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Dioxin Exposure Disrupts the Differentiation of Mouse Embryonic Stem Cells into Cardiomyocytes

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Experimental exposure of fish, birds, and rodents to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) causes multiple Ah receptor–mediated developmental abnormalities, an observation consistent with compelling evidence in human populations that TCDD exposure is responsible for a significant incidence of birth defects. To characterize molecular mechanisms that might explain the developmental effects of dioxin, we have studied the consequences of TCDD exposure on the differentiation of mouse embryonic stem (ES) cells in culture and on the expression of genes, including those coding for homeodomain containing transcription factors, with a role in progression of tissue differentiation and embryonic identity during development. We find that TCDD treatment causes expression changes in a number of homeobox genes concomitant with Ah receptor recruitment to the promoters of many of these genes, whether under naïve or dioxin-activated conditions. TCDD exposure also derails temporal expression trajectories of developmentally regulated genes in a wide diversity of differentiation pathways, including genes with functions in neural and cardiovascular development, self-renewal, hematopoiesis and mesenchymal lineage specification, and Notch and Wnt pathways. Among these, we find that TCDD represses the expression of the cardiac development–specific Nkx2.5 homeobox transcription factor, of cardiac troponin-T and of α- and β-myosin heavy chains, inhibiting the formation of beating cardiomyocytes, a characteristic phenotype of differentiating mouse ES cells in culture. These data identify potential pathways for dioxin to act as a developmental teratogen, possibly critical to cardiovascular development and disease, and provide molecular targets that may help us understand the molecular basis of Ah receptor–mediated developmental toxicity.

Key Words: **Dioxin; Birth Defects; Embryotoxicity; Ah receptor; cardiomyocytes, homeobox genes.**

There is compelling evidence from human population studies in Vietnam that exposure to the defoliant known as Agent Orange is associated with a number of adverse pregnancy effects, including spontaneous abortion, still births, preterm delivery, and birth defects, including "spina bifida" and anencephaly (Dung *et al*., 2007; Ngo *et al*., 2006, 2010; Saito *et al*., 2009; Schecter and Constable, 2006). Agent Orange, a mixture of the N-butyl esters of 2,4-dichlorophenoxyacetic (2,4-D) and 2,4,5-trichlorophenoxyacetic (2,4,5-T) acids, was used as a defoliant by the United States during the war in Vietnam. It was suspected that 2,4,5-T caused birth defects, but it was soon evident that teratogenicity was not caused by 2,4,5-T itself but by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminating by-product of Agent Orange production and storage.

Strong evidence in laboratory animals corroborates the findings in humans. Exposure of mice to TCDD during a specific developmental window causes serious developmental abnormalities, specifically cleft palate and hydronephrosis, and inhibits characteristic events of secondary palate formation, such as osteoblast differentiation and synthesis and mineralization of extracellular matrix (Couture *et al*., 1990). TCDD also disrupts morphogenesis of the rat preimplantation embryo (Hutt *et al*., 2008), and exposure during avian (Bruggeman *et al*., 2005) and zebrafish (Mathew *et al*., 2008) development clearly shows that TCDD is embryotoxic and an experimental developmental teratogen.

TCDD is a ligand of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that regulates the expression of many phase I detoxification genes. AHR forms a heterodimeric complex with the AHR nuclear translocator (ARNT) protein and mediates the transcriptional activation of members of the CYP1 family of cytochrome P450 monooxygenases and of several phase II detoxification enzymes. Although activation of these genes is one of the best characterized effects of Ah receptor function, it does not adequately explain the diversity of effects mediated by the AHR. To identify a broader set of AHR gene targets, we used a genome-wide multipronged approach involving ChIP-on-chip analyses of AHR binding and global gene expression profile signatures. Integration of the information obtained in that work into a prior functional knowledge base revealed that in addition to the xenobiotic metabolism genes, the AHR binds to and regulates a large array of gene clusters involved in

transcriptional regulation, pattern specification, and neural and cardiovascular developmental programs (Sartor *et al*., 2009).

Homozygous ablation of the *Ahr* gene in mice results in numerous age-related pathologies involving multiple organ systems, including an impaired cardiovascular phenotype characterized by the failure of fetal vascular structures in the liver and eye to undergo apoptosis (Lahvis *et al*., 2000), and a fibrotic hepatic phenotype, complicated with premature senescence characteristics (Fernandez-Salguero *et al*., 1995). A growing body of experimental evidence strongly suggests that the AHR also plays a central role in cell proliferation, differentiation, liver and immune system homeostasis, as well as tumor development (Barouki *et al*., 2007). Increasing evidence also indicates that the AHR has endogenous functions with important roles in maintenance of cellular homeostasis that do not require activation by xenobiotic ligands (Bock and Kohle, 2006). Ultimately, the interaction of AHR functions and TCDD adaptive and toxic signals might define an "AHR-TCDD axis" in which the molecular characterization of TCDD teratogenic end points might provide needed clues to define what these AHR homeostatic functions might be. In this context, receptor activation by environmental chemicals may be expected to have the dual effect of disrupting homeostasis while simultaneously triggering the induction of detoxification pathways.

These observations have led us to propose the hypothesis that the AHR possesses developmental regulatory functions that may be targets of environmental injury. To initiate a thorough test of this hypothesis and to probe for a possible developmental basis of environmental disease (Heindel, 2008) associated with dioxin exposure, we have asked whether the interplay of AHR and TCDD perturbs normal gene expression trajectories during *in vitro* differentiation of mouse ES cells. We find that TCDD exposure derails temporal expression of genes in all developmental pathways tested, including cardiovascular and neural pathways. TCDD represses the expression of cardiac troponin-T (cTn-T) and of the cardiac development–specific Nkx2.5 homeobox transcription factor, inhibiting the formation of beating cardiomyocytes, a characteristic of differentiating mouse ES cells in culture. These results identify molecular targets that may help us understand the molecular basis of Ah receptor–mediated developmental toxicity.

MATERIALS AND METHODS

Cell lines, growth conditions, and treatments. WD44 (designated as ES-C57BL/6 by the ATCC; catalog number SRC-1002) and J1 (ATCC catalog number SRC-1010) are embryonic stem (ES) cells from C57BL/6 and 129/Sv mouse strains, respectively, and were a generous gift from Dr Peter Stambrook, University of Cincinnati. Undifferentiated ES cells were maintained in ES medium, consisting of high glucose Dulbecco's minimal essential medium (DMEM) (Gibco) supplemented with 15% ES cell qualified fetal bovine serum (knockout serum replacement; Gibco), 2mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1mM β-mercaptoethanol, and 1000 U/ml ESGRO (LIF, Chemicon international) (Chen *et al*., 2009). Cells were incubated in 0.1% gelatin-coated plates at 37°C, 95% humidity with 5% CO_2, and passaged every second or third day. Cell differentiation was initiated by first forming embryoid bodies in hanging drops. Cells were transferred to DMEM medium lacking LIF supplemented with 15% non-ES qualified fetal bovine serum and suspended at a concentration of 40,000–70,000 cells/ml. Fifty 20-μl aliquots were pipetted onto the inner surface of a bacterial petri dish and the lid was inverted over the bottom plate containing 15 ml PBS to provide humidity. Plates were incubated at 37°C for 3 days, and thereafter, the embryoid bodies were flushed with differentiation medium and incubated in 24-well or in 10-cm plates for varying periods of time. When needed, cultures were treated with TCDD at the standard range of concentrations—between 10pM and 1nM—commonly used for tissue culture work with the high-affinity Ah receptor of mouse cells. Treatments were for the length of time and at the final concentrations specified for each experiment or with the same volume of DMSO vehicle, never to exceed 0.05% of the final volume. Except for the experiments shown in Figure 1A, all other work was done with the C57BL/6 ES cells.

Preparation of whole cell extracts for immunoblotting. For preparation of whole cell extracts, cells were washed and harvested in PBS containing 1× Complete Protease inhibitor and lysed in 300 μl NETN buffer (100mM NaCl, 20mM Tris pH 8.0, 1mM EDTA, 0.5% NP-40, and 1× Complete Protease Inhibitor). After lysis, cells were sonicated on ice three times for 10 s each with a Fisher Scientific Sonic Dismembrator 60. Protein concentrations were measured using the Bradford assay and 20–50 μg of protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis; transferred to Polyvinylidene fluoride; and probed for Oct3/4 (H-134; catalog number sc-9081; Santa Cruz Biotechnology), Nanog, (clone eBioMLC-51; catalog number 14-5761-80; eBioscience), Nkx2.5 (N-19; catalog number sc-8697; Santa Cruz Biotechnology), or β-actin expression (Sigma) after blocking with 3% nonfat dry milk in phosphate buffer saline Tween (PBST) (0.1M PBS with 0.2% Tween 20). After washing, the blots were incubated with the species-appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted in 3% nonfat dry milk in PBST and visualized using chemiluminescence (NanoWest Super Signal, Pierce Rickford, IL).

Analysis of cTn-T by flow cytometry. Troponin-T expression in differentiating ES cells was determined by methods adapted from those used for other proteins in our laboratory (Marlowe *et al*., 2008). Briefly, after allowing for formation of embryoid bodies for 3 days, cells were grown in differentiation medium with the indicated concentrations of TCDD for 10 more days, replacing the medium every 2 days. Thereafter, the cells were trypsinized, collected by centrifugation, washed, suspended at 1×10^6 cells/ml, and fixed in 4% paraformaldehyde in PBS. After fixation, expression of cTn-T was evaluated by incubating the cells with a mouse monoclonal antibody to cTn-T (catalog number AB8295-200; Abcam) or a control mouse IgG, followed by incubation with an fluorescein isothiocyanate-conjugated anti-mouse antibody and analysis in a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems) equipped with a 488-nm argon laser, for measurements of intracellular fluorescence. Mean log fluorescence intensities were determined by the CELLQUEST software program (BD Biosciences). Fluorescent signals were gated at an intensity such that less than 2% of the cells reacted with the negative control antibody would score as positive.

Total RNA isolation, reverse transcription, and real-time RT-PCR. Differentiating or pluripotent ES cells were treated with TCDD or vehicle for the indicated lengths of time, and total cellular RNA was extracted with the RNeasy Mini Kit (Qiagen). First-strand complementary DNAs (cDNAs) were synthesized from 2–20 μg of total RNA in a volume of 10–20 μl containing 1× reverse transcriptase buffer, 7μM random hexamers primer, 0.5mM dNTP mix, 10mM dithiothreitol, 5mM $MgCl_2$, 20 U of RNase inhibitor (RNasin, Promega), and 100 U of SuperScript II RNase H^- reverse transcriptase (Invitrogen). Samples were denatured and annealed to the primer for 10 min at 70°C and reverse transcribed for 3 h at 42°C. Before amplification, the reverse

transcriptase was inactivated by heating to 70°C for 15 min, and RNA was hydrolyzed by incubation with 0.05 N NaOH at 70°C for 10 min, neutralized with 0.05 N HCl, and the cDNA precipitated with ethanol. The resulting cDNA products were dissolved in a final volume of 200 µl, and a 2-µl aliquot was used as template for subsequent quantification by real-time PCR amplification. PCR reactions were conducted in duplicate in a total volume of 25 µl containing SYBR Green PCR Master Mix (Applied Biosystems) and 0.1µM of each primer. Gene-specific primer sets for the various genes tested, i.e., development-specific genes and homeobox genes, are shown in Supplementary tables 1 and 2, respectively. Amplification was performed on an ABI 7500 (Applied Biosystems) where the reaction was heated to 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing-elongation at 60°C for 60 s. Detection of the fluorescent product was carried out during the 72°C extension period, and emission data were quantified using threshold cycle (C_t) values. C_t values for all genes analyzed were determined two to four times, averaged, and means were determined from the average C_t values for each biological duplicate. All means were then normalized to values for β-actin. The relative or fold change from control C_t values was determined for each sample using the equation: fold change $= 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \left(C_t^{\text{Target}} - C_t^{\text{Actin}}\right)_{\text{Test}} - \left(C_t^{\text{Target}} - C_t^{\text{Actin}}\right)_{\text{Control}}$. PCR product specificity from each primer pair was confirmed using melting curve analysis and subsequent polyacrylamide gel electrophoresis.

Chromatin immunoprecipitation analyses. Chromatin immunoprecipitation (ChIP) was performed with minor modifications of procedures previously described (Schnekenburger *et al*., 2007). ES cells grown in differentiation medium for 6, 8, and 12 days from the initiation of embryoid body formation were treated for 90 min with 1nM TCDD or with DMSO vehicle, cross-linked with 1% formaldehyde for 10 min, and the chromatin was sheared to a size range of 0.3–0.6 kb by sonication in a crushed ice/water bath with six 30-s bursts of 200 W with a 30-s interval between bursts using a Bioruptor (Diagenode). Precleared chromatin was incubated overnight on a rotating platform at 4°C with rabbit polyclonal antibodies specific for AHR (Biomol, #SA-210) or nonspecific rabbit IgG (used as an immunoprecipitation control). The immune complexes were recovered by a 2-h incubation at 4°C with a 50% gel slurry of protein A-agarose beads (Upstate). After extensive washing, precipitated chromatin complexes were removed from the beads by incubation with elution buffer (50mM $NaHCO_3$ and 1% SDS), with mild vortexing. This step was repeated and the eluates combined. Cross-linking was reversed and the samples were sequentially digested with RNase A and proteinase K. DNA was purified by chromatography on QIAquick columns (Qiagen), eluted in ddH_2O, and an aliquot was used for analysis by real-time PCR using specific primers covering promoter domains within ± 350 bp from the canonical AHR-binding sites present in the homeobox genes tested, as shown in Supplementary table 3. End-point PCR products were separated by electrophoresis through 8% polyacrylamide gels and visualized after staining with ethidium bromide.

RESULTS

TCDD Treatment Raises an AHR-Dependent Transcriptional Response in ES Cells but Does Not Change the Expression of ES Cell Pluripotent Markers

The AHR is a master regulator of the detoxification of xenobiotic polycyclic aromatic hydrocarbons and responds to a TCDD challenge with the induction of several CYP1 phase I detoxification genes (Nebert *et al*., 2004). To investigate if the AHR was functional in undifferentiated mouse ES cells, we treated C57BL/6 and 129/Sv ES cells with concentrations of TCDD and benzo[a]pyrene (B[a]P) known to induce those genes in cultured mouse hepatoma cells. C57BL/6 and 129/Sv mice are homozygous for the Ahr^{b1} and Ahr^{d} alleles, respectively, of the mouse *Ahr* gene. The Ahr^{b1} allele encodes a high ligand–affinity Ah receptor that responds to ligand concentrations 10–20 times lower than those needed to activate the low-affinity Ah receptor encoded by the Ahr^{d} allele (Swanson and Bradfield, 1993). Eight hours after treatment with 5µM B[a]P, 1nM TCDD, or vehicle, we extracted RNA from the treated ES cells and measured the levels of Cyp1a1, Cyp1a2, and Cyp1b1 messenger RNA (mRNA). In agreement with data recently obtained by Neri *et al*. (2008), we found that both chemicals induced a significant increase of all three mRNAs relative to control in the high-affinity AHR C57BL/6 ES cells. In the low-affinity 129/Sv ES cells, the increase due to TCDD was also statistically significant, but in these cells, B[a]P caused an increase that, although noticeable, did not

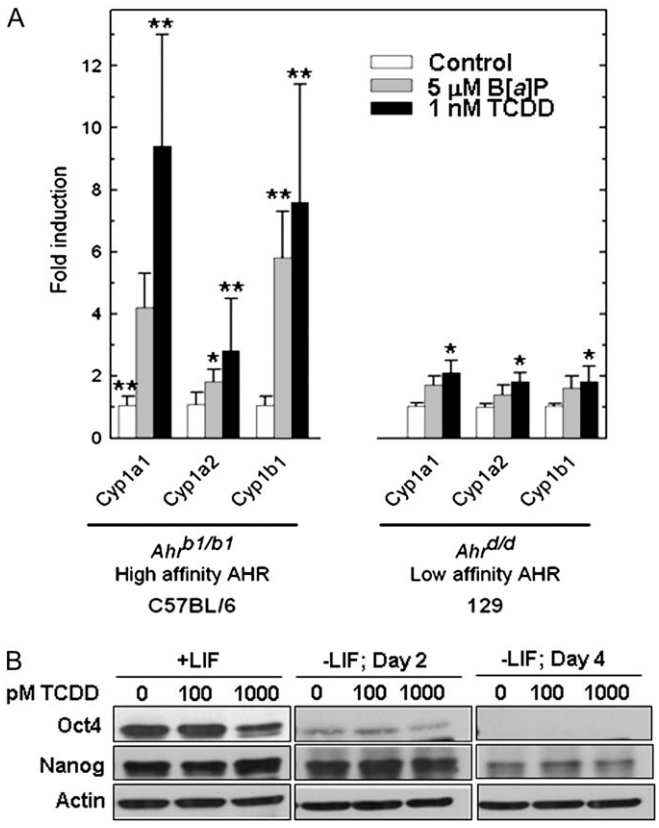

FIG. 1. Mouse ES cells respond to treatment with xenobiotic AHR ligands. (A) ES cells from high-affinity AHR C57BL/6 ($Ahr^{b1/b1}$) and low-affinity 129 ($Ahr^{d/d}$) mice were treated for 8 h with the indicated concentrations of B[a]P, TCDD, or control vehicle and probed for expression of the AHR-dependent *Cyp1a1*, *Cyp1b1*, and *Cyp1a2* genes. Except for the difference between B[a]P treatment and control for all three genes in 129 mice, results of all other treatments are significantly different from controls ($p < 0.05$ for TCDD in 129 mice; $p < 0.01$ for all cases in C57BL/6 mice). (B) Expression of pluripotent ES cell markers is not affected by TCDD treatment. ES cells were maintained in ES medium (+ LIF) or transferred to differentiation medium lacking LIF for 2 and 4 days. In both cases, cells were continuously treated with TCDD concentrations of 0, 100, or 1000pM. Protein extracts were separated by polyacrylamide gel electrophoresis and analyzed for expression of Oct-4 and Nanog with specific antibodies. Expression of β-actin was used as a control. $^{*}p < 0.05$, $^{**}p < 0.01$.

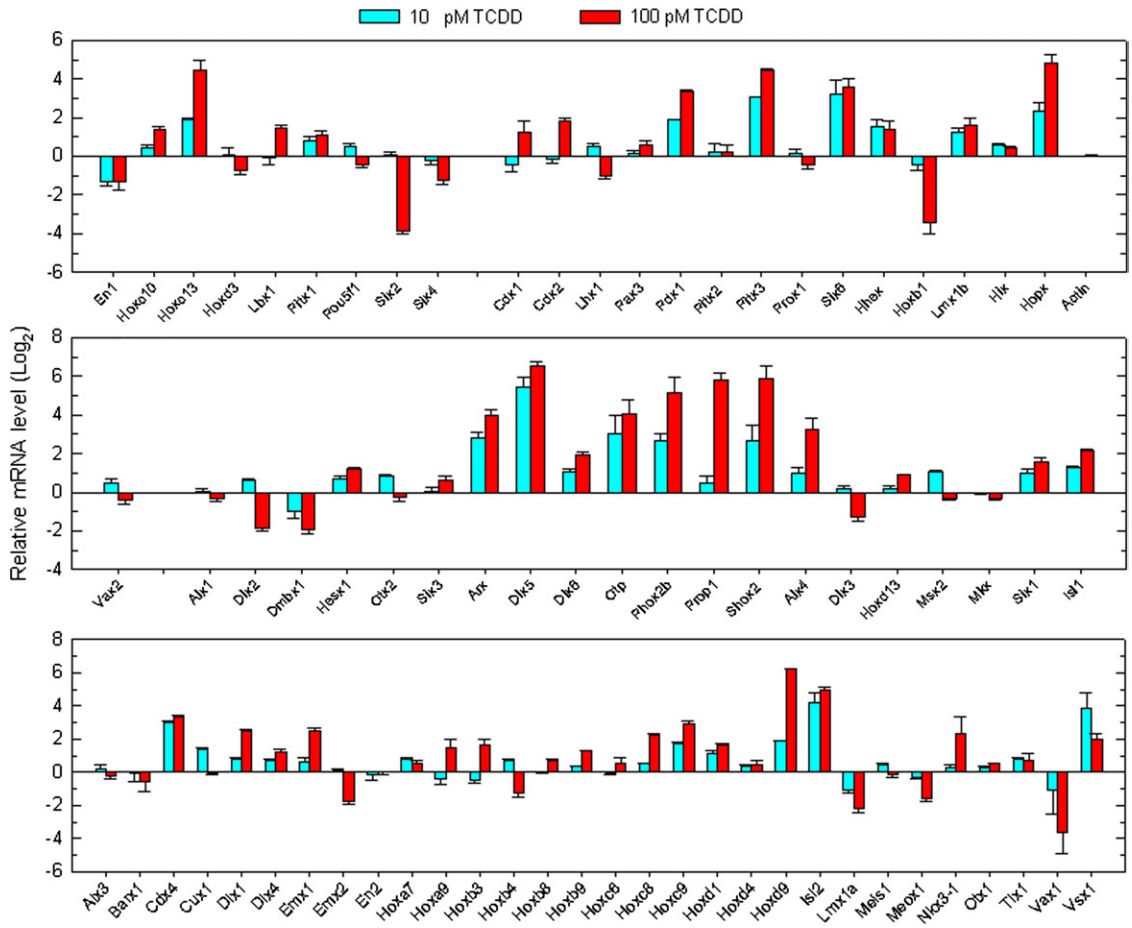

FIG. 2. TCDD treatment deregulates the expression of homeobox genes in ES cells. Differentiating ES cells were treated with 0, 10, or 100pM TCDD for 4 days from the completion of embryoid body formation, and the levels of mRNA expression of 75 homeobox genes in diverse regulatory groups were measured by real-time RT-PCR to determine the effect of TCDD treatment. The ordinate represents relative mRNA levels of TCDD-treated versus control samples.

reach statistically significant levels (Fig. 1A). These values are nearly 10 times lower than those observed in mouse hepatoma Hepa-1 cells, which overexpress AHR, and agree with the lower expression levels that we routinely observed for the AHR in the C57BL/6 ES cells (not shown). Nevertheless, the results demonstrate that the ES cell AHR is active and capable of regulating gene expression and support the concept that AHR activation by TCDD during development may lead to important changes in gene regulation.

Maintenance of the pluripotent phenotype of mouse ES cells is the consequence of the expression of a handful of homeobox genes coding for transcription factors. Critical in this respect is the expression of *Oct-4 (Pouf51)*, *Nanog*, and *Sox2* (Pei, 2009). To evaluate whether exposure to TCDD would change the expression characteristics of these markers either during ES cell undifferentiated growth or during differentiation, we grew ES cells in ES medium or in differentiation medium lacking LIF for 2 and 4 days and continuously treated them with TCDD concentrations of 0, 100, or 1000pM. Thereafter, protein extracts were prepared and analyzed for expression using Western blot analysis with specific antibodies to two of these factors, Oct-4 and Nanog. ES cells grown in complete ES medium showed continued expression of these ES cell markers whether or not they were exposed to TCDD. Similarly, repression of stemness markers by removal of LIF from the medium took place at the same rate, regardless of TCDD treatment (Fig. 1B). Minor fluctuations apparent in Oct-4 expression in some experiments at the highest TCDD concentration of 1000pM did not reach significance when averaged over several replicates. These data suggest that TCDD, and AHR activation, does not cause a significant observable effect in the expression of stem-specific markers either during undifferentiated growth or during the transition from undifferentiated to differentiating blastocysts.

Homeobox Gene Expression is a TCDD Target during Differentiation

Progression of tissue differentiation and embryonic identity during development are specified by transcription factors encoded by homeobox genes (Simon and Kingston, 2009;

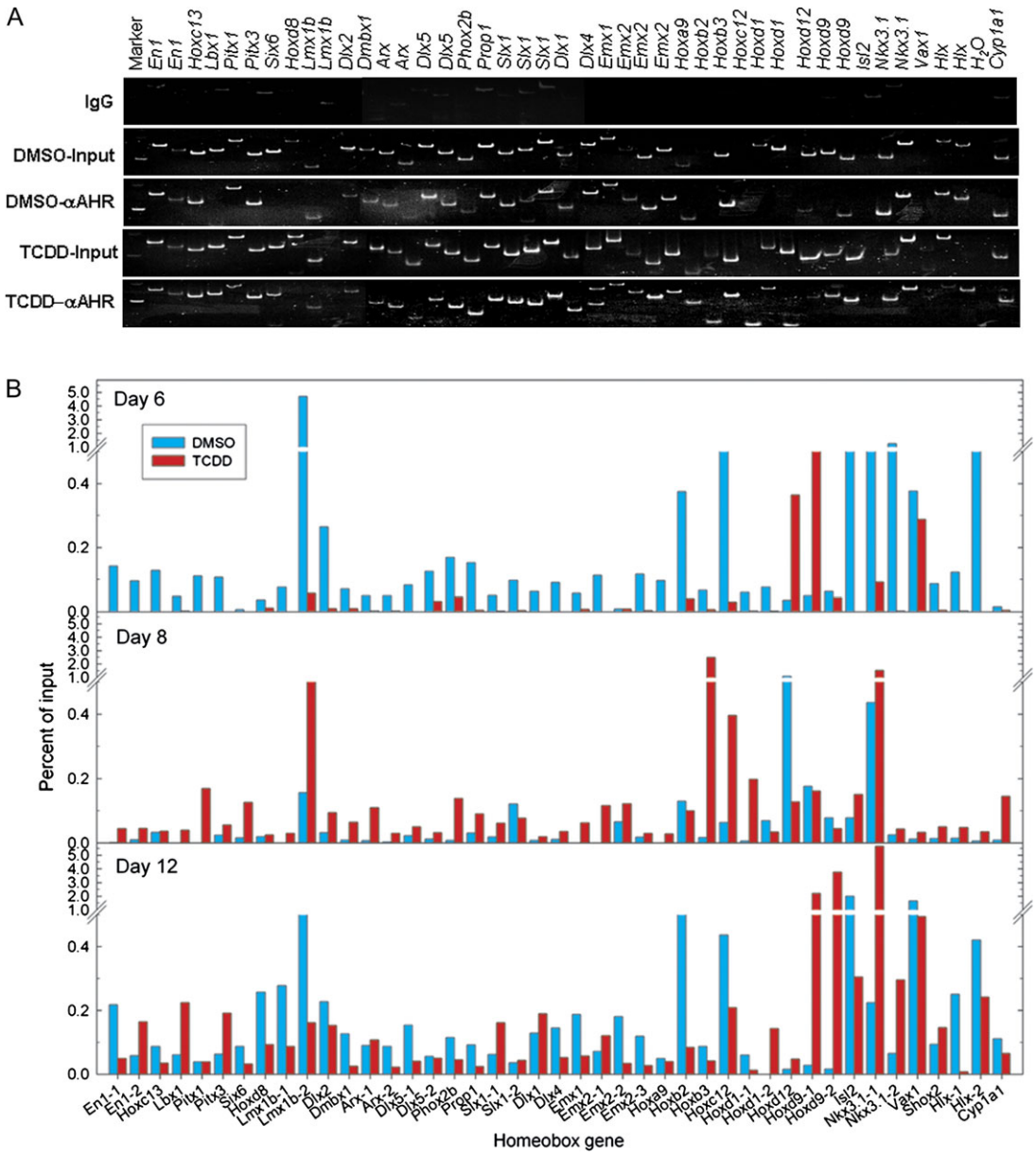

FIG. 3. AHR recruitment to the promoters of homeobox genes. The promoters of the homeobox genes in Figure 4 were scanned for the presence of cognate AHR-binding sites. Of those, 30 showed one to three AHR-binding motifs within 1 kb upstream and 0.2 kb downstream from the transcription start site. ES cells growing in differentiation medium for 6, 8, and 12 days from the initiation of embryoid body formation were treated with 1nM TCDD or vehicle for 90 min and processed for ChIP with rabbit polyclonal anti-AHR antibodies or with control, nonimmune rabbit IgG. Immunoprecipitated chromatin was analyzed by real-time PCR using primer sets for each promoter within ± 350 bp from the AHR-binding motifs. End-point PCR products were separated by electrophoresis through 8% polyacrylamide gels and visualized after staining with ethidium bromide. Panel (A) shows representative micrographs of gels from control, input, and ChIP samples from samples processed on day 12. Panel (B) shows the percent of the input chromatin that was immunoprecipitated from DMSO control and TCDD-treated samples by the anti-AHR antibody. Notice that the recruitment of AHR to the *Cyp1a1* promoter at day 6 is practically nonexistent and that at days 8 and 12 most homeobox gene promoters tested recruit the AHR as efficiently, or more so, as the *Cyp1a1* gene.

Wang *et al.*, 2009). If, as our hypothesis states, the AHR possesses developmental regulatory functions, it would be reasonable to surmise that TCDD treatment might target the expression of homeobox genes. To test this possibility, we treated differentiating ES cells with 0, 10, or 100pM TCDD for 4 days from the completion of embryoid body formation and evaluated the mRNA expression of 75 homeobox genes in different regulatory groups for the effect of TCDD treatment. For the most part, expression of many of these genes showed a significant dose-dependent induction, which in several cases, as for instance in the *Dlx5*, *Hoxd9*, and *Shox2* genes among others, reached levels 50- to 100-fold above control (Fig. 2).

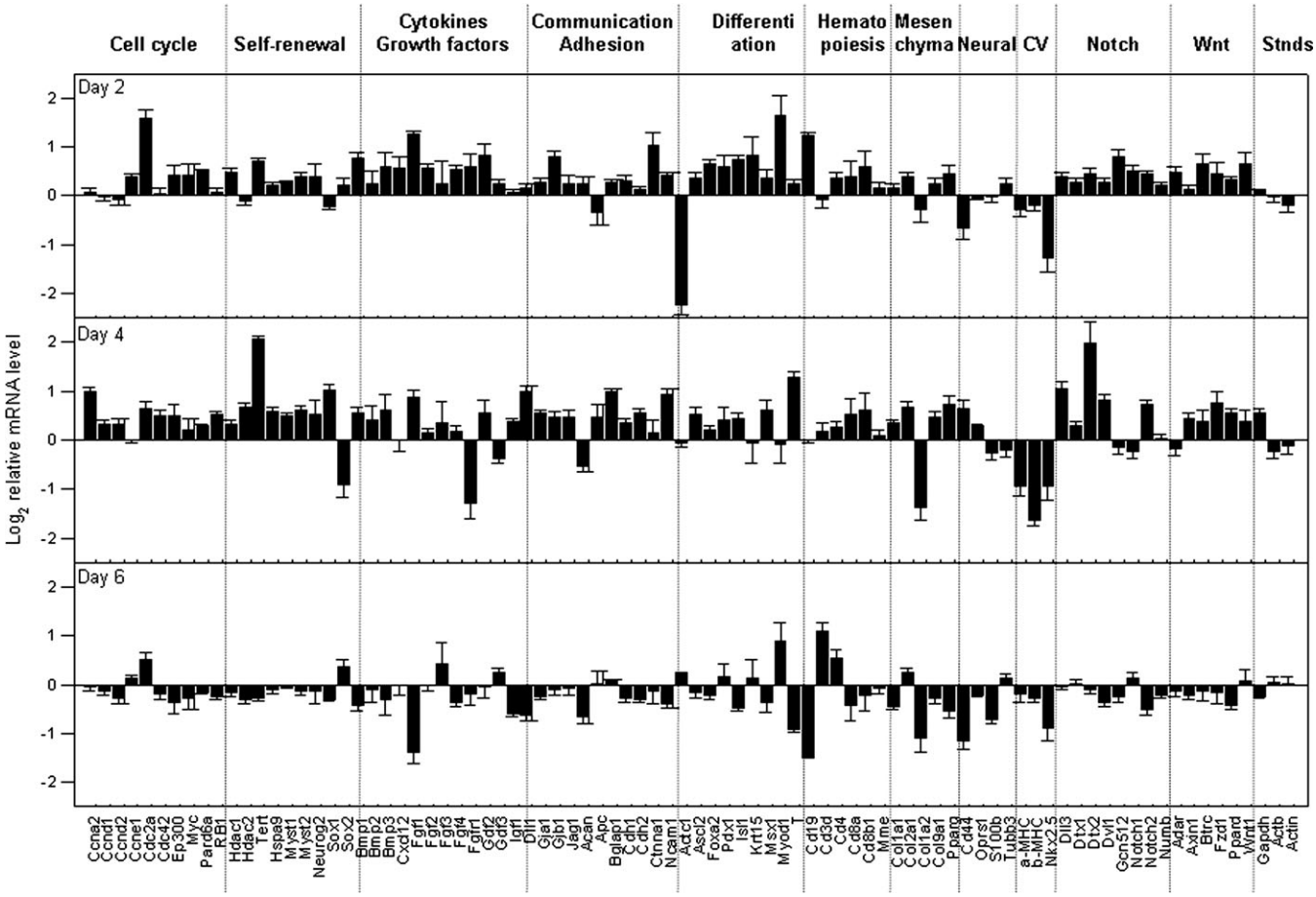

FIG. 4. Expression trajectories of genes involved in embryonic developmental specification are affected by TCDD treatment. At days 2, 4, and 6 after embryoid body formation, differentiating ES cells were probed by real-time RT-PCR for mRNA expression of a set of genes involved in different aspects of development, as indicated. The ordinate represents the $\log_2$ of the ratio of TCDD-treated values over control; hence, a value of 1 corresponds to a doubling of the mRNA expression.

Sequence analysis of the promoters of the homeobox genes tested in these studies indicated that within the span comprised between −1.0 and + 0.2 kb of their transcription start site, several of these promoters harbored canonical AHR-binding sites, frequently as many as 2 or 3 sites. To determine whether the AHR could bind to its cognate site(s) in these promoters and thus explore the possibility that the observed changes in homeobox gene expression could result from AHR-dependent gene regulation, we used ChIP analyses with anti-AHR antibodies. To assess the ability of the activated AHR to bind to DNA, ES cells grown for 6, 8, or 12 days from initiation of embryoid body formation were treated for 90 min with 1nM TCDD or with the same volume of vehicle, and the chromatin processed for immunoprecipitation. Control ChIP with non-immune IgG showed little or no evidence of nonspecific binding, whereas AHR binding was evident at all three experimental points, which showed obvious differences in AHR recruitment characteristics. At day 6, AHR recruitment was strongest in untreated cells, and TCDD treatment seemed to prevent AHR binding to most gene sites, with the exception of sites in *Hoxd12*, *Hoxd9*, and *Vax1*. At days 8 and 12, the reverse was true, and AHR was less frequently found in promoters in untreated cells and to be recruited by TCDD treatment (Figs. 3A–B). The information summarized in Table 1 shows the number of AHR-binding sites in each homeobox gene promoter and whether these sites were occupied in these experiments. Table 1 also shows the extent of the TCDD-dependent change of mRNA expression (expressed as a TCDD:DMSO ratio) shown graphically in Figure 3 and a succinct description of each particular homeobox gene function. These results strongly suggest that AHR targets homeobox gene expression, regulating expression of some of these genes as a naïve receptor, as a TCDD-activated one, or both.

TCDD Treatment Affects the Gene Expression Trajectories of Developmental Markers

The magnitude and diversity of homeobox gene expression changes in response to TCDD treatment raises the possibility

TABLE 1
Homeobox Genes Affected in Their Expression by TCDD Treatment of Differentiating Mouse ES Cells

	mRNA expression		AHR binding			
	TCDD/DMSO fold change					
Gene	Up	Down	Sites in promoter	Bound AHR	Function	Reference
Alx4	10				Aristaless-like homeobox 4. Bone development	Kuijper, *et al.* (2005). Dev. Biol. 285, 533–544.
Arx	16		2	Yes, both	Aristaless-related homeobox. Forebrain development	Fulp, *et al.* (2008). Hum. Mol. Genet. 17, 3740–3760.
Cdx1	4				Caudal-type homeobox 1. Embryonic hematopoiesis	Wang, *et al.* (2008). Proc. Natl. Acad. Sci. U.S.A. 105, 7756–7761.
Cdx2	4				Caudal-type homeobox 2. Trophectoderm specification	Jedrusik, *et al.* (2008). Genes Dev. 22, 2692–2706.
Cdx4	10				Caudal-type homeobox 4. Embryonic hematopoiesis	Lengerke, *et al.* (2007). Ann. N. Y. Acad. Sci. 1106, 197–208.
Cux1	4				Cut-like homeobox 1. Development of cortical interneurons	Cubelos, *et al.* (2008). Dev. Neurobiol. 68, 917–925.
Dlx1	6		1	Yes	Distal-less homeobox 1. Neuronal cell fate in the developing forebrain	Petryniak, *et al.* (2007). Neuron 55, 417–433.
Dlx2		4	1	Yes	Distal-less homeobox 2. Promotes cortical interneuron migration from the basal forebrain	Le, *et al.* (2007). J. Biol. Chem. 282, 19071–19081.
Dlx3		4			Distal-less homeobox 3. Regulates hair follicle differentiation and cycling	Sunwoo, *et al.* (2008). Development 135, 3149–3159.
Dlx4	4		1	Yes	Distal-less homeobox 4. Role in embryogenesis unknown	Coubrough, *et al.* (2006). Exp. Cell Res. 312, 3880–3891.
Dlx5	94		2	Yes, both	Distal-less homeobox 5. Regulates GABAergic interneurons and craniofacial and appendicular skeletal development	Robledo, *et al.* (2002). Genes Dev. 16, 1089–1101.
Dlx6	4				Distal-less homeobox 6. Required for specification of mammalian vestibular apparatus	Robledo, *et al.* (2006). Genesis 44, 425–437.
Dmbx1		4	1	Yes	Diencephalon/mesencephalon homeobox 1. Midbrain and caudal diencephalons differentiation	Broccoli, *et al.* (2002). Mech. Dev. 114, 219–223.
Emx1	6		1	Yes	Empty spiracles homolog homeobox 1. Promotes neuroepithelial cell fate	von Frowein, *et al.* (2006). Dev. Biol. 296, 239–252.
Emx2		4	3	Yes, all 3	Empty spiracles homolog homeobox 2. Repressor of Wnt1 expression in the developing telencephalon	Ligon, *et al.* (2003). Development 130, 2275–2287.
En1		4	2	Yes	Engrailed 1. Modulates calvarial osteoblast differentiation and proliferation. Survival factor for dopaminergic neurons	Sonnier, *et al.* (2007). J. Neurosci. 27, 1063–1071.
Hesx1	2				ES cell homeobox 1. Pituitary morphogenesis	Gaston-Massuet, *et al.* (2008). Dev. Biol. 324, 322–333.
Hhex	4				Hematopoietically expressed homeobox. Repressor of ESM-1, critical for normal function of vascular endothelium	Cong, *et al.* (2006). Biochem. Biophys. Res. Commun. 346, 535–545.
Hlx			2	No	H2.0-like homeobox. Required early in enteric nervous system development	Bates, *et al.* (2006). BMC Dev. Biol. 6, 33–47.
Hopx	30				HOP homeobox. Regulates adult hippocampal and glioblastoma stem cell neurons	De Toni, *et al.* (2008). Neural Dev. 3, 13–22.
Hoxa9	4		1	Yes, weak	Homeobox A9. Required for hematopoietic differentiation of mouse ES cells	Novotny, *et al.* (2009). Mech. Dev. 126, 517–522.
Hoxb1		10			Homeobox B1. Neural crest development into glia of the peripheral nervous system	Arenkiel, *et al.* (2003). Dev. Dyn. 227, 379–386.

TABLE 1—*Continued*

Gene	mRNA expression TCDD/DMSO fold change Up	Down	AHR binding Sites in promoter	Bound AHR	Function	Reference
Hoxb3	4		1	Yes, weak	Homeobox B3. Regulation of stem cell regeneration	Björnsson, *et al*. (2003). Mol. Cell. Biol. 23, 3872–3883.
Hoxb4		2			Homeobox B4. Regulation of stem cell self-renewal	Schiedlmeier, *et al*. (2007). Proc. Natl. Acad. Sci. U.S.A. 104, 16952–16957.
Hoxb9	4				Homeobox B9. Anterior/posterior pattern development	McIntyre, *et al*. (2007). Development 134, 2981–2989.
Hoxc8	6				Homeobox C8. Regulates skeletal development	Juan, *et al*. (2006). Ann. N. Y. Acad. Sci. 1068, 87–94.
Hoxc9	8				Homeobox C9. Antagonizes BMP by binding to Smads and blocking DNA binding	Zhou, *et al*. (2008). Biochim. Biophys. Acta. 1784, 747–752.
Hoxc10	4				Homeobox C10. Establishes lumbar motoneuron columnar, divisional, and motor pool identity	Wu, *et al*. (2008). Development 135, 171–182.
Hoxc13	22		1	Yes	Homeobox C13. Regulates hair follicle differentiation	Pruett, *et al*. (2004). J. Biol. Chem. 279, 51524–51533.
Hoxd1	4		2	Yes, both	Homeobox D1. Controls formation of the limb apical ectodermal ridge via Fgf10	Zakany, *et al*. (2007). Dev. Biol. 306, 883–893.
Hoxd9	74		2	Yes, strong	Homeobox D9. Regulates digit number and identity during limb development	Sheth, *et al*. (2007). Dev. Biol. 310, 430–441.
Isl1	4				Insulin-related protein 1. Role in sensory neuron development and required for progenitor heart cell population	Cai, *et al*. (2003). Dev. Cell 5, 877–889.
Isl2	30		1	Yes	Insulin-related protein 2. Controls binocular vision by repressing a genetic program specifying lateral pathfinding	Pak, *et al*. (2004). Cell 119, 567–578.
Lbx1	4		1	Yes	Ladybird homeobox homolog 1. Specifies a subpopulation of cardiac neural crest necessary for heart development	Schäfer, *et al*. (2003). Circ. Res. 92, 73–80.
Lhx1		2			LIM homeobox protein 1. Essential role in epithelial tubular morphogenesis during kidney organogenesis	Kobayashi, *et al*. (2005). Development 132, 2809–2823.
Lmx1a		5			LIM homeobox 1α. Production of mesencephalic dopamine neurons	Friling, *et al*. (2009). Proc. Natl. Acad. Sci. U.S.A. 106, 7613–7618.
Lmx1b	4		2	Yes, both	LIM homeocox 1β. Controls the isthmic organizer, essential for development of midbrain dopaminergic neurons	Guo, *et al*. (2008). J. Neurosci. 28, 14097–4106.
Meox1		4			Mesenchyme homeobox 1. Essential for specification of mesodermal somites precursors to aorta smooth muscle cells	Wasteson, *et al*. (2008). Development 135, 1823–1832.
Msx2		2			Msh-like homeobox 2. Induces epithelial-mesenchymal transition	di Bari, *et al*. (2009). J. Cell. Physiol. 219, 659–666.
Nkx3-1	6		2	Yes, one very strong	NK-3 transcription factor, locus 1. Contributes to the formation of the axial skeleton	Herbrand, *et al*. (2002). Mech. Dev. 117, 217–224.
Otp	16				Orthopedia homolog. Essential for diencephalic dopaminergic neuron development	Ryu, *et al*. (2007). Curr. Biol. 17, 873–880.
Pdx1	10				Pancreatic and duodenal homeobox 1. Regulation of pancreas development and pancreatic beta-cell growth	Kitamura, *et al*. (2002). J. Clin. Invest. 110, 1839–1847.
Phox2b	35		1	Yes	Paired-like homeobox 2b. Signals from the neural crest to regulate beta-cell mass in the pancreas	Nekrep, *et al*. (2008). Development 135, 2151–2160.

TABLE 1—*Continued*

Gene	mRNA expression: TCDD/DMSO fold change Up	Down	AHR binding: Sites in promoter	Bound AHR	Function	Reference
Pitx1	2		1	Yes, weak	Paired-like homeodomain transcription factor 1. Required for hindlimb bud growth and mandibular morphogenesis	Marcil, *et al*. (2003). Development 130, 45–55.
Pitx3	22		1	Yes	Paired-like homeodomain transcription factor 3. Required for development of ocular lens and skeletal muscles	Qiu, *et al*. (2008). Genesis 46, 324–328.
Prop1	56		1	Yes	Paired-like homeodomain factor 1. Spatial expression during pituitary development	Ward, *et al*. (2007). Mamm. Genome 18, 521–537.
Shox2	58		1	Yes	Short stature homeobox 2. Essential for the differentiation of cardiac pacemaker cells by repressing Nkx2-5	Espinoza-Lewis, R. A., Yu, L., He, F., Liu, H., Tang, R., Shi, J., Sun, X., Martin, J. F., Wang, D., Yang, J., *et al*. (2009). Shox2 is essential for the differentiation of cardiac pacemaker cells by repressing Nkx2-5. Dev. Biol. 327, 376–385.
Six1	4		2	Yes, one weak	Sine oculis–related homeobox 1 homolog. Mediates early neurogenesis in the development of olfactory epithelium	Ikeda, *et al*. (2007). Dev. Biol. 311, 53–68.
Six2		10			Sine oculis–related homeobox 2 homolog. Required for suppression of nephrogenesis in the developing kidney	Self, *et al*. (2006). EMBO J. 25, 5214–5228.
Six4		4			Sine oculis–related homeobox 4 homolog. Regulate the activation of Myf5 expression in embryonic mouse limbs	Giordani, *et al*. (2007). Proc. Natl. Acad. Sci. U.S.A. 104, 11310–11315.
Six6	12		1	Yes	Sine oculis–related homeobox 6 homolog. Regulates retinal and pituitary precursor cell proliferation	Li, *et al*. (2002). Science 297, 1180–1183.
Vax1		10	1	Yes	Ventral anterior homeobox 1. Directs generation of GABAergic interneurons	Taglialatela, *et al*. (2004). Development 131, 4239–4249.
Vsx1	15				Visual system homeobox 1 homolog. Regulates contrast adaptation in the retina	Kerschensteiner, *et al*. (2008). J. Neurosci. 28, 2342–2352.

that these changes may be extended to the genes regulated by each homeobox gene affected, thus causing a broader secondary amplification of the AHR-TCDD regulatory effect. To determine whether other developmental markers were also affected by TCDD treatment, we measured the level of mRNA expression in response to treatment with 100pM TCDD during the first 6 days of differentiation postembryoid body formation. The results show a significant effect of exposure on the temporal expression trajectories of TCDD-mediated changes in regulation of many genes involved in developmental pathways, including cell cycle regulation, genes involved in self-renewal; genes coding for cytokines and growth factors, such as the fibroblast growth factor (FGF) and genes involved in differentiation, hematopoietic and mesenchymal lineages, and neural, cardiovascular, Notch, and Wnt pathways (Fig. 4). A cursory examination of the changes of mRNA expression over the three time points in Figure 4 suggests that as differentiation progresses, the genes under study, to a large extent induced during days 2 and 4 (positive $\log_2$ values), become largely repressed on day 6 (negative $\log_2$ values). These data strongly suggest that TCDD may be responsible for critical changes in the development of a differentiated phenotype and provide significant support for the concept that the AHR-TCDD axis may play a determinant role in the causation of developmental abnormalities.

TCDD Inhibits Cardiomyocyte Differentiation

ES cells are pluripotent, having the potential of generating most embryonic cell lineages (Doetschman *et al*., 1985), including cardiomyocytes (Hescheler *et al*., 1997; Kattman *et al*., 2006; Yamashita *et al*., 2005). Importantly, cardiomyocytes derived from ES cell embryoid bodies beat spontaneously and function in all manner as cardiac cells, forming stable intracardiac grafts when injected into mice (Klug *et al*., 1996). As shown in Figure 4, TCDD repressed mRNA synthesis of α- and β-myosin heavy chain genes and the transcription factor

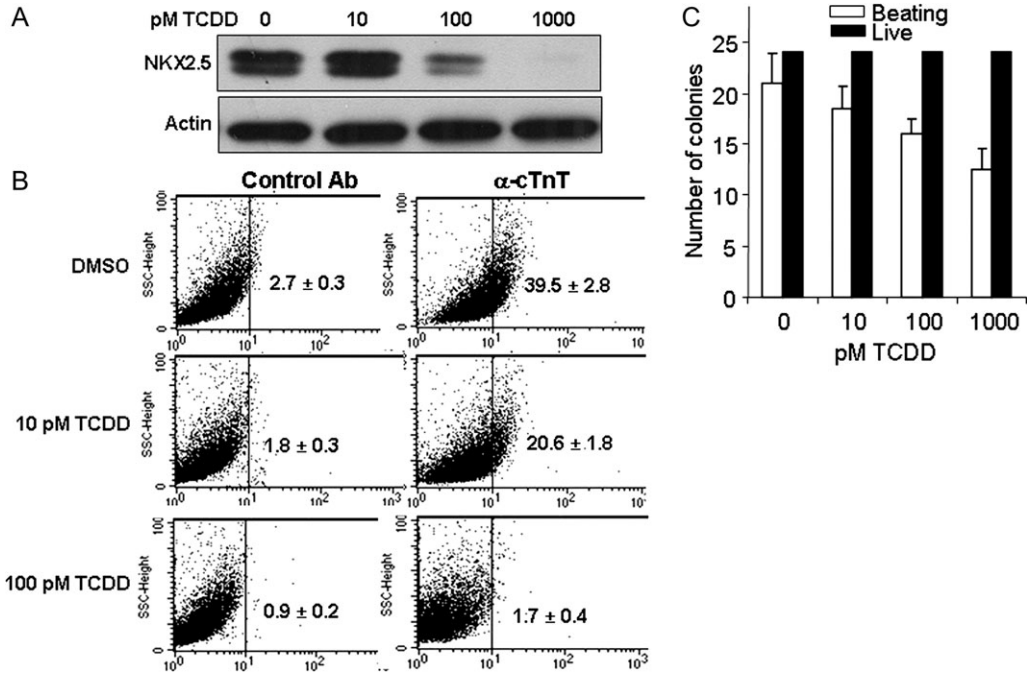

FIG. 5. TCDD treatment inhibits differentiation of ES cells into cardiomyocytes. (A) Expression of the cardiomyocyte-specific homeobox transcription factor Nkx2.5 was assessed by Western blot after treatment of differentiating ES cells with increasing concentrations of TCDD, as indicated, for 10 days postembryoid body formation. (B) Expression of cTn-T was determined by flow cytometry in differentiating ES cells similarly treated with 10 or 100pM TCDD. (C) Embryoid bodies after 3 days as ''hanging drops'' were individually transferred to 24-well plates and allowed to differentiate in the presence of the indicated TCDD concentrations for 14 days. Expansion of the embryoid body and transition to a beating phenotype were determined daily by microscopic examination.

Nkx2.5, which are some of the first cardiomyocyte marker genes to be upregulated upon initiation of cardiomyocyte differentiation (Boheler *et al*., 2002). Nkx2.5 is the major homeobox transcription factor that regulates expression of genes in the cardiomyocyte lineage and a marker of cardiac progenitor cells (Yano *et al*., 2008). Nkx2.5 expression is repressed by the homeodomain transcription factor encoded by the *Shox2* homeobox gene (Espinoza-Lewis *et al*., 2009), and as shown in Figures 2 and 3 and Table 1, one of the major effects of TCDD treatment was a 50-fold induction of *Shox2*, suggesting the possibility that through inhibition of Nkx2.5 expression and of Nkx2.5-regulated cardiovascular markers, TCDD treatment might block attainment of the cardiomyocyte phenotype. To test this hypothesis and to determine the extent of cardiomyocyte marker inhibition by TCDD, we measured expression of Nkx2.5 by Western blot analysis after treating differentiating ES cells for 10 days postembryoid body formation with increasing concentrations of TCDD. The result shows that, in agreement with the mRNA data, 100pM TCDD significantly inhibited, and 1nM completely blocked, Nkx2.5 expression (Fig. 5A). To confirm this result with a second cardiomyocyte marker, we used antibodies to cTn-T to follow its expression during the same experimental treatment protocol and to quantify the number of cells expressing cardiomyocyte markers by flow cytometry. Nearly 40% of the differentiating ES cells treated with vehicle expressed cTn-T, but this number was reduced to half by treatment with 10pM TCDD, and treatment with 100pM TCDD inhibited cTn-T expression in practically all cells (Fig. 5B). Consistent with these results, continued exposure to TCDD from day 3 onwards led to a clear dose-dependent inhibition of the beating phenotype even at concentrations as low as 10pM (Fig. 5C), indicating that TCDD exposure at this developmental stage causes severe alterations of the cardiovascular phenotype.

DISCUSSION

The results presented in this study show that TCDD treatment of differentiating mouse ES cells causes significant changes in the expression of developmental genes and in the regulation of their temporal trajectories. Thus, exposure to TCDD after an initial 3-day period of embryoid body formation results in a fairly complete derailment of the expression of many genes coding for cell cycle regulatory proteins, cytokines and growth factors; development of hematopoietic, mesenchymal, cardiovascular, and neural lineages; and genes in the Notch and Wnt pathways. The pattern of change seems more pronounced in the 4 days immediately following embryoid body formation and for the most part shows twofold to fourfold increases rather than decreases in gene expression. By 6 days, many of the genes involved are now repressed or unaffected (Fig. 4). This massive change in gene expression pattern is accompanied by a concomitant change in the dose-dependent

increase in expression of homeobox genes, many of which, harboring Ah receptor–binding sites in their promoters, recruit AHR to their promoters (Figs. 2 and 3). Of particular relevance is the apparent loss of cardiomyocyte differentiation as a result of TCDD treatment, evidenced by the induction of *Shox2*; the inhibition of Nkx2.5, cTn-T, and α- and β-myosin heavy chain expression; and the loss of the spontaneous beating cardiomyocyte phenotype. Changes in myosin and cTn-T expression have been previously observed in the developing zebrafish heart, with conflicting results. TCDD repressed myosin in one set of microarray experiments (Carney *et al*., 2006) and induced it in another (Handley-Goldstone *et al*., 2005), whereas the later work also obtained conflicting data of induction and repression when they used different probes for cTn-T (Handley-Goldstone *et al*., 2005). In our hands, using reverse transcriptase PCR and protein expression, the data show unequivocally that these genes are repressed by TCDD during mouse ES cell differentiation.

The significant differences observed in TCDD-treated differentiating mouse ES cells relative to vehicle-treated cells are remarkable and so are their implications for the regulation of gene expression. A major hurdle to overcome in the interpretation of these results arises from the regulatory complexity resulting from the lineage diversity of the differentiating ES cells. Within a few days in LIF-depleted differentiating medium, ES cells have given rise to ectoderm, mesoderm, and endoderm layers and soon thereafter to various primordial lineages derived from these layers. As a consequence, the gene expression patterns that we detect do not arise from a homogeneous cell population but from a mixture of cells with different regulatory pathways activated in the different constituent lineages. Hence, ascribing observable phenotypes to gene expression changes to specific biological traits is at best questionable when the cell population is an admixture of such complexity. Phenotypic traits, such as the beating phenotype of cardiomyocytes, can only be defined in molecular terms but cannot be readily followed in specific cell populations because only a fraction of the cells in the expanding embryoid body show the phenotype. Ultimately, these considerations greatly hamper the study of gene-environment interactions in the differentiating ES cells, requiring the development of enrichment techniques that would allow tracking individual lineages to analyze the cellular and molecular process of differentiation and how they are affected by environmental stressors.

Nonetheless, our data on cardiomyocyte development can be interpreted in molecular terms, providing strong support to the growing evidence of a significant role for the AHR in cardiovascular development and disease and to the cardiovascular toxicity of TCDD. In all experimental systems tested to date, the heart has been proven to be a sensitive target of TCDD toxicity and the AHR is a major contributor to cardiovascular homeostasis in all species that have been studied. In developing fish embryos, TCDD reduces blood flow and circulatory functions associated with subcutaneous hemorrhage and pericardial edema (Ivnitski-Steele and Walker, 2005). In avian embryos, TCDD exposure induces dilated cardiomyopathy, myocardial hypoxia, increased vascular endothelium growth factor-A expression, and coronary vascularization, inducing a dose-related reduction in tube outgrowth (Ivnitski-Steele *et al*., 2005). In humans, long-term epidemiologic studies in a retrospective cohort study of more than 1000 individuals have established a strong link between occupational exposure to high doses of TCDD and ischemic heart disease (Flesch-Janys *et al*., 1995). Our previous work in *Apoe*$^{-/-}$ mice (Dalton *et al*., 2001) and the recent findings from Walker and coworkers that *in utero* exposure to TCDD may increase the susceptibility to cardiovascular dysfunction in adult life (Aragon *et al*., 2008; Thackaberry *et al*., 2005) are consistent with the concept that the AHR is a major player in cardiac function. Furthermore, knockout of the *Ahr* gene in mice disrupts cardiovascular homeostasis, causing significant cardiac hypertrophy and elevated levels of expression of cardiovascular markers and increased cardiac fibrosis associated with cardiac hypertrophy (Lund *et al*., 2005), hypertension, and cardiac pathology (Lund *et al*., 2003, 2006).

The invertebrate Ah receptor is a developmental regulator that does not respond to any of the environmental ligands recognized by the vertebrate receptor, although it functions as a transcription factor and binds to the same ARNT dimerization partner and cis-acting response elements as the vertebrate protein. The ability to bind xenobiotic compounds appears to have been acquired only during vertebrate evolution (Hahn, 2002). The *Caenorhabditis elegans* AHR regulates gamma-aminobutyric acidergic neuronal differentiation during worm development (Qin and Powell-Coffman, 2004), a developmental processes independent of exogenous ligand exposure. In *Drosophila*, AHR regulates normal morphogenesis of legs, antennae, and bristles (Adachi-Yamada *et al*., 2005). The mammalian AHR also possesses a developmental role in craniofacial, renal, and cardiovascular morphogenesis (Birnbaum *et al*., 1989; Fernandez-Salguero *et al*., 1997; Lahvis *et al*., 2005), which suggests that its ancestral developmental function has been retained during vertebrate evolution. Having acquired the ability to respond to environmental xenobiotic ligands, the vertebrate AHR may be a developmental regulator that fulfills the dual functions of xenobiotic detoxification and maintenance of homeostasis.

SUPPLEMENTARY DATA

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

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