行政院國家科學委員會補助專題研究計畫期中進度報告

single-minded 基因與環境荷爾蒙戴奧辛對斑馬魚頭骨發育的影響

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 91－2313－B－019－033
執行期間：　91 年 08 月 01 日 至　92 年 07 月 31 日

計畫主持人：胡清華
共同主持人：
計畫參與人員：王文德，柯靜宜，鍾欣怡，楊惠雯，吳建勳，陳宜群

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立台灣海洋大學生物科技所

中　華　民　國　92　年　06　月　24　日

中英文摘要

在本期研究中，我們針對了 single-minded 基因對斑馬魚頭骨發育的情形利用 morpholino 的方式進行該基因的抑制後觀察到頭股的發育發生了相當嚴重的影響，目前正以各種標示基因觀察受 single-minded 基因抑制影響的下游基因與頭骨發育間的關係。除此之外，對於環境荷爾蒙與頭骨發育間的關係，我們已完成詳細的觀察，並利用各種標示基因觀察受戴奧辛影響的情形。這項工作已完成論文撰寫的工作，目前已投稿於 Molecular Pharmacology 接受審查中。在下一期的研究中，我們將進一步探討 single-minded 基因與其他胚胎發育調控基因間的互動關係，以釐清該基因在胚胎發育中所扮演的角色。

To investigate the function of *single-minded* gene in vertebrate development, we have microinjected *single-minded 2*-specific morpholino into zebrafish embryo at 1-cell stage and observed the effects on head skeleton development. By using various markers and Alcian blue staining, we have observed severe cranial cartilage defects in *sim2*-morpholino injected embryos. In addition, the cranial cartilage development was severely disrupted by waterborne 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Specifically, the expression profiles of cartilage markers *chm1*, *col2a1* and the *col2a1* upstream regulator *sox9a,* were all disturbed, but not eliminated, by TCDD exposure. It suggested that the TCDD disrupted the process of precartilage condensation, but not chondrogenesis, during cartilage morphogenesis.

Introduction

Halogenated aromatic hydrocarbons, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzo-furans (PCDFs), and biphenyls (PCBs), comprise a family of bioaccumulative xenobiotics that are resistant to biological degradation (Safe, 1994). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of these chemicals and has been used as the prototype compound to investigate the toxicity effect of aromatic hydrocarbons (Whitlock, 1999). Presumably most, if not all, of these pathological effects are mediated by the cytosolic aryl hydrocarbon receptor (AHR) signaling pathway (Wilson and Safe, 1998). Exposure to these exogenous compounds leads to activating a basic-helix-loop-helix PAS (bHLH-PAS) factor, aryl hydrocarbon receptor (AHR). Consequently, the liganded AHR associates with another bHLH-PAS factor, aryl hydrocarbon receptor nuclear translocator (ARNT), to form a DNA binding complex in the nucleus and modulates various downstream genes (Denison and Nagy, 2003; Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). Induction of cytochrome P4501A1 (CYP1A1) mRNA is the most recognized biochemical effect of the AhR-related signaling pathway (Whitlock, 1999).

Cytochrome P450 comprises a heme protein family that oxygenate a variety of endogenous and exogenous compounds, including steroids, neurohormones, fatty acids, prostaglandins, and a number of drugs and environmental chemicals (Gonzalez, 1988). The highly conserved CYP1A is a subfamily of cytochrome P450 family that oxygenates a variety of halogenated polycyclic hydrocarbon xenobiotics (Gonzalez, 1988). There are multiple forms of CYP1A genes in both of mammals and teleost (Smith et al., 1998; Stoilov et al., 2001). However, the biological functions of these redundant genes are unclear.

In adult vertebrate, the predominant TCDD-mediated CYP1A1 transcription occurs in the liver. However, the extrahepatic transcription of CYP1A1 has also been found in heart, brain, intestine, kidney, gill, skin and vascular endothelium (Stegeman et al., 1991). The strong CYP1A1 induction in the liver is consistent with the role of hepatocytes in biotransformation of xenobiotics (Whitlock, 1999). In addition to adult fish, the embryos and larvae of teleost are also sensitive to the xenobiotics (Dong et al., 2002; Guiney et al., 1997; Henry et al., 1997; Toomey et al., 2001). Previously, we demonstrated that TCDD induced significant amount of CYP1A1 transcription in zebrafish embryos and larvae (Wang et al., 1998). It suggests that both of AhR and ARNT factors exist in forms that can accurately respond to the proper ligand in fish embryos (Wang et al., 1998).

TCDD exposure causes a variety of toxicological defects in fish embryo, such as hemorrhages, pericardial and meningial edema, cardiovascular dysfunction, craniofacial malformations, erythropoiesis disruption, midbrain circulation failure, and apoptosis of renal tubules, hepatocytes, pancreas and all major brain regions (Dong et al., 2001; Dong et al., 2002; Guiney et al., 1997; Henry et al., 1997; Teraoka et al., 2002; Toomey et al., 2001).

To investigate the pathological effects of TCDD in fish embryos, here we examine the changes of transcription profile of CYP1A1 in TCDD-exposed zebrafish embryo by means of whole-mount RNA in situ hybridization. The toxicity effect of TCDD in cranial cartilage development was examined by Alcine blue stain and in situ hybridization analyses of cartilage markers, *col2a1*, *chm1*, *sox9a*, and the endodermic marker *shh*.

Materials and Methods

Zebrafish embryos and TCDD exposure

Wild-type (A B strain) zebrafish embryos were maintained at 28.5°C and were staged by hours postfertilization (hpf) (Kimmel et al., 1995). For TCDD treatment, newly fertilized embryos were continuously exposed to the waterborne TCDD (10 nM) or vehicle (0.03% DMSO) at 28.5°C.

CYP1A1 cloning and in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Westerfield, 1995). For the CYP1A1 probe, a digoxigenin-labeled cRNA fragment containing 998 bp (nt 217 to 1214) of the zebrafish CYP1A1 gene (GenBank.AF210727) fragment was synthesized with the DIG RNA Labeling Kit (Sp6/T7) from Roche (USA). The cartilage-specific probes have been previously described elsewhere: *col2a1* (Yan et al., 1995), *chm1* (Sachdev et al., 2001), *sox9a* (Chiang et al., 2001), and *shh* (Krauss et al., 1993). The nomenclature and atlas of blood vessels was described elsewhere (Isogai et al., 2001).

Alcian-blue staining

The head cartilages were stained with Alcian blue as previously described (Kimmel et al., 1998). 4% Neutralized formaldehyde fixed embryos were stained overnight in 0.1% Alcian blue overnight. After a series of washes, the preparations were rehydrated and the tissue was softened by digestion with trypsin.

Results

Constitutive and TCDD-induced CYP1A1 transcription in zebrafish embryo

Previous studies have shown that fish embryos are highly sensitive to TCDD exposure (Dong et al., 2001; Dong et al., 2002; Guiney et al., 1997; Henry et al., 1997; Teraoka et al., 2002; Toomey et al., 2001). The major events of TCDD exposure in fish embryos include causing developmental defects and stimulating transcription of cytochrome P450 1A (CYP1A) family via aryl hydrocarbon receptor (AHR) and its associated factor, AHR nuclear translocator (ARNT). However, the tissue distribution of CYP1A1 mRNA in the developing fish embryos is unclear. By using DIG-labeled CYP1A1 RNA as probe, here we examined the changes of CYP1A1 expression pattern in control and TCDD-treated zebrafish embryos by *in situ* hybridization (Fig. 1).

In normal developed embryos, a constitutive level of CYP1A1 mRNA was first detected in the integument of wild-type embryo at 24 hpf (Fig. 1A). Subsequently, the CYP1A1 mRNA was mainly expressed in the circulatory loop, especially at mandibular aortic arches (AA1), lateral dorsal aortas (LDA), the primitive internal carotid arteries (PICA), basal communicating artery (BCA) and posterior communicating segment (PCS) at 36 hpf. In addition, CYP1A1 transcripts also appeared in several cranial veins, including the primordial hindbrain channel (PHBC), primordial midbrain channel (PMBC) (Fig. 1C, 1E). At 48 hpf, the constitutive CYP1A1 mRNA was remained in the cranial vessels, such as LDA, AA1, PICA, optic artery (OA), the caudal division of the internal carotid artery (CaDI), optic vein (OV), posterior cerebral vein (PCeV), metencephalic artery (MtA) and middle cerebral vein (MCeV) (Fig 1G, 1I, 1J). In addition to the cranial vessels, the CYP1A1 mRNA also appeared in the trunk and tail vessels, including the dorsal artery (DA), caudal artery (CA), intersegmental vessel (Se) and dorsal longitudinal anastomotic vessel (DLAV) at 36 hpf and 48 hpf stages (Fig 1C, 1G, 1K). However, we did not observe CYP1A1 transcripts in heart and common cardinal vein (duct of Cuvier) in control embryos and the transcriptional signals in the cranial veins were generally weaker than which in the arteries.

In waterborne TCDD-treated embryos, the CYP1A1 transcription in the integument and vascular endothelial cells was markedly enhanced as early as 24 hpf throughout 48 hpf (Fig. 1B, 1D, 1F, 1H). Despite the integument and circulation system, a novel transcription of CYP1A1 was also induced in pronephric duct (PND), pronephric tubule (PNT) by TCDD at 36 hpf (Fig. 1D, 1F, 1L 1M). Later on the CYP1A1 mRNA was also induced in eyes and heart by TCDD at 48 hpf (Fig 1N, 1O).

Malformation of head skeleton

In fish embryos, TCDD elicits various types of toxic responses, such as hemorrhages, cardiovascular dysfunction, craniofacial malformation, midbrain apoptosis and other pathological responses (Dong et al., 2001; Dong et al., 2002; Henry et al., 1997; Teraoka et al., 2002; Toomey et al., 2001). Figure2 shows that TCDD reduced the longitude axis of head and disturbed the cranial cartilage development. Consequently the ethmoid plate (ep) and trabecula (tr) cartilage in the TCDD-treated embryos was reduced and the lower part of anterior pharyngeal arches, including the Meckel's (m) and ceratohyal (ch) cartilages, were aligned posteriorly and ventrally. From the ventral view, it appeared that the ceratohdyal and ceratobranchials (cb1-5) in the TCDD-treated embryos were orientated perpendicularly to the longitude axis, instead of being a

sharp angle observed in the wild type embryos. Ceratobranchials became progressively smaller posteriorly and only a few chondrocytes were present in the last two arches in TCDD-treated embryos. The basihyal (bs) and basibranchial (bb) cartilages, that connect the ceratohyal and ceratobranchial arches, did not develop completely in TCDD-exposed embryo. In addition, the cartilages were thinner in TCDD-exposed embryos.

TCDD effects on transcription of cranial cartilage markers

To determine the molecular mechanism of TCDD mediated cranial cartilage malformation, we have investigated the spatial expression pattern of cranial cartilage markers by in situ hybridization. The type II collagen (Col2a1) is the predominant protein in cartilage extracellular matrix and it is required for proper chondrogenesis. Transcription of *col2a1* marks differentiating chondrocytes (Yan et al., 1995). Chondromodulin-1 (ChM-1) is a secreted glycoprotein that stimulates the growth and maturation of chondrocytes and inhibits vascular invasion into cartilage during endochondral bone formation. The zygotic expression of *chm1* was first observed in notochord at 10-somite stage and subsequently appeared in the surrounding area of otic vesicles as well as the craniofacial cartilage elements at following stages (Sachdev et al., 2001). Here we examined the development of cranial cartilages by the spatial patterns of *col2a1* and *chm-1* (Fig 3). It was noted that the transcripts of both genes were highly reduced or even lost in the ceratohdyal and ceratobranchials in TCDD-treated embryos, but remained strong transcription in the Meckel's cartilage (Fig 3D, 3J). However, the shape of Meckel's cartilage was distorted by TCDD treatment. The length of the Meckel's cartilage was shorten in the TCDD-exposed embryos and came to lie further to the posterior position and the anterior end did not fuse together completely (Fig 3D, 3J). Furthermore, the basihyal and basibranchial were highly reduced or missed in the TCDD-exposed embryos. Consequently, the bilateral ceratohyal arch and the first ceratobranchials (cb1) were not connected along the midline. At 60 hpf, the anterior region of trabeculae did not fuse together to form the ethmoid plate properly in the TCDD-treated embryo (Fig. 3G, 3H). Our results show that the malformation of cranial skeleton in the TCDD-exposed larvae could be reflected by the distortion of *col2a1* and *chm-1* expression patterns during 48-60 hpf.

The HMG-domain transcription factor Sox9 is a critical upstream regulator of *col2a1* gene and it is required for cartilage development (Akiyama et al., 2002; Bi et al., 1999; Yan et al., 2002). There are two types of *sox9* genes, *sox9a* and *sox9b*, that are expressed in zebrafish chondrogenic cell lineages (Chiang et al., 2001). The *sox9a* mutation causes craniofacial defects and loses cartilage elements of the neurocranium, pharyngeal arches and pectoral girdle (Yan et al., 2002). In TCDD-exposed embryos, the *sox9a* transcription in the trabeculae is inhibited and its transcription in the anterior pharyngeal arches, including the Meckel's and ceratohyal, was protruded from the oral cavity (Fig 3K-3N). This observation consists with the phenotype of craniofacial defects in TCDD-exposed embryos, in which the Meckel's and ceratohyal arches were orientated in perpendicular to the anterior-posterior axis.

The Hedgehog (HH) signaling pathway is involved in patterning and development of a variety of organ systems, including the nervous system, the skeletal system, the craniofacial structures, and the gastrointestinal tract (Ingham and McMahon, 2001). At larval stage (60-72 hpf), the *shh* transcription in the oral ectoderm (oe) was eliminated in the TCDD-exposed embryos (Fig 3O-3R). However, the rest part of *shh* expression domains in the TCDD-exposed

embryos remained indistinguishable from normal embryos.

Discussion

Transcription of *CYP1A1* in zebrafish

In vertebrate, the ubiquitous bHLH-PAS factors, aryl hydrocarbon receptor (AHR and AHR2) and their associate factors aryl hydrocarbon receptor nuclear translocator (ARNT and ARNT2), play important functions in embryonic development (Abbott et al., 1999; Hosoya et al., 2001; Maltepe et al., 1997). Mouse strains deficient in the AHR protein have severe defects in liver development and mutation of the murine *arnt* locus results in placental, vascular, and hematopoietic defects. However, the mechanism that drives the autonomic AHR-ARNT pathway in embryos has not been clarified yet. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypic AHR agonist that stimulates *CYP1A1* transcription in fish embryos and causes developmental defects (Dong et al., 2001; Dong et al., 2002; Henry et al., 1997; Teraoka et al., 2002; Toomey et al., 2001). It was hypothesized that the developmental toxicity of TCDD is mediated by AHR, since the earliest signs of TCDD toxicity correlate with expression and activation of AHR in fish larvae and mutation of *ahr* results in loss of teratogenic response to TCDD (Vorderstrasse et al., 2001; Wilson and Safe, 1998).

Recently, it was shown that AHR2 and ARNT2 were coexpressed in embryonic vascular system (Andreasen et al., 2002). In zebrafish embryos, TCDD-treatment induced CYP1A1 transcription first in skin and vascular system at 24 hpf and later on it appeared at heart, kidney, and liver (Andreasen et al., 2002). However, the CYP1A1 mRNA was scarcely detectable in control embryos by conventional Northern analysis (Wang et al., 1998). To investigate the primordial response of TCDD in fish embryos, in this study we examined the spatial expression pattern of CYP1A1 in zebrafish embryos by whole mount in situ hybridization analysis. Unlike previous studies, here we observed a basal level of CYP1A1 transcripts in the primitive vessels and integument, but not in heart and common cardinal vein (Cuvier duct) at stages between 24-48 hpf. Like a number of vascular markers, such as *notch5*, *flk1*, *gridloc*, *tbx20* and *deltaC* (Lawson and Weinstein, 2002), CYP1A1 is preferentially transcribed in the arteries rather than in veins. Presumably the skin and vascular cells, especially the arterial epithelium cells, contain an uncharacterized AHR agonist, which in turn drives the autonomic AHR-ARNT pathway and mediates the constitutive CYP1A1 transcription. It was shown that both of skin and vascular endothelium were sensitive to TCDD and produced high level of CYP1A1 transcription (DeVito et al., 1997). The vascular system is a major initial site affected by TCDD in lake trout early life stages (Guiney et al., 1997). The function of AHR-ARNT pathway in these cells is unclear. It was noted that a basal level of CYP1A1 mRNA also appeared in liver during larva stage (data not shown).

Despite the constitutive transcription, TCDD enhanced CYP1A1 transcription in the integument and vascular endothelial cells as well as induced novel CYP1A1 transcription in lens, heart and pronephric ducts in embryos. Similar stimulation of CYP1A1 transcription was also observed in other species (Toomey et al., 2001). Accordingly, it suggests that both of the AHR and ARNT factors existed in all above embryonic tissues, but only the integument and vascular system gave constitutive level of CYP1A1 transcripts.

The biological function of AHR-mediated pathway has not been fully characterized. Interestingly, *ahr* -/- null mice developed age-related lesions in cardiovascular, liver and skin in the absence of exogenous ligand (Fernandez-Salguero et al., 1997). It suggested that that the

autonomic AHR-ARNT pathway plays important function in physiological homeostasis. Here, our observation of the basal CYP1A1 transcription in embryonic vessels, skin and liver provide evidences that the autonomic AHR-ARNT pathway take parts in the physiological functions of these tissues.

TCDD induces cranial skeleton defects

Exposure to TCDD causes a variety of developmental pathogenesis in fish embryos, such as hemorrhages, pericardial and meningial edema, cardiovascular dysfunction, craniofacial malformations, regional ischemia, growth retardation, erythropoiesis disruption, midbrain circulation failure, and apoptosis of renal tubules, hepatocytes, pancreas and all major brain regions (Dong et al., 2001; Dong et al., 2002; Henry et al., 1997; Teraoka et al., 2002; Toomey et al., 2001). Here we investigate the toxicity effect of TCDD in head skeleton development by in situ hybridization of cartilage markers. The earliest signs of craniofacial malformation appeared at 48 hpf, where the *sox9a* transcriptions in the neurocranium and anterior pharyngeal arches of TCDD-treated embryos were either inhibited or distorted. Subsequently, the distorted expression patterns of cartilage markers *col2a1* and *chm1* at 60 and 72 hpf stages provide further evidences of the skeleton malformation in TCDD-treated embryos. However, the strong transcriptional activities of cartilage markers suggested that the chondrocytes derived from neural crest cells were not reduced in these TCDD-exposed embryos. Therefore TCDD disturbed the process of precartilage condensation during cartilage morphogenesis, but not the process of chondrogenesis (Kimmel et al., 2001).

shh is expressed in the pharyngeal endoderm and encodes a potent signaling molecule. TCDD-treatment changed the size of pharyngeal endoderm, but not the intensity of *shh* transcription in the pharyngeal endoderm. It suggested that the distortion of *shh* was not the major cause of craniofacial malformation in TCDD-treated embryos. However, we noted that *shh* expression in oral ectoderm was inhibited in TCDD-treated embryos. Recently, it was shown that the SHH and its related signal pathway in oral ectoderm and ventral forebrain have important roles in pituitary development (Herzog et al., 2003; Sbrogna et al., 2003). Missing of *shh* transcripts at oral ectoderm suggested that the pituitary development might be disturbed in TCDD-treated embryos. It will be interesting to know whether the regulation of pituitary hormones is disturbed in TCDD-treated embryos.

References

Abbott BD, Schmid JE, Pitt JA, Buckalew AR, Wood CR, Held GA and Diliberto JJ (1999) Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. *Toxicol Appl Pharmacol* **155**:62-70.

Akiyama H, Chaboissier MC, Martin JF, Schedl A and de Crombrugghe B (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev* **16**:2813-2828.

Andreasen EA, Spitsbergen JM, Tanguay RL, Stegeman JJ, Heideman W and Peterson RE (2002) Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure. *Toxicol Sci* **68**:403-419.

Bi W, Deng JM, Zhang Z, Behringer RR and de Crombrugghe B (1999) Sox9 is required for cartilage formation. *Nat Genet* **22**:85-89.

Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J and Chung B (2001) Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev Biol* **231**:149-263.

Denison MS and Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Auun Rev Pharmacol Toxicol* **43**:309-334.

DeVito MJ, Diliberto JJ, Ross DG, Menache MG and Birnbaum LS (1997) Dose-response relationships for polyhalogenated dioxins and dibenzofurans following subchronic treatment in mice. I. CYP1A1 and CYP1A2 enzyme activity in liver, lung, and skin. *Toxicol Appl Pharmacol* **147**:267-280.

Dong W, Teraoka H, Kondo S and Hiraga T (2001) 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. *Neurosci Lett* **303**:169-272.

Dong W, Teraoka H, Yamazaki K, Tsukiyama S, Imani S, Imagawa T, Stegeman JJ, Peterson RE and Hiraga T (2002) 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol Sci* **69**:191-201.

Fernandez-Salguero PM, Ward JM, Sundberg JP and Gonzalez FJ (1997) Lesions of aryl-hydrocarbon receptor-deficient mice. *Vet Pathol* **34**:605-614.

Gonzalez FJ (1988) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**:243-288.

Guiney PD, Smolowitz RM, Peterson RE and Stegeman JJ (1997) Correlation of 2,3,7,8-tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. *Toxicol Appl Pharmacol* **143**:256-273.

Henry TR, Spitsbergen JM, Hornung MW, Abnet CC and Peterson RE (1997) Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (Danio rerio). *Toxicol Appl Pharmacol* **142**:56-68.

Herzog W, Zeng X, Lele Z, Sonntag C, Ting JW, Chang CY and Hammerschmidt M (2003) Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. *Dev Biol* **254**:36-49.

Hosoya T, Oda Y, Takahashi S, Morita M, Kawauchi S, Ema M, Yamamoto M and Fujii-Kuriyama Y (2001) Defective development of secretory neurones in the hypothalamus of Arnt2-knockout mice. *Genes Cells* **6**:361-374.

Ingham PW and McMahon AP (2001) Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* **15**:3059-3087.

Isogai S, Horiguchi M and Weinstein BM (2001) The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol* **230**:278-301.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B and Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* **203**:253-310.

Kimmel CB, Miller CT, Kruze G, Ullmann B, BreMiller RA, Larison KD and Snyder HC (1998) The shaping of pharyngeal cartilages during early development of the zebrafish. *Dev Biol* **203**:245-263.

Kimmel CB, Miller CT and Moens CB (2001) Specification and morphogenesis of the zebrafish larval head skeleton. *Dev Biol* **233**:239-257.

Krauss S, Concordet JP and Ingham PW (1993) A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**:1431-1444.

Lawson ND and Weinstein BM (2002) Arteries and veins: making a difference with zebrafish. *Nat Rev Genet* **3**:674-682.

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA and Simon MC (1997) Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* **386**:403-407.

Rowlands JC and Gustafsson JA (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Critical Reviews in Toxicology* **27**:109-134.

Sachdev SW, Dietz UH, Oshima Y, Lang MR, Knapik EW, Hiraki Y and Shukunami C (2001) Sequence analysis of zebrafish chondromodulin-1 and expression profile in the notochord and chondrogenic regions during cartilage morphogenesis. *Mech Dev* **105**:157-162.

Safe SH (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* **24**:87-149.

Sbrogna JL, Barresi MJ and Karlstrom RO (2003) Multiple roles for Hedgehog signaling in zebrafish pituitary development. *Dev Biol* **254**:19-35.

Schmidt JV and Bradfield CA (1996) Ah receptor signaling pathways. *Annual Review of Cell and Developmental Biology* **12**:55-89.

Smith G, Stubbins MJ, Harries LW and Wolf CR (1998) Molecular genetics of the human cytochrome P450 monooxygenase superfamily. *Xenobiotica* **28**:1129-1165.

Stegeman JJ, Smolowitz RM and Hahn ME (1991) Immunohistochemical localization of environmentally induced cytochrome P450IA1 in multiple organs of the marine teleost Stenotomus chrysops (Scup). *Toxicol Appl Pharmacol* **110**:486-504.

Stoilov I, Jansson I, Sarfarazi M and Schenkman JB (2001) Roles of cytochrome p450 in development. *Drug Metabol Drug Interact* **18**:33-55.

Teraoka H, Dong W, Ogawa S, Tsukiyama S, Okuhara Y, Niiyama M, Ueno N, Peterson RE and Hiraga T (2002) 2,3,7,8-Tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: altered regional blood flow and impaired lower jaw development. *Toxicol Sci* **65**:192-199.

Toomey BH, Bello S, Hahn ME, Cantrell S, Wright P, Tillitt DE and Di Giulio RT (2001) 2,3,7,8-Tetrachlorodibenzo-p-dioxin induces apoptotic cell death and cytochrome P4501A expression in developing Fundulus heteroclitus embryos. *Aquat Toxicol* **53**:127-138.

Vorderstrasse BA, Steppan LB, Silverstone AE and Kerkvliet NI (2001) Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. *Toxicol Appl Pharmacol* **171**:157-164.

Wang WD, Chen YM and Hu CH (1998) Detection of Ah receptor and Ah receptor nuclear translocator mRNAs in the oocytes and developing embryos of zebrafish (Danio rerio). *Fish Physiol Biochem* **18**:49-57.

Westerfield M (1995) *The Zebrafish Book.* University of Oregon Press, Eugene.

Whitlock JP (1999) Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* **39**:103-125.

Wilson CL and Safe S (1998) Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol Pathol* **26**:657-671.

Yan YL, Hatta K, Riggleman B and Postlethwait JH (1995) Expression of a type II collagen gene in the zebrafish embryonic axis. *Dev Dyn* **203**:363-376.

Yan YL, Miller CT, Nissen RM, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos PA, Amsterdam A, Chung BC, Westerfield M, Haffter P, Hopkins N, Kimmel C, Postlethwait JH and Nissen R (2002) A zebrafish sox9 gene required for cartilage morphogenesis. *Development* **129**:5065-5079.

Footnotes

This work was supported in part by the National Science Council (Taiwan, ROC) through grants 89-2313-B-019-001 and 91-2313-B-019-033 to C.-H.H.

Address correspondence to: Prof. Chin-Hwa Hu, Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 202-24, Taiwan, ROC. E.mail: chhu@mail.ntou.edu.tw

Legends for Figures:

Figure 1. Spatial transcription pattern of *CYP1A1* in zebrafish embryo. Whole mount in situ hybridization was carried out using a digoxigenin-labeled cRNA probe for zebrafish *CYP1A1* (GeneBank number AF210727), as described in Materials and Methods. (A, C, E, G, I-K), control embryos; (B, D, F, H, L-O), waterborne TCDD (10 nM) exposed embryos. (A, B), lateral view at 24 hpf; (C-F), lateral (C, D) and dorsal (E, F) views at 36 hpf; (G, H), lateral view at 48 hpf; (I, J), lateral (I) and dorsal (J) views of embryonic head at 48 hpf; (K) lateral view of trunk and tail regions at 48 hpf; (L, M), head (L) and trunk (M) transverse sections of TCDD-exposed embryo at 36 hpf; (N, O), sagittal sections of TCDD-exposed embryos at 48 hpf. Abbreviations: AA1, mandibular aortic arches; BCA, basal communicating artery; CA, caudal artery; CaDI, the caudal division of the internal carotid artery; DA, dorsal artery; DLAV, dorsal longitudinal anastomotic vessel; LDA, lateral dorsal aortas; MCeV, middle cerebral vein; MtA, metencephalic artery; OA, optic artery; OV, optic vein; PCeV, posterior cerebral vein; PCS, posterior communicating segment; PHBC, primordial hindbrain channel; PICA, primitive internal carotid arteries; PMBC, primordial midbrain channel; PND, pronephric duct; PNT, pronephric tubule; Se, intersegmental vessel.

Figure 2 TCDD caused craniofacial malformation. The structure of head cartilages was observed by Alcian blue staining at 5 dpf. (A, B), control larva; (C, D), TCDD-treated larva. (A, C), lateral views, (B, D), ventral views. Abbreviations: bb, basibranchial; cb1-5, ceratobranchials 1-5; ch, ceratohyal; ep, ethmoid plate; hs, hyosymplectic; m, Meckel's ; pch, parachordal; tr, trabecula.

Figure 3. Spatial expression patterns of craniofacial markers were distorted by TCDD. (A-D), ventral views of *col2a1* expression at 60 hpf (A, B) and 72 hpf (C, D); (E-J), lateral (E, F), dorsal (G, H) and ventral (I, J) views of *chm1* expression at 60 hpf (E-H) and 72 hpf (I, J); (K-N), lateral views of *sox9a* expression at 48 hpf (K, L) and 60 hpf (M, N); (O-R), lateral views of *shh* expression at 60 hpf (O, P) and 72 hpf (Q, R). (A, C, E, G, I, K, M, O, Q), control embryos; (B, D, F, H, J, L, N, P, R), waterborne TCDD (10 nM)-treated embryos. Abbreviations: oe, oral ectoderm; cb1-5, ceratobranchials 1-5; ch, ceratohyal; ep, ethmoid plate; m, Meckel's ; tr, trabecula.

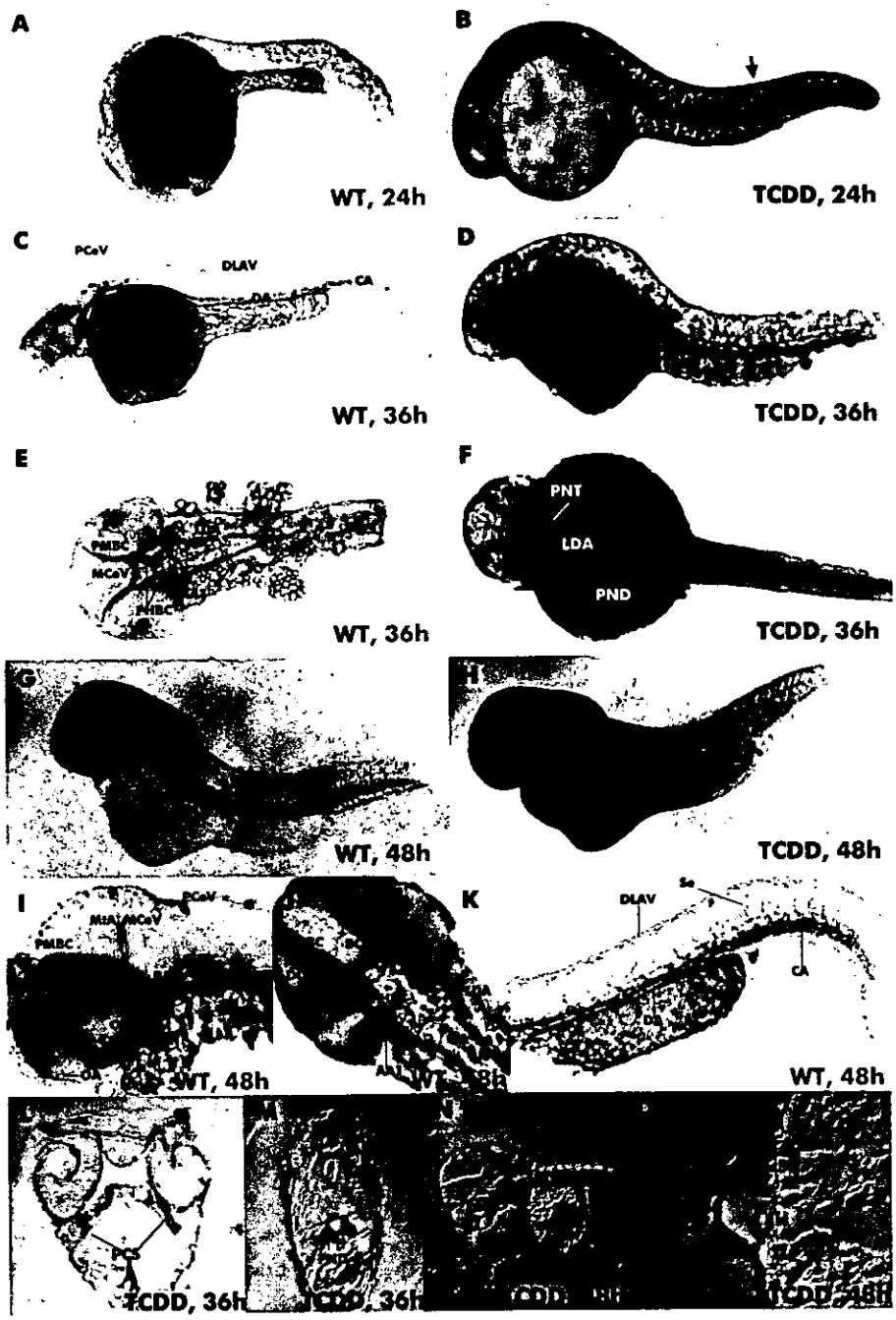
A
WT, 24h
B
TCDD, 24h
C
PCeV
DLAV
CA
WT, 36h
D
TCDD, 36h
E
PMBC
MCeV
WT, 36h
F
PNT
LDA
PND
TCDD, 36h
G
WT, 48h
H
TCDD, 48h
I
PCeV
PMBC
WT, 48h
K
DLAV
CA
WT, 48h
TCDD, 36h
TCDD, 36h
TCDD, 48h

Figure 1

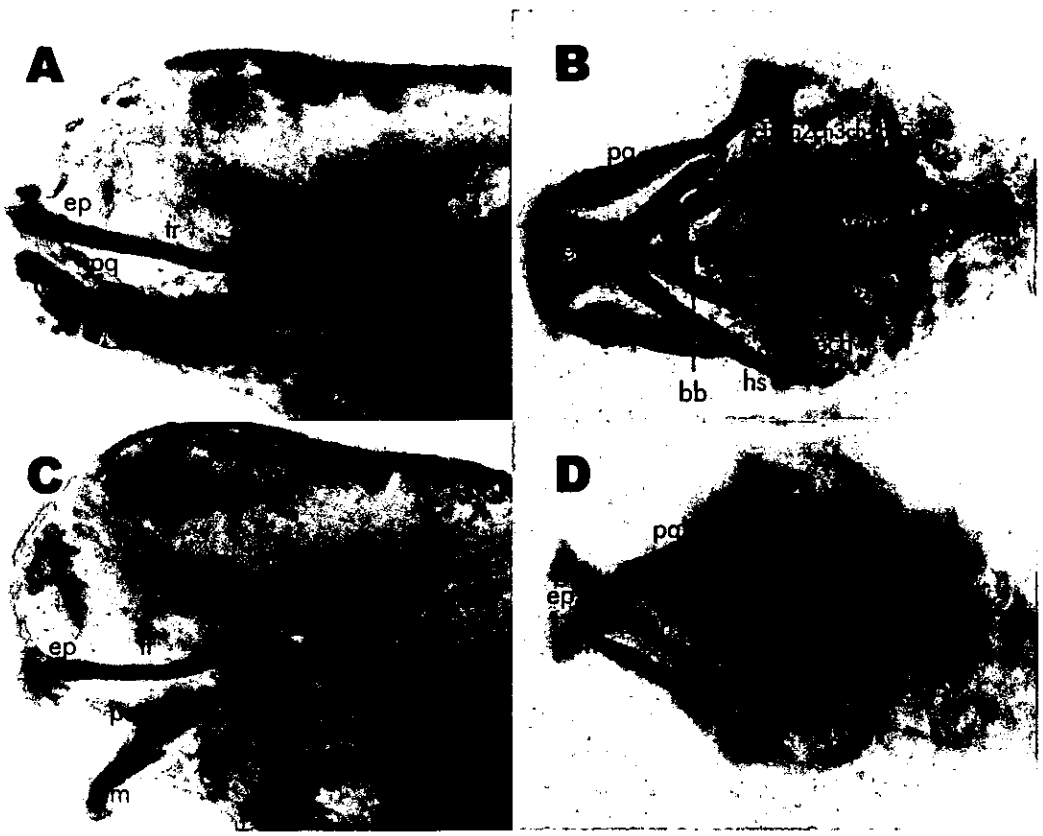

Figure 2

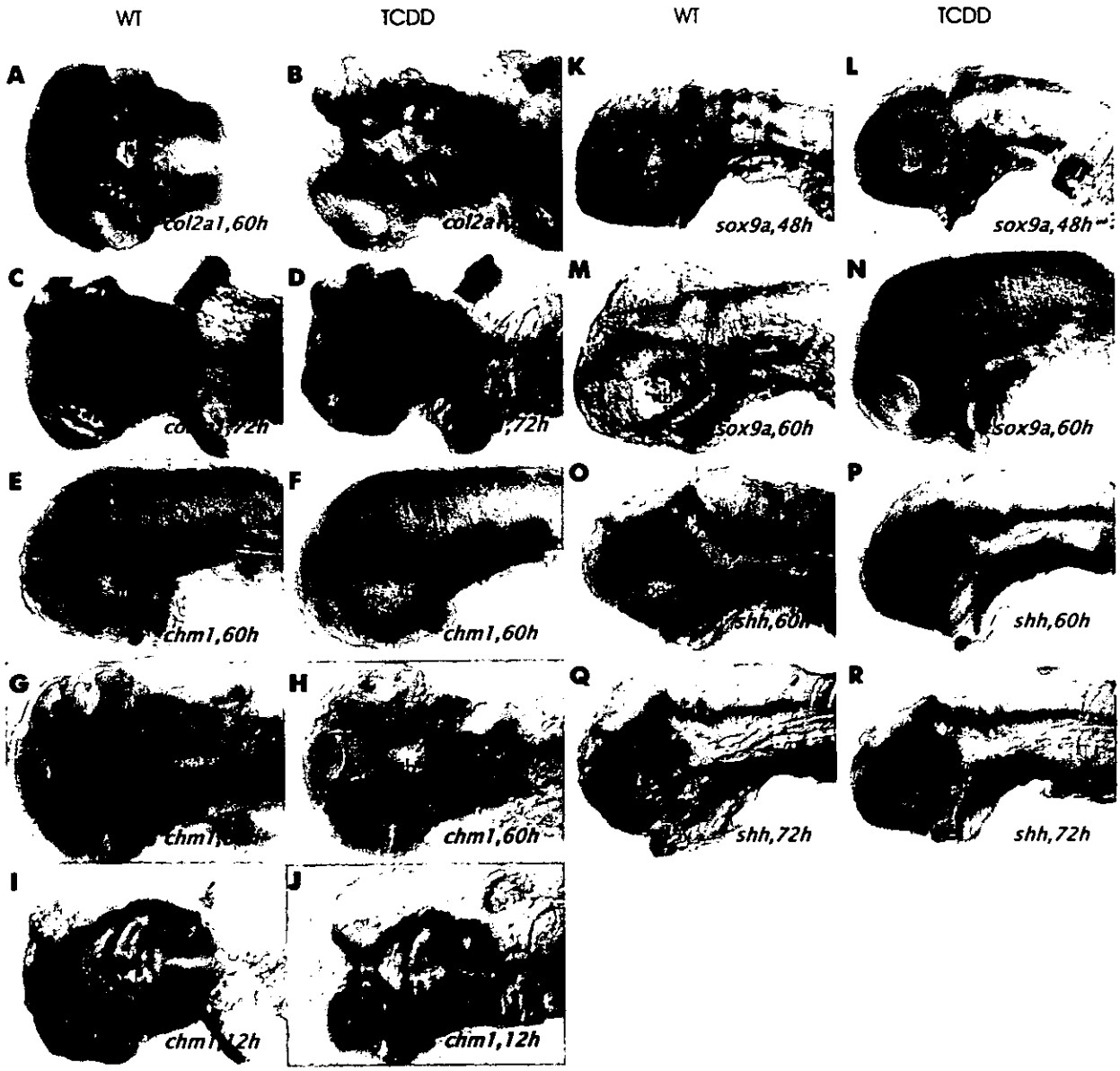
WT
TCDD
WT
TCDD
A
col2a1,60h
B
col2a1,60h
K
sox9a,48h
L
sox9a,48h
C
col2a1,72h
D
col2a1,72h
M
sox9a,60h
N
sox9a,60h
E
chm1,60h
F
chm1,60h
O
shh,60h
P
shh,60h
G
chm1,60h
H
chm1,60h
Q
shh,72h
R
shh,72h
I
chm1,12h
J
chm1,12h

Figure 3