf, while a string-like and elongated heart tube was observed at 48 hpf in the Aroclor 1254-treated group (Fig. 7c). Accordingly, we hypothesized that Aroclor 1254 exposure could impair cardiac chamber development, which we verified histologically. In agreement with this hypothesis, wall thinning because of a reduced myocardium and chamber dilation were observed in heart sections at 96 hpf, while the valve in the PCB-exposed embryos was indistinguishable from that in control group (Fig. 8d). Taken together, this data demonstrates that Aroclor 1254 exposure can induce significant toxic effects on cardiac chamber development, but not on heart valve development.

The effect of PCB exposure on RA, AHR and WNT signaling pathways in zebrafish

In order to better understand the mechanism underlying the effect of PCBs on cardiac development, we studied three signaling pathways important in this process, including AHR, WNT, and RA signaling. Recent studies suggest that RA signaling may participate in defining the posterior limit of the second heart field formation, which is only one of its important regulatory roles in early development [32]. Using whole mount in situ hybridization, which was confirmed by

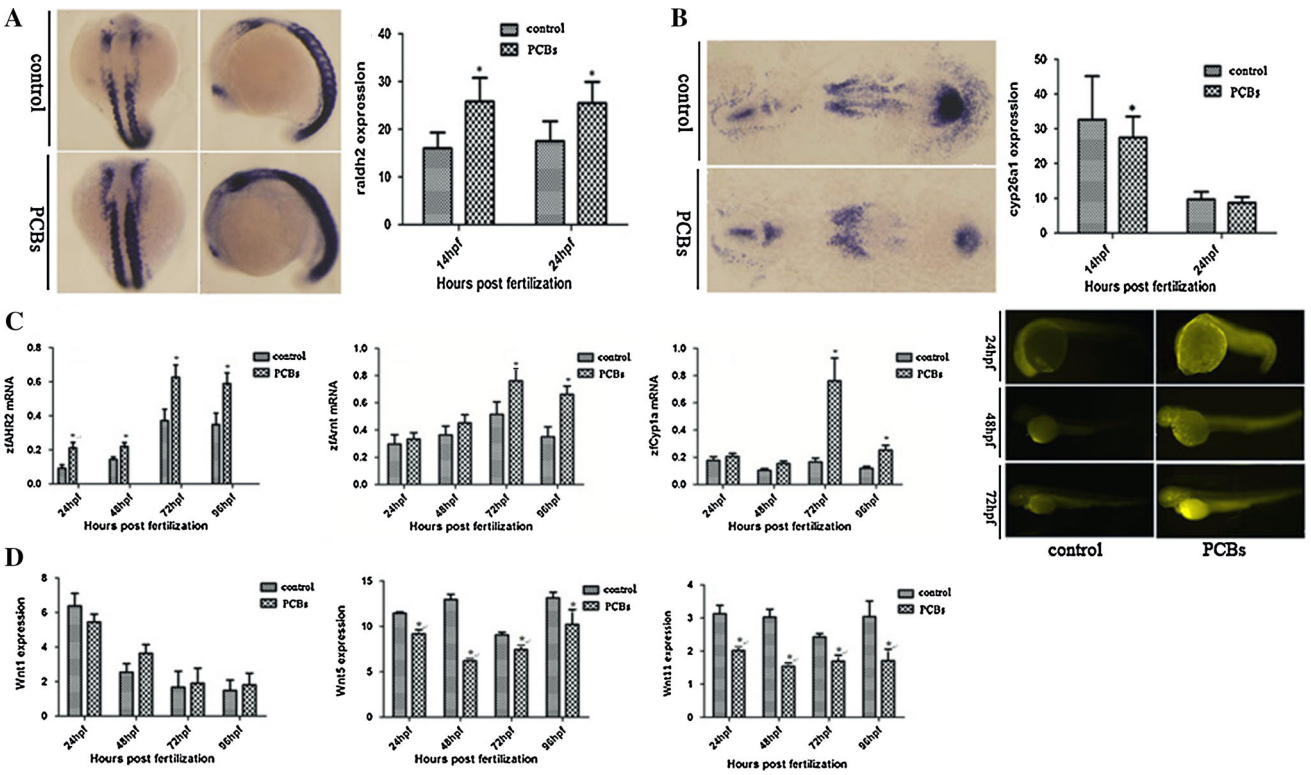

Fig. 8 The effect of PCB exposure on *RA, AHR and WNT* signaling pathways in zebrafish. **a** PCB exposure alters the expression of Raldh2. The expression of Raldh2 was detected by whole mount in situ hybridization at 14 hpf (*left panel*) and real-time PCR at 14 and 24 hpf (*right panel*). **b** PCB exposure alters the expression of Cyp26a1. The expression of Cyp26a1 was detected by whole mount in situ hybridization at 20 hpf (*left panel*) and real-time PCR at 14 and 24 hpf (*right panel*). **c** PCB exposure alters the expression of Ahr2 (*left panel*), Arnt (*middle panel*) and Cyp1a1 (*right panel*) in AHR signaling pathway at 24, 48, 72 and 96 hpf. **d** PCB exposure alters the expression of wnt1 (*left panel*), wnt5 (*middle panel*) and wnt11 (*right panel*) in WNT signaling pathway at 24, 48, 72 and 96 hpf. $*P < 0.05$

real-time PCR at 14 and 24 hpf, we detected changes in the expression of Raldh2, whose protein product is an enzyme that catalyzes the synthesis of RA from retinaldehyde, and cyp26a1, which encodes a member of the cytochrome P450 superfamily of enzymes regulating the cellular level of RA. The expression of Raldh2 was significantly up-regulated in PCB-exposed embryos at 14 and 24 hpf, particularly along the back and the somites (Fig. 8a). The expression level of Cyp26a1 was down-regulated compared in PCB-exposed embryos, which was significant at 14 and 24 hpf (Fig. 8b). These results indicate that PCB exposure influences the RA signaling pathway through up-regulation of Raldh2 and down-regulation of Cyp26a1, which contributes to abnormal cardiac development.

We next studied important members of the AHR signaling pathway in control and PCB-exposed embryos, including the expression level of Ahr2, Arnt and Cyp1a1 by real-time PCR at 24, 48, 72 and 96 hpf (Fig. 8c). The expression of Ahr2 was significantly up-regulated in PCB-exposed embryos at each time point. Moreover, we found that the expression of Arnt was up-regulated in PCB-exposed embryos at 72 and 96 hpf, but the increase was not significant at 24 and 48 hpf when compared with control embryos. The expression of Cyp1a was examined by real-time PCR and immunofluorescence. Our real-time PCR results show that the expression of Cyp1a was up-regulated in PCB-exposed embryos at each time point, although this was not significant at 24 and 48 hpf. This result was confirmed by immunofluorescence (Fig. 8c; right panel). Taken together, these results show that up-regulation of molecules in the AHR signaling pathway might contribute to the toxic effects of PCBs.

Last, we studied the WNT signaling pathway, which is divided into the canonical and non-canonical pathways (Fig. 8d). The expression of WNT1, which is one of the canonical WNT signaling molecules, showed no significant change in expression between control and PCB-exposed embryos, indicating that the canonical WNT signaling pathway was unaffected. The expression level of WNT5 and WNT11, which are both members of the non-canonical WNT signaling pathway, were significantly down-regulated in PCB-exposed embryos when compared with control embryos. These results indicate that inhibition of the non-canonical WNT signaling pathway might contribute to

Table 2 The mortality rates of zebrafish embryos and larvae co-treated with PCBs and different concentrations of DEAB for various exposure times

Concentration	Time				
	12 hpf (%)	24 hpf (%)	48 hpf (%)	72 hpf (%)	96 hpf (%)
Control	0.30	1	1	1	1.70
1 mg/L PCBs	12.30	20	27.7	36.30	40.30
5 nM DEAB	0.30	1	1	1	1.70
10 nM DEAB	0.30	1	1	1	1.70
25 nM DEAB	1.33	3	1	3	3.67
1 mg/L PCBs + 5 nM DEAB	9.60[a]	13.30[a]	19[a]	24[a]	35.80[a]
1 mg/L PCBs + 10 nM DEAB	7[a]	13[a]	15[a]	23.30[a]	33[a]
1 mg/L PCBs + 25 nM DEAB	7.60[a]	15.80[a]	18.80[a]	28.80[a]	40[a]

[a] Compared with embryos just treated with 1 mg/L PCBs, $P < 0.05$

the abnormal cardiac developmental in the PCB-exposed embryos.

The toxic effects of PCBs on zebrafish heart development are partially rescued by inhibition of RA signaling pathway

As shown in the study above-mentioned, we hypothesized that increased levels of endogenous RA played an important role in the toxic effects of PCBs on zebrafish embryonic development, especially the heart, so we examined whether inhibiting RA signaling could rescue PCB-exposed embryos. This was achieved using the RA synthesis inhibitor DEAB. We found that the mortality rate of embryos treated with 5, 10, and 25 nmol/L DEAB and 1 mg/L Aroclor 1254 were significantly decreased when compared with embryos just treated with 1 mg/L Aroclor 1254 (Table 2), showing that the toxic effects of PCBs on zebrafish heart development are partially rescued by appropriate inhibition of RA signaling pathway.

Discussion

PCBs are global, persistent organic pollutants with high multi-system toxicity, a long half-life and bioaccumulative characteristics, which have attracted wide attention [5]. Moreover, as the first organ to form during vertebrate embryogenesis, the heart has long been the focus of many scientific studies [33]. Thus, we focus on the toxic effects of PCBs on cardiac development.

The zebrafish heart has a single ventricle and single atrium, which is different from the four-chambered mammalian heart. However, cardiovascular development in early development is very similar between zebrafish and mammals [34]. Thus we used the zebrafish as a model to study the toxic effects of PCBs on cardiac development. We found that PCB exposure increased the embryonic lethality rate, coupled with a decreased hatching rate of embryos. We next showed that zebrafish exposed to PCBs during the early developmental period exhibited gross malformations, such as smaller size, pericardial edema and yolk sac edema, which were consistent with previous studies in zebrafish [35]. Additionally, an abnormal embryonic heart phenotype was observed during early heart development; specifically, PCB-exposed embryos exhibited defects in cardiac looping, wall thinning due to a reduced myocardium and chamber dilation. In addition to the structural defects described above, a decreased heart rate was observed in PCB-exposed embryos. However, the trajectory of heart rate was increased in PCB-exposed embryos. This indicated that PCB exposure impaired the function of the heart, which exhibited developmental delay. In summary, these results provide experimental confirmation of the hypothesis that early PCB exposure impacts normal cardiac development, resulting in congenital heart disease.

In addition to characterizing the effects of PCB exposure on zebrafish embryonic heart development, we also wanted to study its molecular mechanism. It is known that multiple signaling pathways converge to regulate heart development, such as RA, WNT, and AHR signaling [36, 37]. RA signaling has been shown to play an important role in regulating the size of the heart field in zebrafish [21], and excess or reduced levels of RA are known to cause cardiac defects. We detected an increased expression level of Raldh2 and decreased expression of the RA-metabolizing enzyme Cyp26a1 in PCB-exposed embryos, indicating that an increase in RA levels may lead to cardiac malformations and developmental abnormalities in PCB-exposed embryos. Given this result, we hypothesized that the cardiac toxicity of PCBs could be rescued by inhibition of the RA signaling pathway. To test this hypothesis, we examined the mortality rates after treatment with DEAB in PCB-

exposed embryos. We found that the mortality associated with PCB exposure was significantly decreased at lower concentrations of DEAB, indicating a partial rescue effect, indicating that PCB exposure influences the RA signaling pathway, resulting in abnormal cardiac development.

With regard to the WNT signaling pathway, it has been shown that WNT signaling is critical in cardiac development. The WNT proteins are a group of secreted lipid-modified signaling proteins of 350–400 amino acids, which activate various pathways in the cell that can be categorized into the canonical and noncanonical WNT pathways [38]. It has been confirmed that the canonical WNT pathway regulates the differentiation and proliferation of cardiac progenitor cells in the second heart field and outflow tract [39], and it is antagonized for heart induction to occur [40]. The non-canonical WNT pathway is required for the genesis of electrical gradient coupling across the developing ventricular myocardium in the zebrafish heart [41], and may promote cardiac tissue formation from the early mesoderm in mesoderm explants [42]. Thus, to determine whether the cardiac toxicity of PCBs is associated with the WNT pathway, we measured the expression level of canonical WNT signaling molecules (WNT1) and non-canonical WNT signaling molecules (WNT5 and 11) after PCB exposure. Our results indicated that inhibition of the non-canonical WNT signaling pathway might contribute to cardiac developmental abnormalities in PCB-exposed embryos.

The AHR is a member of the basic helix-loop-helix Per-ARNT-Sim protein family of developmental regulators and environmental sensors, and is a soluble ligand-activated transcription factor known to bind dioxins including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related planar aromatic hydrocarbons, including PCBs. Following activation by a ligand, the AHR moves into the nucleus where it interacts with the aryl hydrocarbon receptor nuclear translocator, forming a heterodimer that binds to cis-acting elements called AhR-responsive element (AHREs) or xenobiotic-responsive element (XREs), upstream of genes encoding biotransformation enzymes such as cytochrome P450 1A (Cyp1a) [43, 44]. In our studies we showed increased expression of several critical signaling molecules in this pathway, such as AHR2, ARNT and Cyp1a1. These results verified that the AHR signaling pathway is important in the cardiac toxicity of PCB exposure.

In summary, these studies demonstrate that PCBs are toxic toward zebrafish embryonic development, particularly with regard to cardiac development. We were also able to delineate the molecular mechanisms underlying this toxicity, leading to the conclusion that PCBs cause toxic effects that impede normal heart development in zebrafish through multiple mechanisms. We found evidence the PCBs acted through disrupting the RA, AHR and WNT signaling pathway to disrupt heart development. However, the functions of the RA pathway are more widely discussed than the rest in our study, so more studies about AHR and WNT pathway are still needed in the further work.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (Grant No. 30973213), the Key Medical Personnel Foundation of Jiangsu Province (Grant No. RC2011021), Nanjing Medical Science and Technique Development Foundation, and the Science and Technology Development Foundation of Nanjing Medical University (Grant No. 2011NJMU209).

References

1. Dolk H, Loane M, Garne E (2011) Congenital heart defects in Europe: prevalence and perinatal mortality, 2000 to 2005. Circulation 123:841–849. doi:10.1161/CIRCULATIONAHA.110.958405
2. Bruneau BG (2008) The developmental genetics of congenital heart disease. Nature 451:943–948. doi:10.1038/nature06801
3. Mone SM, Gillman MW, Miller TL, Herman EH, Lipshultz SE (2004) Effects of environmental exposures on the cardiovascular system: prenatal period through adolescence. Pediatrics 113:1058–1069
4. Pompa G, Caloni F, Fracchiolla ML (2003) Dioxin and PCB contamination of fish and shellfish: assessment of human exposure. Review of the international situation. Vet Res Commun 27(Suppl 1):159–167
5. Crinnion WJ (2011) Polychlorinated biphenyls: persistent pollutants with immunological, neurological, and endocrinological consequences. Altern Med Rev 16:5–13
6. Guvenius DM, Aronsson A, Ekman-Ordeberg G, Bergman A, Noren K (2003) Human prenatal and postnatal exposure to polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorobiphenylols, and pentachlorophenol. Environ Health Perspect 111:1235–1241
7. Solomon GM, Weiss PM (2002) Chemical contaminants in breast milk: time trends and regional variability. Environ Health Perspect 110:A339–A347
8. Shen H, Main KM, Virtanen HE, Damggard IN, Haavisto AM, Kaleva M, Boisen KA, Schmidt IM, Chellakooty M, Skakkebaek NE et al (2007) From mother to child: investigation of prenatal and postnatal exposure to persistent bioaccumulating toxicants using breast milk and placenta biomonitoring. Chemosphere 67:S256–S262
9. Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, Tinglestad J, Tully M (1986) Neonatal effects of transplacental exposure to PCBs and DDE. J Pediatr 109:335–341
10. Taylor PR, Lawrence CE, Hwang HL, Paulson AS (1984) Polychlorinated biphenyls: influence on birthweight and gestation. Am J Public Health 74:1153–1154
11. Wang YP, Hong Q, Qin DN, Kou CZ, Zhang CM, Guo M, Guo XR, Chi X, Tong ML (2012) Effects of embryonic exposure to polychlorinated biphenyls on zebrafish (*Danio rerio*) retinal development. J Appl Toxicol 32:186–193. doi:10.1002/jat.1650
12. Stainier DY (2001) Zebrafish genetics and vertebrate heart formation. Nat Rev Genet 2:39–48
13. Lam SH, Winata CL, Tong Y, Korzh S, Lim WS, Korzh V, Spitsbergen J, Mathavan S, Miller LD, Liu ET et al (2006) Transcriptome kinetics of arsenic-induced adaptive response in zebrafish liver. Physiol Genomics 27:351–361
14. Ung CY, Lam SH, Hlaing MM, Winata CL, Korzh S, Mathavan S, Gong Z (2010) Mercury-induced hepatotoxicity in zebrafish: in vivo mechanistic insights from transcriptome analysis,

phenotype anchoring and targeted gene expression validation. BMC Genom 11:212. doi:10.1186/1471-2164-11-212
15. Hill AJ, Teraoka H, Heideman W, Peterson RE (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86:6–19
16. Jones HS, Panter GH, Hutchinson TH, Chipman JK (2010) Oxidative and conjugative xenobiotic metabolism in zebrafish larvae in vivo. Zebrafish 7:23–30. doi:10.1089/zeb.2009.0630
17. Teraoka H, Dong W, Hiraga T (2003) Zebrafish as a novel experimental model for developmental toxicology. Congenit Anom 43:123–132
18. Gittenberger-de Groot AC, Bartelings MM, Deruiter MC, Poelmann RE (2005) Basics of cardiac development for the understanding of congenital heart malformations. Pediatr Res 57:169–176
19. Kwon C, Cordes KR, Srivastava D (2008) Wnt/beta-catenin signaling acts at multiple developmental stages to promote mammalian cardiogenesis. Cell Cycle 7:3815–3818
20. Niessen K, Karsan A (2008) Notch signaling in cardiac development. Circ Res 102:1169–1181. doi:10.1161/CIRCRESAHA.108.174318
21. Keegan BR, Feldman JL, Begemann G, Ingham PW, Yelon D (2005) Retinoic acid signaling restricts the cardiac progenitor pool. Science 307:247–249
22. Westerfield M (1993) The Zebrafish Book: a guide for the laboratory use of Zebrafish (*Danio rerio*) [M].Edition 4. Eugene OR: Univ. of Oregon Press
23. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310
24. Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 3:59–69. doi:10.1038/nprot.2007.514
25. Chen JN, Fishman MC (1996) Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. Development 122:3809–3816
26. Yelon D, Horne SA, Stainier DY (1999) Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214:23–37
27. Ohyama T, Mohamed OA, Taketo MM, Dufort D, Groves AK (2006) Wnt signals mediate a fate decision between otic placode and epidermis. Development 133:865–875
28. Park SS, Miller H, Klotz AV, Kloepper-Sams PJ, Stegeman JJ, Gelboin HV (1986) Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): cross reactivity with 3-methylcholanthrene induced rat cytochrome P-450. Arch Biochem Biophys 249:339–350
29. Grimes AC, Erwin KN, Stadt HA, Hunter GL, Gefroh HA, Tsai HJ, Kirby ML (2008) PCB126 exposure disrupts zebrafish ventricular and branchial but not early neural crest development. Toxicol Sci 106:193–205
30. Yutzey KE, Rhee JT, Bader D (1994) Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. Development 120:871–883
31. Rottbauer W, Wessels G, Dahme T, Just S, Trano N et al (2006) Cardiac myosin light chain-2: a novel essential component of thick-myofilament assembly and contractility of the heart. Circ Res 99:323–331
32. Ryckebusch L, Wang Z, Bertrand N, Lin SC, Chi X, Schwartz R, Zaffran S and Niederreither K (2008) Retinoic acid deficiency alters second heart field formation. Proceedings of the National Academy of Sciences of the United States of America 105: 2913–2918. doi:10.1073/pnas.0712344105
33. Lescroart F, Meilhac SM (2012) Cell lineages, growth and repair of the mouse heart. Results Probl Cell Differ 55:263–289. doi:10.1007/978-3-642-30406-4_15
34. Quaife NM, Watson O, Chico TJ (2012) Zebrafish: an emerging model of vascular development and remodelling. Curr Opin Pharmacol 12:608–614. doi:10.1016/j.coph.2012.06.009
35. Sisman T, Geyikoglu F, Atamanalp M (2007) Early life-stage toxicity in zebrafish (*Danio rerio*) following embryonal exposure to selected polychlorinated biphenyls. Toxicol Ind Health 23:529–536
36. Rochais F, Mesbah K, Kelly RG (2009) Signaling pathways controlling second heart field development. Circ Res 104:933–942
37. Puga A (2011) Perspectives on the potential involvement of the AH receptor-dioxin axis in cardiovascular disease. Toxicol Sci 120:256–261. doi:10.1093/toxsci/kfq393
38. Willert K, Nusse R (2012) Wnt proteins. Cold Spring Harb Perspect Biol 4:a007864. doi:10.1101/cshperspect.a007864
39. Mohamed OA, Clarke HJ, Dufort D (2004) Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. Dev Dyn 231:416–424
40. Schneider VA, Mercola M (2001) Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. Genes Dev 15:304–315
41. Panakova D, Werdich AA, Macrae CA (2010) Wnt11 patterns a myocardial electrical gradient through regulation of the L-type Ca(2+) channel. Nature 466:874–878. doi:10.1038/nature09249
42. Eisenberg CA, Eisenberg LM (1999) WNT11 promotes cardiac tissue formation of early mesoderm. Dev Dyn 216:45–58
43. Safe SH (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87–149
44. Denison MS, Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43:309–334