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Dioxin and AHR impairs mesoderm gene expression and cardiac differentiation in human embryonic stem cells

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Abstract

Dioxin and dioxin-related polychlorinated biphenyls are potent toxicants with association with developmental heart defects and congenital heart diseases. However, the underlying mechanism of their developmental toxicity is not fully understood. Further, different animals show distinct susceptibility and phenotypes after exposure, suggesting possible species-specific effects. Using a human embryonic stem cell (ESC) cardiomyocyte differentiation model, we examined the impact, susceptible window, and dosage of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on human cardiac development. We showed that treatment of human ESCs with TCDD at the ESC stage inhibits cardiomyocyte differentiation, and the effect is largely mediated by the aryl hydrocarbon receptor (AHR). We further identified genes that are differentially expressed after TCDD treatment by RNA-sequencing, and genomic regions that are occupied by AHR by chromatin immunoprecipitation and high-throughput sequencing. Our results support the model that TCDD impairs human ESC cardiac differentiation by promoting AHR binding and repression of key mesoderm genes. More importantly, our study demonstrates the toxicity of dioxin in human embryonic development and uncovered a novel mechanism by which dioxin and AHR regulates

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Author Contributions:

BZ, GH conceived the study. HF, LW, GH carried out the experiments. JW, BB, JL carried out the data analysis. HF, BZ, GH wrote the manuscript.

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The authors declare that there are no competing financial interests.

Supplemental Information:

Supplemental Figures

Supplemental Tables

lineage commitment. It also illustrates the power of ESC-based models in the systematic study of developmental toxicology.

Graphical Abstract

Keywords

Human Embryonic Stem Cells; AHR; Dioxin; Mesoderm; Cardiac Differentiation

1. Introduction

Congenital heart defects (CHDs) are the most common type of birth defect, affecting close to 1% of live births worldwide¹. They are thought to be caused by a combination of genetic and environmental factors². However, only a small fraction of CHDs can be attributed to heritable gene mutations and the same CHD mutations can cause a variety of disease phenotypes, suggesting the involvement of modifier genes as well as non-genetic factors^{3–5}.

In human populations, epidemiology studies showed that occupational or accidental exposure to environmental chemicals such as dioxins can lead to increased congenital defects in newborns and ischemic heart diseases in adults^{6–9}. In addition, epidemiology evidence linking prenatal dioxin exposure and birth defects, miscarriage, and stillbirth has also been well documented^{10–12}. In a recent review of the Seveso cohort, it was reported that out of 52 Seveso children with prenatal dioxin exposure, 5 showed abnormalities in the heart and 3 showed abnormalities in the vascular system. But the small number of cases did not provide sufficient statistical power to draw strong correlations¹³. Thus, whether prenatal TCDD exposure directly contributes to congenital heart diseases remains unknown. In animals, experiments showed that the developing heart is sensitive to dioxins and dioxin-like polychlorinated biphenyls (PCBs)¹⁴, as dioxin impairs cardiac development, morphology, and/or function in different species^{15–23}. While this is consistent with findings in human populations, the underlying mechanism has not been fully delineated.

The teratogenic and carcinogenic effects of dioxin are thought to be mediated by AHR. AHR is a ligand-activated transcription factor and is highly conserved from invertebrates to

vertebrates. It contains a ligand binding domain, and can interact with many environmental compounds. Upon ligand binding, AHR binds to the dioxin-responsive elements (DREs) in gene promoters and enhancers, and regulates downstream transcription²⁴. In addition to xenobiotic response, AHR also plays critical roles in normal development and other physiological functions, as AHR deletion mice show defects in organ and vasculature development, reduced fertility, and cardiac hypertrophy^{25–27}. It has been proposed that AHR originally evolved to regulate embryonic development and only acquired the ability to bind xenobiotic chemicals during the evolution of vertebrates²⁸.

Embryonic stem cells (ESCs) are pluripotent stem cells that can differentiate into all cell types in the adult body. In vitro differentiation of ESCs into the cardiac lineage has been found to largely mimic the early steps in heart development, and has been successfully used as a culture model to investigate the regulatory mechanisms during cardiogenesis. In addition, it has also been used to test the developmental toxicity of environmental chemicals²⁹. Here, we employed an advanced monolayer-based human ESC cardiomyocyte differentiation protocol³⁰ to investigate the role of dioxin and AHR in human cardiac development. Our results indicate that dioxin impairs the induction of mesoderm genes via AHR, thereby inhibiting mesoderm and cardiac differentiation. Our study provides experimental support for the toxicity of dioxin in human cardiac development, and uncovers a novel epigenetic mechanism by which AHR regulates developmental gene expression. It also illustrates the power of ESC-based in vitro models in the systematic study of environmental health science questions.

2. Materials and Methods

2.1 Cell culture and cardiomyocyte differentiation

Human ESC lines H1(WA01) and H9 (WA09) were obtained from WiCell Research Institute. Human ESC line Me11 was kindly provided by Dr. Edouard G Stanley. In this cell line, EGFP expression is driven by the regulatory elements of the cardiac progenitor gene *Nkx2.5*, and can thus be used to monitor the differentiation process. Cells were routinely maintained in TeSR™-E8™ (Stemcell Technologies) on growth factor-reduced Matrigel (Corning) following published protocols³¹.

Human ESCs were cultured in E8 to 90~100% confluency before induction. To induce cardiac differentiation, the medium was replaced with cardiac differentiation basal medium 1 (RPMI1640 containing 1 × B27 minus insulin supplement) with 5 μM GSK3 inhibitor CHIR99021 (SelleckChem) for 2 days, and changed to basal medium 1 without the inhibitor for another 2 days. Cells were then cultured in basal medium 1 with 5 μM Wnt inhibitor IWR-1 (SelleckChem) for 2 days, followed by basal medium 1 only for 2 additional days. After that, cells were cultured in cardiac differentiation basal medium 2 (RPMI1640 containing 1 × B27 supplement) for 6 more days with medium refreshment every 2 days. During this differentiation process, ESCs were induced to cardiomyocyte through the mesoderm, cardiac mesoderm, cardiomyocyte progenitor, and cardiomyocyte stages.

2.2 Chemical exposure

2,3,7,8-Tetrachlorodibenzodioxin (TCDD, Sigma), AHR inhibitor CH223191 (Sigma) and induction reagents were dissolved in DMSO. In dose titration, TCDD is added to a final concentration of 0.1, 1, 2, or 10 nM as indicated. In other experiments, TCDD is added to a final concentration of 2 nM based on a previous study³². The AHR inhibitor CH223191 is added to a final concentration of 1 μ M. For control experiments, equivalent volume of DMSO is added. The final DMSO concentration in both the control and experimental groups were kept at 0.1% to minimize the impact on cell growth.

2.3 Immunofluorescence staining and Western blotting

Immunofluorescence staining was carried out as described before³³. Cells at different stages were stained with primary antibodies against T (TBXT) (Santa Cruz sc17745, 1:200), ISL-1 (ISLET1) (Developmental Studies Hybridoma Bank 39.4D5, 1:200), ACTN2 (ACTININ2) (Abcam ab9465, 1:300), TNNT2 (TROPONIN T2) (Abcam ab8295, 1:300) and cell nuclei were counterstained with DAPI (Invitrogen). Images were taken with Zeiss Axiovert 40 inverted microscope.

Cells were lysed in cell lysis buffer (Thermo Scientific 78501) containing 1 $\times$ protease inhibitor tablet (Sigma 11836170001) and blotted using standard protocols. Antibody against AHR (b-11, Santa Cruz) was used at 1:1000 dilution.

2.4 RNA isolation, reverse transcription, and RT-qPCR

Total RNA was isolated using the GeneJet RNA purification kit (Thermo Scientific), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) to generate cDNA. qPCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad) on the Bio-Rad CFX-384 Real-time PCR Detection System. Primers used in the study are listed in Table S5. Actin (ACTB) was used for normalization, and values were plotted as mean $\pm$ standard error.

2.5 CRISPR-Cas9 mediated gene inactivation

DNA oligos (Table S5) were annealed and cloned into pLentiCRISPR (Addgene 49535), to target exon 2 of human AHR. Lentivirus was prepared by transfecting 293T cells using a standard protocol (<https://www.addgene.org/protocols/lentivirus-production/>). Human ESCs were transduced with lentivirus and selected for viral integration with 2 μ g/ml puromycin. AHR expression was determined by western blot.

2.6 ChIP-seq & RNA-seq

Human ESCs were treated with DMSO or 2 nM TCDD for 4 days, and ChIP-seq was carried out similarly as described before³⁴ with the AHR antibody (H-211, Santa Cruz). RNA-seq was carried out using the Truseq RNA Library Prep Kit V2 (Illumina) according to the manufacturer's instructions. All libraries were sequenced on the NextSeq sequencer (Illumina). Two replicates were carried out for both the ChIP-seq and RNA-seq experiments.

2.7 Data analysis

For RNA-seq, reads were first filtered with cutadapt (v1.9.1), and only those with quality scores higher than 20 were retained for further analysis. Filtered reads were aligned to reference hg19 with STAR (v020201) with parameters: --outFilterMismatchNoverLmax 0.04. Genomic feature at gene level were counted with featureCounts (v1.5.1). Package edgeR were used to calculate the differential expressed genes (DEGs). DEGs were selected with fold change 1.5 and adjusted p-value 0.05. For Figure 5E, GSE85331 was used and mesoderm genes were defined as those that are up-regulated in mesoderm differentiation with FDR < 0.01, Log2 Fold-change > 3, and Log2 Counts per million > 3. For AHR ChIP-seq, filtered reads were uniquely aligned to hg19 with bowtie (v1.1.2) with parameters: -v 2 -m 1 --best --strata. SICER(v1.1) were used for peak calling with gap 600, FDR 10e-6. Peaks were filtered with fold change > 2, and then were assigned to the nearest genes within 20 kb.

3. Results

3.1 Impact of Dioxin on human ESC cardiac differentiation

While dioxin is known to have deleterious effects on cardiac development and function, it shows different impact in different species^{35, 36}. In addition, the expression levels of AHR and AHR co-factors are different in mouse and human ESCs³⁷. Therefore, we used a human ESC-based in vitro culture model to test whether and how dioxin may affect human cardiac development. The monolayer-based protocol we use allows highly efficient differentiation into cardiomyocytes and the differentiation process closely mimics cardiac development in vivo³⁰. To monitor the efficiency of cardiac differentiation, we carried out the experiments in the Nkx2.5::EGFP reporter human ESC line. In this cell line, EGFP expression is driven by the regulatory elements of the cardiac progenitor gene Nkx2.5, and can thus be used to monitor the differentiation process. We verified the robustness of the differentiation process by RT-qPCR of known cardiac markers such as ACTN2, TNNT2, MYL2, etc.

Using this system, we treated the cells with DMSO or TCDD throughout the differentiation protocol. We initially exposed the cells to 2 nM TCDD. The concentration of TCDD was chosen based on the following considerations: 1) a previous study in human exposure³² showed that the dioxin-like polychlorinated biphenyls in umbilical cord serum in people residing near waste sites were found to range from 0.60 ~ 16.98 ng/mL (equivalent to 0.2 ~ 5.05 nM TCDD based on the toxic equivalency factor); 2) 1–10 nM TCDD is commonly used in most *in vitro* studies. We found that TCDD treatment led to cell loss during the differentiation process. More importantly, TCDD induced a clear inhibition in cardiac differentiation, as indicated by the reduction in both the EGFP reporter fluorescence and the expression of key cardiac lineage markers, including myocyte (ACTN2, TNNT2, TNNI3, MYH6, MYL7, MYL2, IRX4), ion channel (HCN1, HCN4, KCNH2, KCNQ1), Atrial (NPPA, SLN) and nodal cell (TBX18) markers (Figure 1). Experiments on several hESC cell lines generate similar result (Figure S1). These results support the notion that TCDD has cardiac toxicity in humans.

3.2 Role of dioxin in mesoderm differentiation in human ESCs

To further identify the window of susceptibility, we treated the cells with TCDD at the following four different stages during cardiomyocyte differentiation: ESC (day (–3) to day (0)), mesoderm (day (1) to day (4)), cardiac mesoderm (day (5) to day (8)), cardiac progenitor (day (9) to day (14)) (Figure 2A). We then examined the expression of cardiomyocyte markers ACTN2, TNNT2, and MYL2 expression at the end of the differentiation protocol. By RT-qPCR, we found that TCDD treatment at the ESC or the mesoderm stages both reduced cardiomyocyte differentiation efficiency, with the treatment at the ESC stage showing the strongest effect (Figure 2B). In contrast, treatment at the cardiac mesoderm and cardiac progenitor stages showed no inhibition. Consistent with RT-qPCR, immunofluorescence staining showed that TCDD treated cells show reduced marker expression at the protein and cellular level as well (Figure 2C). Together, our results suggested that TCDD inhibits cardiomyocyte differentiation by inhibiting the commitment to the mesoderm and cardio-mesoderm lineage.

To further support the above observations, we treated the cells at the ESC stage, and examined the consequence of the treatment at different time points during cardiac differentiation. We found that TCDD treatment did not affect the proliferation, cell morphology, and expression of pluripotency genes such as NANOG, OCT4, and SOX2 in human ESCs (Figure S2), suggesting that it doesn't impair ESC maintenance and pluripotency. In contrast, TCDD treated cells showed reduced expression of mesoderm markers T and GSC, as determined by RT-qPCR (Figure 3A). Consistently, immunofluorescence staining showed reduced marker expression at the protein and cellular level in TCDD treated cells (Figure 3B). Subsequently, TCDD also led to impaired expression of cardiac markers ISL-1, TBX5, and Nkx2.5, as well as cardiomyocyte markers TNNT2 and ACTN2 (Figure 3A, 3B). This result confirmed our conclusion that TCDD inhibits human ESC differentiation into the mesoderm lineage. Finally, we titrated the dosage and duration of TCDD treatment. We found that 2 nM and 4 days of TCDD treatment at the ESC stage is sufficient to elicit the strongest inhibition of mesoderm differentiation based on the expression of mesoderm marker T (Figure 3C, 3D).

3.3 Role of AHR in dioxin developmental toxicity

AHR is the main receptor for TCDD and mediates most of the effects caused by TCDD. To test whether AHR is responsible for TCDD's inhibition of mesoderm commitment in human ESCs, we first examined its expression by RT-qPCR. Interestingly, we found that human ESCs express a significantly higher amount of AHR than mouse ESCs (Figure S3A). Next we examined the response of a well-characterized AHR target gene, CYP1A1, after TCDD treatment. As expected, TCDD treatment of human ESCs led to a significant increase in CYP1A1 expression (Figure 4A, 4B). Thus, TCDD treatment that impairs mesoderm differentiation can also induce AHR target gene expression, in agreement with the idea that AHR mediates the activity of TCDD in mesoderm inhibition (Figure 3C, 3D).

Next, we treated human ESCs with the AHR antagonist CH223191 in conjunction with TCDD, and then induced mesoderm differentiation. We found that CH223191 blocked the inhibitory effect of TCDD based on the expression of the mesoderm marker T (Figure 4C),

suggesting that AHR plays a critical role. To further test this hypothesis, we generated AHR deletion human ESCs using CRISPR/Cas9-mediated genome editing (Figure 4D). Consistent with CH223191 treatment, AHR deletion greatly reduced the inhibitory effect of TCDD based on RT-qPCR (Figure 4E). Immunofluorescence staining further confirmed that Ahr deletion led to increased expression of T, indicating enhanced mesoderm differentiation efficiency. Together, these results support the conclusion that TCDD inhibits mesoderm differentiation in human ESC via the activation of AHR.

3.4 Role of AHR in mesoderm gene regulation

To further explore the mechanism by which TCDD and AHR regulates mesoderm differentiation, we treated ESCs with TCDD for four days, induced mesoderm differentiation, and carried out RNA-seq. We plotted the Log2 fold-changes of gene expression in DMSO- vs. TCDD-treated cells in a scatter plot, and the normalized RNA-seq signals for selected genes in the genome browser tracks. Similar to the RT-qPCR results (Figure 3B), we found that TCDD treatment resulted in the down-regulation of key developmental genes such as T, GSC, and EOMES (Figure 5A, 5B, Table S1), which have been shown to play critical roles in mesoderm differentiation and development³⁸. Specifically, T is expressed at the onset of gastrulation in response to mesoderm inducing-signals, and its expression is restricted in the primitive streak and mesoderm cells emerging from the streak^{39, 40}. It is required for mesoderm formation in human ESCs and its overexpression promote mesoderm gene expression⁴¹. GSC is briefly expressed during early gastrulation: initially at the anterior end of the developing primitive streak and later at the anteriormost mesoderm⁴². It plays important roles in the Spemann Organizer and mesoderm patterning^{43, 44}, and has been successfully used as a marker to characterize mesoderm differentiation in ESCs⁴⁵. EOMES is first expressed in the early primitive streak, and later in the nascent mesoderm and the anterior visceral endoderm⁴⁶. It promotes mesoderm formation⁴⁷, presumably by repressing ectoderm gene expression⁴⁸. It also promotes cardiac mesoderm specification and cardiovascular progenitor formation⁴⁹. Thus, our results suggested that TCDD inhibits the up-regulation of master mesoderm genes such as T, GSC, and EOMES.

To test whether these mesoderm genes are regulated by TCDD via AHR, we carried out chromatin-immunoprecipitation followed by high-throughput sequencing (ChIP-seq) for AHR in TCDD-treated ESCs. We identified a total of 6722 AHR peaks, which are associated with 2216 nearby genes (Table S2). Reassuringly, the known AHR binding motif was re-discovered in the AHR peaks (Figure 5C), suggesting the validity of the data. Gene ontology analysis showed that many AHR-bound genes are involved in transcription regulation and cardiac differentiation (Figure 5D, Table S3). Furthermore, there is a significant overlap between AHR-bound genes and those that are involved in mesoderm commitment (Figure 5E, Table S4), including several master regulators such as T, GSC, and EOMES (Figure 5F) as shown by the ChIP-seq signals in the genome browser tracks. Finally, the AHR-bound regions in mesoderm regulators are also co-occupied by histone marks H3K4m1 and H3K27m1. These histone marks are commonly found at gene regulatory regions⁵⁰, and their presence strongly suggests that AHR may play an important role in the direct regulation of the downstream genes. Together with the above gene

expression analysis, our results support the model that TCDD inhibits mesoderm gene via AHR in human ESCs.

4. Discussion

Human population studies indicate that TCDD exposure is associated with increased mortality from heart diseases⁶. However, the underlying mechanism is not fully understood. In this work, we established a human ESC based *in vitro* culture system to investigate the role of TCDD and AHR in cardiac differentiation, and uncovered a mechanistic link in TCDD exposure, AHR activation, and mis-regulation in gene expression during human embryogenesis. Our study bridges previous epidemiology and experimental observations, and provides useful information for human health risk assessment.

Consistent with previous reports in animal models and mouse ESCs, we showed that TCDD exposure impairs ESC cardiac differentiation and the effect of TCDD is largely mediated by AHR. Interestingly, unlike mouse ESCs, we found that the inhibition of cardiomyocyte generation is most obvious when human ESCs are exposed at the ESC stage. Furthermore, the AHR protein is readily detectable in human ESCs, and TCDD treatment or AHR does not affect pluripotency gene expression. In comparison, it has been shown that in the absence of TCDD AHR is largely repressed in mouse ESCs, and its expression reduces pluripotency gene expression⁵¹. In addition, TCDD inhibits mouse ESC cardiac differentiation when cells were exposed during embryoid body formation by disrupting Activin, BMP, and WNT signaling and altering homeobox transcription factor expression^{29, 52–54}. These differences suggest that human and mouse ESCs show distinct susceptibility to TCDD toxicity, possibly due to the species-specific expression pattern of AHR and its co-factors³⁷. In addition, they also suggest that AHR can regulate different target genes in different species or cellular contexts^{35, 36}, highlighting the relevance of our study.

AHR has long been recognized as a mediator of drug metabolism, but its xenobiotic-independent function and its role in normal cellular physiology is still not fully understood. Our results showed that TCDD can interfere with normal cellular differentiation and development, and AHR binding inhibits mesoderm gene activation and thereby mesoderm differentiation. Consistently, it was recently reported that AHR interacts with the NuRD (nucleosome remodeling and deacetylation) complex to regulate differentiation-specific genes⁵⁵. Thus, our findings reveal a previously uncharacterized role of AHR in the regulation of developmental genes, and provide insights for its physiological function in normal development.

Finally, the approach we described in this study can be easily adapted to investigate the impact of other environmental chemicals in human development. It can nicely complement existing strategies and has the following advantages: 1) Guided differentiation of human ESCs have been well established and closely mimics development *in vivo*; 2) Human ESCs and differentiated cells can more faithfully represent human physiology than other model organisms; 3) *In vitro* differentiation allows easy and systematic examination of the cell type and developmental window that is susceptible to the perturbation; 4) Cultured cells facilitate

the use of biochemical, genetic, genomic approaches for mechanistic investigations, and are also amenable for high throughput screens; 5) With the advance in CRISPR/Cas9-mediated genome editing techniques, human ESCs and their derivatives can be used to investigate the interactions between genetic and environmental factors. Thus, we propose that the human ESC differentiation system, as we demonstrated in this study, can serve as a platform for the systematic dissection of the developmental toxicity of environmental compounds in human.

Conclusions:

Our study demonstrated the toxicity of dioxin in human cardiac differentiation and uncovered a novel mechanism by which dioxin and AHR regulates mesoderm lineage commitment during early development. It illustrates the power of ESC-based models in the systematic study of environmental health science questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Junghare SW; Desurkar V, Congenital heart diseases and anaesthesia. *Indian J Anaesth* 2017, 61, (9), 744–752. [PubMed: 28970633]
2. Catana A; Apostu AP, The determination factors of left-right asymmetry disorders- a short review. *Clujul Med* 2017, 90, (2), 139–146. [PubMed: 28559696]
3. Muntean I; Toganel R; Benedek T, Genetics of Congenital Heart Disease: Past and Present. *Biochem Genet* 2017, 55, (2), 105–123. [PubMed: 27807680]
4. Sarmah S; Muralidharan P; Marrs JA, Common congenital anomalies: Environmental causes and prevention with folic acid containing multivitamins. *Birth Defects Res C Embryo Today* 2016, 108, (3), 274–286. [PubMed: 27718306]
5. Fahed AC; Gelb BD; Seidman JG; Seidman CE, Genetics of congenital heart disease: the glass half empty. *Circ Res* 2013, 112, (4), 707–20. [PubMed: 23410880]
6. Humblet O; Birnbaum L; Rimm E; Mittleman MA; Hauser R, Dioxins and cardiovascular disease mortality. *Environ Health Perspect* 2008, 116, (11), 1443–8. [PubMed: 19057694]
7. Bertazzi PA; Consonni D; Bachetti S; Rubagotti M; Baccarelli A; Zocchetti C; Pesatori AC, Health effects of dioxin exposure: a 20-year mortality study. *Am J Epidemiol* 2001, 153, (11), 1031–44. [PubMed: 11390319]
8. Revich B; Aksel E; Ushakova T; Ivanova I; Zhuchenko N; Klyuev N; Brodsky B; Sotskov Y, Dioxin exposure and public health in Chapaevsk, Russia. *Chemosphere* 2001, 43, (4–7), 951–66. [PubMed: 11372889]

9. Ketchum NS; Michalek JE, Postservice mortality of Air Force veterans occupationally exposed to herbicides during the Vietnam War: 20-year follow-up results. *Mil Med* 2005, 170, (5), 406–13. [PubMed: 15974208]
10. Noel JK; Namazi S; Haddock RL, Disparities in Infant Mortality Due to Congenital Anomalies on Guam. *Hawai'i journal of medicine & public health : a journal of Asia Pacific Medicine & Public Health* 2015, 74, (12), 397–402.
11. Schmidt C, The Fog of Agent Orange. *Scientific American* 2016, 314, (6), 70–5.
12. Kishi R; Araki A; Minatoya M; Hanaoka T; Miyashita C; Itoh S; Kobayashi S; Ait Bama Y; Yamazaki K; Miura R; Tamura N; Ito K; Goudarzi H; members of The Hokkaido Study on, E.; Children's, H., The Hokkaido Birth Cohort Study on Environment and Children's Health: cohort profile-updated 2017. *Environmental health and preventive medicine* 2017, 22, (1), 46. [PubMed: 29165157]
13. Eskenazi B; Warner M; Brambilla P; Signorini S; Ames J; Mocarelli P, The Seveso accident: A look at 40years of health research and beyond. *Environment international* 2018, 121, (Pt 1), 71–84. [PubMed: 30179766]
14. Kopf PG; Walker MK, Overview of developmental heart defects by dioxins, PCBs, and pesticides. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2009, 27, (4), 276–85. [PubMed: 19953399]
15. Cantrell SM; Lutz LH; Tillitt DE; Hannink M, Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): the embryonic vasculature is a physiological target for TCDD-induced DNA damage and apoptotic cell death in Medaka (*Orizias latipes*). *Toxicol Appl Pharmacol* 1996, 141, (1), 23–34. [PubMed: 8917672]
16. Hornung MW; Spitsbergen JM; Peterson RE, 2,3,7,8-Tetrachlorodibenzo-p-dioxin alters cardiovascular and craniofacial development and function in sac fry of rainbow trout (*Oncorhynchus mykiss*). *Toxicol Sci* 1999, 47, (1), 40–51. [PubMed: 10048152]
17. Guiney PD; Walker MK; Spitsbergen JM; Peterson RE, Hemodynamic dysfunction and cytochrome P4501A mRNA expression induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin during embryonic stages of lake trout development. *Toxicol Appl Pharmacol* 2000, 168, (1), 1–14. [PubMed: 11000095]
18. Antkiewicz DS; Burns CG; Carney SA; Peterson RE; Heideman W, Heart malformation is an early response to TCDD in embryonic zebrafish. *Toxicol Sci* 2005, 84, (2), 368–77. [PubMed: 15635151]
19. Walker MK; Catron TF, Characterization of cardiotoxicity induced by 2,3,7, 8-tetrachlorodibenzo-p-dioxin and related chemicals during early chick embryo development. *Toxicol Appl Pharmacol* 2000, 167, (3), 210–21. [PubMed: 10986012]
20. Sommer RJ; Hume AJ; Ciak JM; Vannostrand JJ; Friggens M; Walker MK, Early developmental 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure decreases chick embryo heart chronotropic response to isoproterenol but not to agents affecting signals downstream of the beta-adrenergic receptor. *Toxicol Sci* 2005, 83, (2), 363–71. [PubMed: 15564318]
21. Thackaberry EA; Nunez BA; Ivnitiski-Steele ID; Friggins M; Walker MK, Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on murine heart development: alteration in fetal and postnatal cardiac growth, and postnatal cardiac chronotropy. *Toxicol Sci* 2005, 88, (1), 242–9. [PubMed: 16120746]
22. Aragon AC; Kopf PG; Campen MJ; Huwe JK; Walker MK, In utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: effects on fetal and adult cardiac gene expression and adult cardiac and renal morphology. *Toxicol Sci* 2008, 101, (2), 321–30. [PubMed: 17975115]
23. Carreira VS; Fan Y; Kurita H; Wang Q; Ko CI; Naticchioni M; Jiang M; Koch S; Zhang X; Biesiada J; Medvedovic M; Xia Y; Rubinstein J; Puga A, Disruption of Ah Receptor Signaling during Mouse Development Leads to Abnormal Cardiac Structure and Function in the Adult. *PLoS One* 2015, 10, (11), e0142440. [PubMed: 26555816]
24. Denison MS; Soshilov AA; He G; DeGroot DE; Zhao B, Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci* 2011, 124, (1), 1–22. [PubMed: 21908767]
25. Fernandez-Salguero PM; Ward JM; Sundberg JP; Gonzalez FJ, Lesions of aryl-hydrocarbon receptor-deficient mice. *Vet Pathol* 1997, 34, (6), 605–14. [PubMed: 9396142]

26. Schmidt JV; Su GH; Reddy JK; Simon MC; Bradfield CA, Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc Natl Acad Sci U S A* 1996, 93, (13), 6731–6. [PubMed: 8692887]
27. Thackaberry EA; Gabaldon DM; Walker MK; Smith SM, Aryl hydrocarbon receptor null mice develop cardiac hypertrophy and increased hypoxia-inducible factor-1alpha in the absence of cardiac hypoxia. *Cardiovasc Toxicol* 2002, 2, (4), 263–74. [PubMed: 12665660]
28. Hahn ME; Karchner SI; Merson RR, Diversity as Opportunity: Insights from 600 Million Years of AHR Evolution. *Curr Opin Toxicol* 2017, 2, 58–71. [PubMed: 28286876]
29. Jiang Y; Wang D; Zhang G; Wang G; Tong J; Chen T, Disruption of cardiogenesis in human embryonic stem cells exposed to trichloroethylene. *Environ Toxicol* 2016, 31, (11), 1372–1380. [PubMed: 25847060]
30. BurrIDGE PW; Matsa E; Shukla P; Lin ZC; Churko JM; Ebert AD; Lan F; Diecke S; Huber B; Mordwinkin NM; Plews JR; Abilez OJ; Cui B; Gold JD; Wu JC, Chemically defined generation of human cardiomyocytes. *Nat Methods* 2014, 11, (8), 855–60. [PubMed: 24930130]
31. Chen G; Gulbranson DR; Hou Z; Bolin JM; Ruotti V; Probasco MD; Smuga-Otto K; Howden SE; Diol NR; Propson NE; Wagner R; Lee GO; Antosiewicz-Bourget J; Teng JM; Thomson JA, Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 2011, 8, (5), 424–9. [PubMed: 21478862]
32. Grumetto L; Ortosecco G; Russo G; Guida M; Ferranti P; Nasi A; Barbato F, Dioxin-like PCB levels in maternal and umbilical cord sera of people living near dump sites in southern Italy: a pilot study of biomonitoring. *Environ Monit Assess* 2015, 187, (3), 88. [PubMed: 25663396]
33. Zheng X; Yang P; Lackford B; Bennett BD; Wang L; Li H; Wang Y; Miao Y; Foley JF; Fargo DC; Jin Y; Williams CJ; Jothi R; Hu G, CNOT3-Dependent mRNA Deadenylation Safeguards the Pluripotent State. *Stem Cell Reports* 2016, 7, (5), 897–910. [PubMed: 27746116]
34. Wang L; Du Y; Ward JM; Shimbo T; Lackford B; Zheng X; Miao YL; Zhou B; Han L; Fargo DC; Jothi R; Williams CJ; Wade PA; Hu G, INO80 facilitates pluripotency gene activation in embryonic stem cell self-renewal, reprogramming, and blastocyst development. *Cell stem cell* 2014, 14, (5), 575–91. [PubMed: 24792115]
35. Dere E; Lee AW; Burgoon LD; Zacharewski TR, Differences in TCDD-elicited gene expression profiles in human HepG2, mouse Hepa1c1c7 and rat H4IIE hepatoma cells. *BMC Genomics* 2011, 12, 193. [PubMed: 21496263]
36. Suzuki T; Nohara K, Regulatory factors involved in species-specific modulation of arylhydrocarbon receptor (AhR)-dependent gene expression in humans and mice. *J Biochem* 2007, 142, (4), 443–52. [PubMed: 17652329]
37. Ginis I; Luo Y; Miura T; Thies S; Brandenberger R; Gerecht-Nir S; Amit M; Hoke A; Carpenter MK; Itskovitz-Eldor J; Rao MS, Differences between human and mouse embryonic stem cells. *Dev Biol* 2004, 269, (2), 360–80. [PubMed: 15110706]
38. Showell C; Binder O; Conlon FL, T-box genes in early embryogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists* 2004, 229, (1), 201–18. [PubMed: 14699590]
39. Smith JC; Price BM; Green JB; Weigel D; Herrmann BG, Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* 1991, 67, (1), 79–87. [PubMed: 1717160]
40. Beddington RS; Rashbass P; Wilson V, Brachyury--a gene affecting mouse gastrulation and early organogenesis. *Development* 1992, 157–65.
41. Faial T; Bernardo AS; Mendjan S; Diamanti E; Ortmann D; Gentsch GE; Mascetti VL; Trotter MW; Smith JC; Pedersen RA, Brachyury and SMAD signalling collaboratively orchestrate distinct mesoderm and endoderm gene regulatory networks in differentiating human embryonic stem cells. *Development* 2015, 142, (12), 2121–35. [PubMed: 26015544]
42. Blum M; Gaunt SJ; Cho KW; Steinbeisser H; Blumberg B; Bittner D; De Robertis EM, Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* 1992, 69, (7), 1097–106. [PubMed: 1352187]

43. Cho KW; Blumberg B; Steinbeisser H; De Robertis EM, Molecular nature of Spemann's organizer: the role of the *Xenopus homeobox* gene *goosecoid*. *Cell* 1991, 67, (6), 1111–20. [PubMed: 1684739]
44. Artinger M; Blitz I; Inoue K; Tran U; Cho KW, Interaction of *goosecoid* and *brachyury* in *Xenopus* mesoderm patterning. *Mechanisms of development* 1997, 65, (1–2), 187–96. [PubMed: 9256355]
45. Tada S; Era T; Furusawa C; Sakurai H; Nishikawa S; Kinoshita M; Nakao K; Chiba T; Nishikawa S, Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* 2005, 132, (19), 4363–74. [PubMed: 16141227]
46. Ciruna BG; Rossant J, Expression of the T-box gene *Eomesodermin* during early mouse development. *Mechanisms of development* 1999, 81, (1–2), 199–203. [PubMed: 10330500]
47. Russ AP; Wattler S; Colledge WH; Aparicio SA; Carlton MB; Pearce JJ; Barton SC; Surani MA; Ryan K; Nehls MC; Wilson V; Evans MJ, *Eomesodermin* is required for mouse trophoblast development and mesoderm formation. *Nature* 2000, 404, (6773), 95–9. [PubMed: 10716450]
48. Nelson AC; Cutty SJ; Niini M; Stemple DL; Flicek P; Houart C; Bruce AE; Wardle FC, Global identification of *Smad2* and *Eomesodermin* targets in zebrafish identifies a conserved transcriptional network in mesendoderm and a novel role for *Eomesodermin* in repression of ectodermal gene expression. *BMC biology* 2014, 12, 81. [PubMed: 25277163]
49. Costello I; Pimeisl IM; Drager S; Bikoff EK; Robertson EJ; Arnold SJ, The T-box transcription factor *Eomesodermin* acts upstream of *Mesp1* to specify cardiac mesoderm during mouse gastrulation. *Nature cell biology* 2011, 13, (9), 1084–91. [PubMed: 21822279]
50. Rada-Iglesias A; Bajpai R; Swigut T; Brugmann SA; Flynn RA; Wysocka J, A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* 2011, 470, (7333), 279–83. [PubMed: 21160473]
51. Ko CI; Fan Y; de Gannes M; Wang Q; Xia Y; Puga A, Repression of the Aryl Hydrocarbon Receptor Is Required to Maintain Mitotic Progression and Prevent Loss of Pluripotency of Embryonic Stem Cells. *Stem Cells* 2016, 34, (12), 2825–2839. [PubMed: 27374890]
52. Wang Q; Chen J; Ko CI; Fan Y; Carreira V; Chen Y; Xia Y; Medvedovic M; Puga A, Disruption of aryl hydrocarbon receptor homeostatic levels during embryonic stem cell differentiation alters expression of homeobox transcription factors that control cardiomyogenesis. *Environ Health Perspect* 2013, 121, (11–12), 1334–43. [PubMed: 24058054]
53. Carreira VS; Fan Y; Wang Q; Zhang X; Kurita H; Ko CI; Naticchioni M; Jiang M; Koch S; Medvedovic M; Xia Y; Rubinstein J; Puga A, Ah Receptor Signaling Controls the Expression of Cardiac Development and Homeostasis Genes. *Toxicol Sci* 2015, 147, (2), 425–35. [PubMed: 26139165]
54. Wang Q; Kurita H; Carreira V; Ko CI; Fan Y; Zhang X; Biesiada J; Medvedovic M; Puga A, Ah Receptor Activation by Dioxin Disrupts Activin, BMP, and WNT Signals During the Early Differentiation of Mouse Embryonic Stem Cells and Inhibits Cardiomyocyte Functions. *Toxicol Sci* 2016, 149, (2), 346–57. [PubMed: 26572662]
55. Gialitakis M; Tolaini M; Li Y; Pardo M; Yu L; Toribio A; Choudhary JS; Niakan K; Papayannopoulos V; Stockinger B, Activation of the Aryl Hydrocarbon Receptor Interferes with Early Embryonic Development. *Stem Cell Reports* 2017, 9, (5), 1377–1386. [PubMed: 29107595]

Highlights

- Established an *in vitro* model to study cardiac toxicity using human embryonic stem cells
- Defined the role of AhR in mesoderm development and lineage specification
- Uncovered TCDD toxicity and AhR function in early developmental stages in human

Figure 1. TCDD impairs human ESC cardiomyocyte differentiation.

(A) The NKX2.5::EGFP reporter human ESCs were treated with DMSO or TCDD (2 nM) during cardiomyocyte differentiation from day (−3) to day (14). EGFP fluorescence was detected in live cells at the end of the differentiation on day (14) by fluorescence microscopy. (B) The expression of cardiomyocyte marker genes was examined by RT-qPCRs on day (14). All experiments were repeated for at least three times, and one representative result was shown. p-values were calculated by two-tailed Student's t-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 2. TCDD exposure at the ESC stage has the most significant impact on cardiomyocyte differentiation.

(A) Illustration of the developmental stages and treatment windows during human ESC cardiomyocyte differentiation. (B-C) Cells were treated with DMSO or TCDD (2 nM) at the indicated windows during differentiation. At the end of the 14-day differentiation protocol, the effect of the treatments at the indicated windows were determined by the expression of cardiomyocyte marker genes based on RT-qPCR of the indicated cardiomyocyte markers (B) and immunofluorescence staining of the cardiomyocyte marker ACTN2 (C). All experiments were repeated for at least three times, and one representative result was shown. p-values were calculated by two-tailed Student's t-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3. TCDD exposure inhibits mesoderm commitment.

(A) ESCs were treated with DMSO or TCDD (2 nM) from day (–3) to day (0), and induced to differentiate into cardiomyocytes. Cells were collected at the indicated time points, and the expression of marker genes was determined by RT-qPCR (A) or immunofluorescence staining (B). (C) ESCs were treated with TCDD (2 nM) for one to four days, and then induced to differentiate into the mesoderm lineage. Two days later, mesoderm differentiation efficiency was determined by T expression using RT-qPCR. (D) ESCs were treated with TCDD (2 nM) at the indicated concentrations for 4 days, and then induced to differentiate into the mesoderm lineage. Two days later, mesoderm differentiation efficiency was determined by T expression using RT-qPCR. All experiments were repeated for at least three times, and one representative result was shown. p-values were calculated by two-tailed Student's t-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 4. AHR mediates the inhibitory effect of TCDD.

ESCs were treated with TCDD at the indicated concentration for 4 days (A), or with TCDD (2 nM) for the indicated time period (B). The expression of the AHR target gene CYP1A1 was determined by RT-qPCR. (C) ESCs were treated with DMSO, TCDD (2 nM), the AHR inhibitor CH223191 (abbreviated as CH), or TCDD (2 nM) and the AHR inhibitor CH223191 for 4 days, and then induced to differentiate into the mesoderm lineage. Two days later, mesoderm differentiation efficiency was determined by T expression using RT-qPCR. (D) A clonal ESC line with AHR deletion (AHR-KO) was generated using CRISPR/Cas9-mediated gene inactivation. AHR expression in wild type (Control) or AHR-KO cells was determined by western blot. (E-F) Wild type (Control) or AHR-KO cells were treated with DMSO or TCDD (2 nM) for 4 days, and then induced to differentiate into the mesoderm lineage. Two days later, mesoderm differentiation efficiency was determined by T expression using RT-qPCR (E) or immunofluorescence (F). All experiments were repeated for at least three times, and one representative result was shown. p-values were calculated by two-tailed Student's t-test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 5. AHR regulates mesoderm gene expression in human ESCs.

(A) ESCs were treated with DMSO or TCDD (2 nM) for four days, and then induced to differentiate into the mesoderm lineage. Cells were harvested two days after mesoderm induction, and gene expression changes between DMSO vs. TCDD group were determined by total RNA sequencing from two replicates. Differentially expressed genes were highlighted (red: up-regulated; blue: down-regulated), and selected genes were labeled with gene symbols. (B) Genome browser tracks for selected mesoderm genes were captured to show their decreased expression in TCDD comparing to DMSO treated cells, using data from (A). Y-axis: normalized RNA-seq signals (reads per million per kilobase, RPKM) that correspond to the gene expression level. (C) AHR ChIP-seq was performed from ESCs treated with 2 nM TCDD for four days in duplicates to identify genomic regions occupied

by AHR. Enriched motif in AHR-occupied genomic regions was identified using the MEME algorithm. (D) Ingenuity pathway analysis was carried out for AHR-bound genes to identify enriched gene categories. Log10(-p-value) of selected gene categories were plotted, and the complete list of enriched gene categories was included in Table S3. (E) Overlap between mesoderm genes (see methods for details) and AHR-bound genes. (F) Genome browser tracks to show AHR occupancy and histone marks H3K27me3 and H3K4me1 at key mesoderm genes. Y-axis: normalized ChIP-seq signals that indicate the occupancy of AHR and histone marks.