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TCDD-Induced Alterations in Gene Expression Profiles of the Developing Mouse Paw Do Not Influence Morphological Differentiation of This Potential Target Tissue

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The aryl-hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates the toxicity of certain halogenated aromatic hydrocarbons including 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD). These compounds are potent developmental toxicants that can alter gene expression and disrupt processes of proliferation and differentiation. It has not yet been determined which tissues during development are most sensitive to these compounds, nor which genes are directly associated with the toxicities. We developed a transgenic (TG) mouse model to delineate the temporal and spatial context of transcriptionally active AhR by utilizing a dioxin responsive element–linked *LacZ* reporter system. The present study focuses on the pattern of TCDD-induced transgene expression localized to the footpad and digits of the paws between gestational days (GD) 13 and 18. Paw morphology was evaluated at several developmental stages following TCDD exposure. Gene expression profiles acquired by microarray technology were evaluated in the paws of fetuses exposed at GD 14.5. The results showed that TCDD exposure *in utero* induced LacZ expression in the developing paws. This expression appeared to be localized to the mesenchymal cell layer. Gross morphological changes were not observed in the paws prior to or after birth following TCDD exposure *in utero*. However, significant alterations in gene expression profiles in the developing paws were observed at 24 h following TCDD exposure *in utero*. These results indicate that the developing paw is a target tissue of TCDD in terms of altered gene expression, further validating the use of this AhR responsive reporter gene TG mouse model in studying AhR ligand-mediated responsiveness. However, the linkage of these changes to detectable biological outcomes in the paw remains unclear.

Key Words: **Developing paw; gene expression; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; transgenic mouse model.**

Experimental animal data and epidemiological studies in human populations exposed to aryl-hydrocarbon receptor (AhR) agonists suggest that developing tissues are susceptible to affects on growth and differentiation (Birnbaum, 1995; Weisglas-Kuperus, 1998). Embryo lethality, cleft palate, hydronephrosis, thymic atrophy, immunodeficiency, growth retardation, and reproductive abnormalities are among the species-specific responses of the developing organism to 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD) and related compounds (Birnbaum, 1995). However, the mechanism of toxicity and the direct target tissues are not clearly defined. Studies in mice show that 0.01–0.03% of the total dose of TCDD is transferred to each fetus (Weber and Birnbaum, 1985), suggesting a direct effect in developing tissues. It has been hypothesized that the developmental, immunosuppressive, and reproductive effects of the xenobiotics that bind the AhR are caused by the inappropriate modulation of AhR-regulated genes. In order to determine the relationship between modulated molecular events and adverse tissue responses, it is important to identify the most sensitive target tissues/cells and at what stages during development they are vulnerable.

The AhR-AhR nuclear translocator (ARNT) transcription factor complex interacts with DNA sequences known as dioxin responsive elements (DREs), which regulate transactivation of dioxin responsive genes (Hankinson, 1995). AhR and ARNT have been identified in fetal mouse tissues between gestational day (GD) 10 and 16 (Abbott and Probst, 1995; Abbott *et al.*, 1995). AhR messenger RNA (mRNA) was also detected at the eight-cell stage in the murine preimplantation embryo (Peters and Wiley, 1995). The expression of the gene regulatory proteins AhR and ARNT in a developmental stage- and tissue-specific manner suggests that these proteins have a functional purpose.

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As a means to identify potential tissue- and cell-specific targets of both AhR functionality and TCDD toxicity, we utilized a transgenic (TG) mouse model. This model takes advantage of the DRE to activate a β-galactosidase (β-gal) reporter gene (*lacZ*) driven by a transcriptionally active AhR. The DRE-lacZ construct has been successfully incorporated into the mouse genome and determined to be responsive to TCDD and other AhR agonists *in vivo* (Willey *et al.*, 1998; Bemis *et al.*, 2005). Using this model, the spatial and temporal expression of transcriptionally active AhR during development can be determined. Activation of the transgene would also indicate that the necessary constituents for the AhR signaling pathway are present in these tissues.

Work previously published on this DRE-lacZ mouse model demonstrated TCDD-induced transcriptionally active AhR in several developing tissues including the genital tubercle, palate, and paws (Willey *et al.*, 1998). The finding that the paw exhibited a high level of TCDD-induced β-gal activity was surprising since there have been no publications noting any gross or functional alterations to the limbs in exposed animals or humans. Some deformities, altered pigmentation of nails, and stiffness or callosity of palms and soles were reported in children exposed *in utero* to polychlorinated biphenyls and polychlorinated dibenzofurans (Hsu *et al.*, 1994; Lu and Wu, 1985; Masuda, 1994). Hyperpigmentation and deformation of the nails also have been reported in nonhuman primates (Allen *et al.*, 1977) but there are no reports of significant alterations elicited by TCDD or other dioxin-like compounds to the limbs of mice or other rodents. Several teratogens targeting human limb development are documented (Klaassen, 2001). Thalidomide is a historical reminder of the sensitivity of limb development to teratogens. Retinoic acid causes limb defects in a developmental stage–dependent manner. Both limb teratogens share a common theme, i.e., the developmental stage of exposure plays a significant role in susceptibility.

Given that several environmental contaminants, such as the teratogens indicated above, as well as TCDD, exploit sensitive developmental stages, our continued investigations had several goals. We sought to determine, using our DRE-lacZ model, the temporal and spatial response of the AhR to TCDD during the critical period in which distal limb development occurs. Another goal was to determine if gross or histopathological indicators of paw development were affected by TCDD exposure *in utero*. Finally, we examined TCDD-dependent alterations of gene expression profiles in the developing paw that resulted from activation of the AhR signaling pathway. Results from these investigations indicate that while the developing paw is certainly a direct target of TCDD, via the AhR, to alter gene expression profiles, these molecular alterations did not appear to result in any significant morphological changes in paw development. However, these studies also suggest that mesenchymal cells may be a primary site of TCDD-modulated AhR activity. This particular finding may have significant relevance for the possible disruption of critical mesenchymal-epithelial interactions in several developing tissues that have been shown to be targets of TCDD-elicited toxicity.

MATERIALS AND METHODS

Generation and screening of TG animals. The generation of this TG line has been described previously (Willey *et al.*, 1998). Briefly, the *Sal*I-*Not*I fragment from the p2Dlac construct containing two DREs, a minimal ovalbumin TATA box promoter and the bacterial *lacZ* gene, was microinjected into fertilized C57Bl/6 × SJL F_2 hybrid eggs by DNX, Inc. (Princeton, NJ). The construct has been determined to contain only DRE and TATA box interaction sequences following a search of Genbank and Swiss-Prot for known binding elements (Willey *et al.*, 1998). The founder lines were identified by PCR amplification of *lacZ* from DNA in tail snips.

DNA isolated from fetal tail snips was digested in a lysis solution (0.5M ethylenediaminetetraacetic acid [EDTA], 1% sodium dodecyl sulfate [SDS], 50mM Tris-HCl [pH 7.4], 100mM NaCl) supplemented with 0.6 mg/ml Proteinase K for 2.5 h at 55°C. Protein was precipitated by the addition of 100 ml of 5M potassium acetate, vortexed, and incubated on ice for 6 min. After centrifugation, DNA was precipitated with 300 μl of isopropanol. The DNA pellet was washed with 70% ethanol and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0). The PCR reaction consisted of 1× PCR buffer (10mM Tris-HCl, pH 9, 50mM KCl, 0.1% Triton X-100), 2mM $MgCl_2$, 0.2mM of each deoxy-nucleotide triphosphate, 25 pmol of each *lacZ*-specific primer (forward: 5′-CTTAATCGCCTTGCAGCACATCC-3′, reverse, 5′-CTTCCAGATAACTGCCGTCACTCC-3′) (Buzy *et al.*, 1995), 1 U of *Taq* polymerase, template DNA (1 μg), and filtered, deionized water to a total volume of 50 ml. The PCR thermoprofile consisted of a denaturing temperature of 94°C for 1 min, an annealing temperature of 65°C for 45 s, and an extension temperature of 72°C for 2 min, for 40 cycles followed by a final extension period of 5 min at 72°C. PCR product was electrophoresed on an agarose gel and visualized using ethidium bromide.

Animals and chemical treatment. Reporter gene-positive animals were bred as heterozygote (+/−) matings to generate timed-pregnant females with litters consisting of both TG-positive (+/− and +/+) offspring and transgene-negative (−/−) littermate controls. Mice were given food and water *ad libitum* and housing was provided in accordance with standards used in the Vivarium, an accredited animal facility at the University of Rochester.

Timed-pregnant animals were generated by overnight pairings, followed by removal of the male and examination for a sperm plug. The first day after pairing was termed GD 0. The GD designations in the text, e.g., GD 14.5, indicate the GD at the time of treatment. Pregnant dams were treated with a single dose of TCDD or olive oil (vehicle) i.p. 24 h prior to embryo collection. The highest dosage of TCDD used (30 μg/kg) has been shown to produce maximal induction of AhR activity in C57BL/6J mice (Poland and Glover, 1980) and strong transgene activation in the DRE-lacZ mouse model (Bemis *et al.*, 2005; Willey *et al.*, 1998).

Analysis of DRE-dependent β-gal in fetuses. Fetuses were washed in cold phosphate-buffered saline (PBS) and fixed in a solution of PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde for 2 h, followed by rinsing and incubation in X-gal staining solution (1 mg/ml X-gal, 5mM potassium ferrocynide, 5mM potassium ferricynide, 2mM $MgCl_2$, and 0.02% NP-40) for 12 h at 35°C. Whole fetuses were washed and transferred to 70% ethanol for image acquisition and storage. Fetuses older than GD 13 were sectioned after fixation to facilitate access of the staining solution to internal organs. Paws from fetuses that were stained *in situ* with X-gal were sectioned for histologic examination. X-gal precipitate is soluble in xylene, thus xylene use was minimized. Paws were dehydrated with 95% ethanol for 20 min, twice, then 100% ethanol for 20 min, twice, xylene for 6 min, twice, and embedded in paraffin for 20 min. Five micron sections were rehydrated

through a series of xylene and alcohol dilutions and counterstained with eosin.

To determine β-gal by a luminescent assay, paws from fetuses exposed to TCDD or vehicle control were washed in PBS and incubated in a red blood cells lysis solution (0.17M ammonium chloride, 10mM potassium bicarbonate, and 1mM EDTA) for 5 min to improve assay sensitivity (Nazarenko *et al.*, 2001a). Afterward, paws were washed with PBS and placed in a lysis solution (100mM potassium phosphate and 0.2% Triton-X 100, supplemented with 1mM dithiothreitol [DTT], 0.2mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin) for 5 min on ice. The paws were then homogenized between two frosted glass slides. The homogenate was transferred to a microcentrifuge tube and centrifuged for 2 min at 12,000 × g. The supernatant was heated at 48°C for 60 min to inactivate mammalian (endogenous) β-gal activity and stored at − 20°C. The protocol from the Galacto-Light plus kit (Tropix, Bedford, MA) was used to detect luminescence.

Gel shift assay. Paws were cut into small pieces and placed in a microcentrifuge tube, spun down briefly and the supernate was decanted. Whole cell extracts were harvested as previously described (Henry *et al.*, 1994). Briefly, three to five tissue volumes of HEDG buffer (25mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1.5mM EDTA, 1mM DTT, 10% [v/v] glycerol, pH 7.6) containing PMSF and leupeptin were added to the pellet. The tissue was resuspended in buffer, allowed to swell by incubation on ice for 10 min, and homogenized by 10 stokes with a tissue grinder. The salt concentration was adjusted to 0.4M NaCl, and samples were extracted on ice for 45 min. The suspension was then centrifuged at 20,000 × g for 20 min and the supernate stored at − 70°C. A 25-μl aliquot of sample was incubated with ^{32}P-DRE, complexes resolved on a nondenaturing polyacrylamide gel and quantified by densitometry on a phosphorimager analyzer.

RNA isolation for microarray analysis. Timed-pregnant females were dosed with either TCDD or olive oil as described above and the fetuses were harvested 24 h later. Two fetuses each were collected from each of the two positions along the lateral regions of the two uterine horns and two fetuses directly adjacent to the cervix. Thus, six fetuses were collected per litter. Paws were removed, pooled between the paired fetuses, and maintained as separate tissue samples according to both uterine (cervical or either lateral positions) and paw position (fore or hind) throughout the remaining procedures. For RNA extraction, paws were homogenized mechanically in Trizol reagent (Gibco, Gaithersburg, MD) and processed as described by the manufacturer. RNA was resuspended in 20 μl of diethylpyrocarbonate treated water.

Microarray analysis. RNA samples were delivered to the Functional Genomics Center at the University of Rochester. Samples were labeled and hybridized onto MU74Av2 (Affymetrix, Santa Clara, CA) GeneChip arrays and scanned.

Data analysis. Normalized intensity data were analyzed on GeneSpring v5.0 (Silicon Genetic, Palo Alto, CA). Normalized signal intensity ratios were compared between vehicle control and TCDD-exposed samples in fore paws and hind paws from lateral and cervical uterine positions. The ''draw gene'' command coupled with the ''find similar'' command, using a correlation factor of 0.95, were used to compile a list of genes. These genes were placed in biologically functional categories based on information provided by Netaffx (Affymetrix) and published literature. All microarray data were normalized using Robust Multichip Analysis (RMA) using the untreated control as a baseline. All biological replicates were statistically analyzed using a two class unpaired comparison using variance stabilization. A false discovery rate was calculated using a Benjamini Hochberg correction, and all analysis described were performed in Iobion GeneTraffic Software. Gene lists of significant genes were annotated using both public and commercial databases prior to cluster analysis. Genes demonstrating a change of $<$ or $>$ 1.5-fold with a p value of < 0.05 were considered as being significantly altered.

Real-time reverse transcription–PCR. Validation of the responses of selected genes was performed at the Functional Genomics Center using reverse transcription–PCR (RT-PCR) using the assay-on-demand (AOD) system (Applied Biosystems, Foster City, CA). Primer/probe sets were designed using Primer Express (Applied Biosystems). All samples were run in triplicate to determine reaction to reaction variability, and all experiments were repeated a total of three times. This resulted in nine data points on each sample for each gene. Differential gene expression was measured using the delta Ct method and was performed within the SDS v2.3 software. The standard deviation (SD) was calculated for all replicates, and statistical significance of fold change differences were calculated by *t*-tests for all comparisons.

Evaluation of paw morphology. Paw morphology was evaluated at postnatal day (PND) 0 and PND 2 following GD 15.5 treatment with 30 or 3 μg/kg TCDD, respectively. Results were compared to fetuses from olive oil (vehicle)–treated dams. Evaluation was performed without knowledge of experimental treatment but with identification of age and sex. Hind and fore paws from at least five fetuses were examined separately. Paws were fixed with 10% neutral-buffered formalin paraffin embedded, sectioned to 5 μm, and stained with hematoxylin and eosin. Images of sections were captured using Image Pro Plus (v4.0) for morphometric data collection.

RESULTS

Reporter Gene Activation in the Paws

Previous studies indicated that fetuses exposed to TCDD (30 μg/kg) *in utero* demonstrated X-gal staining in developing paws (Willey *et al.*, 1998). In the mouse, limb buds develop and become prominent between GD 9–12. Much of the development of the distal limb, including paws and digits, occurs between GD 13 and 18 (Kaufman and Bard, 1999). No significant X-gal staining was detected in the developing limbs when TCDD exposures occurred prior to GD 13 (not shown). A time-course determining the spatial and temporal staining of X-gal in response to TCDD was conducted between GD 13.5 and 17.5. In the hind and fore paws, thin lines of staining were observed along the edges of the digits when dams were treated on GD 13.5 and 14.5 (Fig. 1A). For treatments on GD 14.5 and 15.5, the staining was more intense in the interdigital region at the base of the digits, and a circular pattern of staining was observed on the foot pads (Fig. 1B). A similar pattern of staining, albeit less intense, was observed on GD 15.5 in litters exposed to TCDD concentrations as low as 15 and 1 μg/kg *in utero* (data not shown). A more diffuse staining, primarily at the base of the digits and between the digits, was observed when treatment occurred on GD 16.5 (Fig. 1C) for fetuses of dams exposed to 30 μg/kg. Only minimal staining was evident in the footpad for exposures on GD 17.5 (Fig. 1D). Reporter gene activity in paws was not observed in vehicle-treated animals (Fig. 1B, inset) or in transgene-negative littermates (data not shown). The circular pattern observed in the paws of TCDD-exposed fetuses appears to involve primarily subdermal mesenchymal cells that may be involved in footpad development (Fig. 2B). Significant staining in the epithelial cells was not apparent. Histological evaluation also showed staining in the interdigital mesenchyme (Fig. 2A). Separate biochemical assays were employed to assess transcriptionally active AhR and DRE-binding potential in paws from fetuses exposed to TCDD *in utero*. Increased β-gal activity resulting from

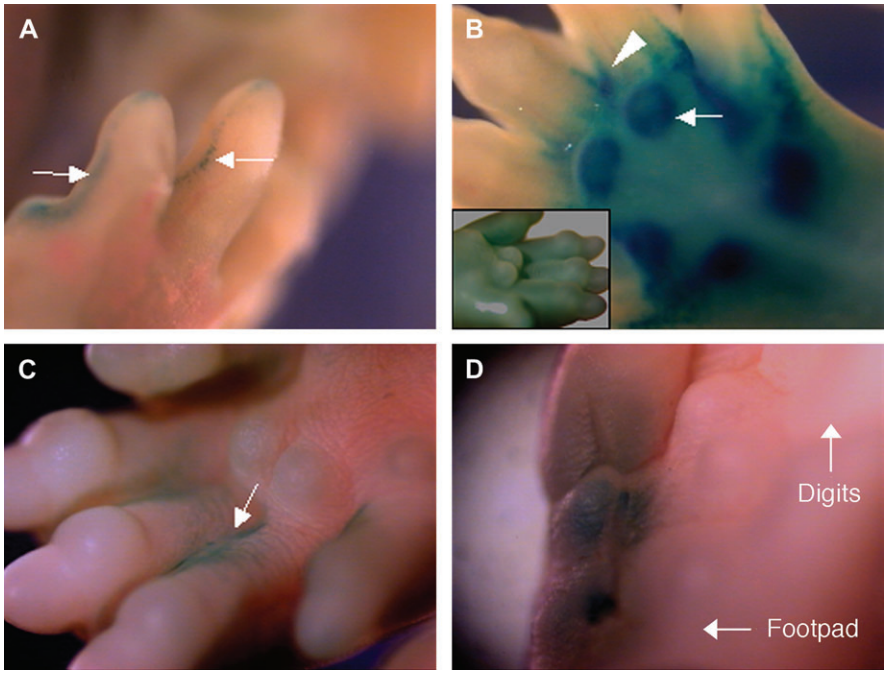

FIG. 1. Determination of transgene activation *in situ*: the paw. Blue stain indicates transgene activation and the presence of active AhR/ARNT complex. All tissues except the inset in "B," are from TCDD-treated, transgene-positive animals. Images are ×40. (A) A thin line of staining is observed along the edges of the digits (arrows) from fetuses treated at GD 13.5–14.5 and examined 24 h later. (B) Following treatments at GD 14.5–15.5, a circular pattern of staining is observed in both the fore and hind paw, perhaps associated with the developing footpad (arrow). Staining is also noted in the interdigital space (arrow head). (B, inset) Vehicle-treated GD 14.5 paw. (C) Following treatment on GD 16.5, a more diffuse pattern is observed, with staining between the digits and at the base of the digits (arrow). (D) Only minimal staining is observed in paws from fetuses treated at GD 17.5. For each treatment GD, paws from a minimum of five fetuses were examined. Representative results are shown.

transcriptionally active AhR was measured by luminescence and was increased in paw homogenates from fetuses exposed to TCDD relative to control counterparts (Fig. 3A). The DRE-binding potential of the AhR-ARNT complex in paws was assessed by electrophoretic mobility shift assay. Consistent with increased reporter gene activity, DRE binding was increased in paw homogenates from fetuses exposed to TCDD relative to controls (Fig. 3B).

Morphology in the Paw

The above data indicate that β-gal activity was maximally induced in the developing paw within the exposure period between GD 14.5 and 16.5 (i.e., at 24 h following treatment times of 13.5 and 15.5). Paw morphology was evaluated at GD 18 and PND 0 in fetuses from dams exposed to 30 μg TCDD/kg at GD 15.5. TCDD at dosages of 5 μg/kg or greater has been shown to inhibit lactation resulting in poor pup survival following birth (Vorderstrasse *et al.*, 2004). As such, paw morphology was also evaluated at PND 2 in fetuses from dams exposed to 3 μg TCDD/kg. Evaluation of paw morphology from GD 18 or PND 0 pups from dams exposed to TCDD (30 μg/kg) or vehicle illustrated no gross or histopathological differences between exposed and unexposed pups (data not shown). In particular, no change in epithelial thickness was observed. PND 2 fetuses, exposed to TCDD (3 μg/kg) *in utero*, also showed no alterations in paw morphology (data not shown).

Gene Expression Profiles in the Paw

Given that TCDD-induced transcriptionally active AhR, as determined by β-gal activity, in the paws maximally when treatments occurred between GD 14.5 and GD 15.5, alterations in gene expression associated with this time point were examined. In response to TCDD given at GD 14.5, numerous genes were induced and relatively fewer genes were reduced. These genes were placed in categories based on known or suspected biological function (Table 1). Notably, results were found to be very consistent when data generated from fore or hind paws or from fetuses at different uterine positions were compared. Since there was no basis for indicating that any differences observed were significant or biologically meaningful, the data from all determinations based on paw or uterine position were pooled to make up the data presented in Table 1. Genes of interest selected based on their role in regulating skin, vascular, and immune systems were validated using real-time RT-PCR (Tables 2 and 3). Microarray data for all the MU74Av2 GeneChips used in the experiments discussed in these studies were placed in a public repository, National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

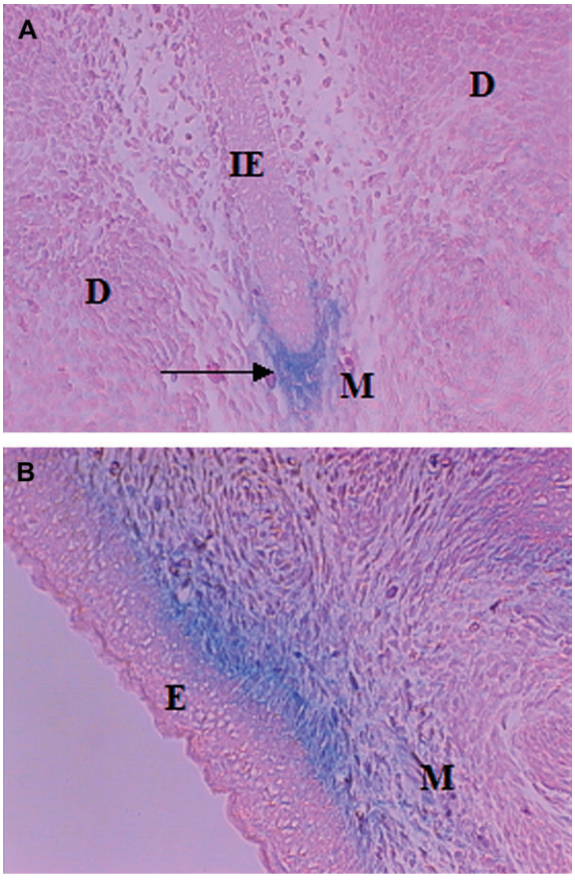

FIG. 2. Histologic evaluation of the paw. (A) Staining in the interdigital mesenchyme of the GD 15.5 treatment paw (arrow). (B) Transgene activation in the subdermal layers of the developing palm of a GD 15.5–treated mouse (A = ×100, B = ×200) IE = interdigital epithelium, D = digit, M = mesenchyme, E = epithelium). Representative sections of paws from a minimum of five separate fetuses are shown.

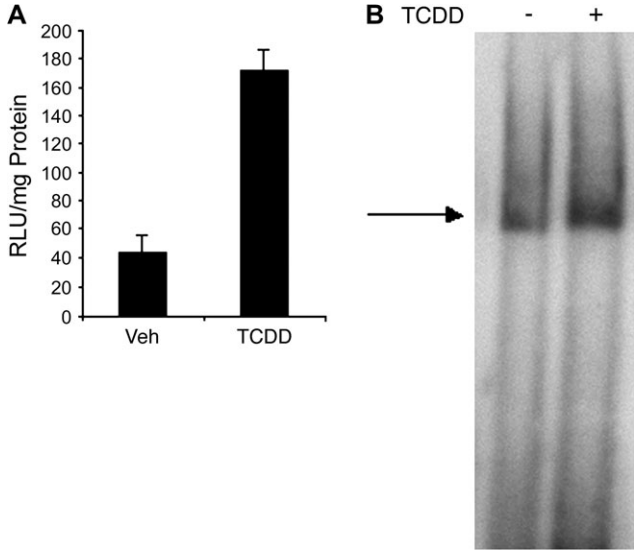

FIG. 3. Confirmation of transgene induction in the paw. (A) Transgene induction of about four-fold is detected in TCDD-exposed paws as compared to vehicle treated at GD 15. Tissue lysates were generated from the tissues and evaluated using the luminescent β-gal assay. Results are as mean ± SD of triplicate analyses. (B) Gel shift analysis using 32P-DRE indicates the presence of a DNA binding form of the AhR in the TCDD-treated paw (arrow). Representative data from two separate experiments are shown.

DISCUSSION

AhR mRNA and protein have been identified in developing mouse embryos, specifically in the palate, genital tubercle, adrenals, liver, thymus, heart, skin, and neuroepithelium (Abbott *et al.*, 1995; Jain *et al.*, 1998). Interestingly, these levels decreased in most of these tissues during late organogenesis, suggesting a transitory role during development. ARNT mRNA and protein were colocalized in palate, liver, heart, and neuroepithelium. However, noncoordinate expression between AhR and ARNT was identified in the adrenal, skeletal muscle, hindbrain, and the choroid plexus of the first and fourth ventricles (Abbott and Probst, 1995). Thus, the presence of AhR or ARNT expression alone does not necessarily indicate that the AhR signaling pathway will be active in the presence of AhR agonists. Detection of high levels of transcriptionally active AhR in the paws between GD 14 and 16 is of particular interest because paws have not been previously identified as a major target tissue of TCDD-induced toxicity in experimental animals.

In the footpad and plantar region, TCDD-induced transcriptionally active AhR, as detected by LacZ activity, was primarily found at a stage when epithelial keratinization is thought to occur. The sensitivity of these epithelial cells to teratogens has prompted investigators to use changes in footpad patterning as a marker for teratogenic exposure (Mori *et al.*, 2000; Shimokuni *et al.*, 1995). At the molecular level, the greater than five-fold increased expression of those genes encoding proteins involved in skin differentiation (Table 1), e.g., small proline-rich protein (Sprr), several keratin proteins (Krt), and the skin cell terminal differentiation markers loricrin (Lor) and involucrin (Ivl) (Presland and Dale, 2000), suggest that the developing skin epithelium is affected by TCDD exposure. However, as indicated by histologic evaluation, transcriptionally active AhR was localized primarily in the mesenchymal cells, and significant X-gal staining in epithelial cells of the paws was not observed (Fig. 2). Both AhR and ARNT have been identified in epithelial cells (FitzGerald *et al.*, 1996) and these cells are suggested as direct targets for AhR agonists evidenced by the occurrence of chloracne and other ectodermal dysplasias in exposed adult humans and some animal species. However, considering the vital role of epithelial-mesenchymal interactions in skin cell differentiation, migration, and tissue development (Maas-Szabowski *et al.*, 2000), it is possible that mesenchymal-directed disruption in these interactions may alter normal patterns of epithelial proliferation and differentiation. Notably, the edges of the digits, mesenchymal cells in the interdigital regions, and those mesenchymal cells in the foot pads where induced β-gal

TABLE 1
TCDD-Mediated Alterations in Mouse Paw mRNA

Gene name	Accession number	Fold change	*p* value (RMA analysis)
	Xenobiotic metabolism genes		
Cyp1a1	K02588	28	0.02
Cyp1b1	X78445	22	0.001
Gpx3	U13705	5.3	0.01
Ugt1a6	U16818	3.2	0.02
Sult2b1	AF026072	2.8	0.02
Adh3	U20257	1.9	0.05
	Developmental genes		
Psca		11.5	0.03
Pp11r	M95545	4.3	0.04
Il1f5	AJ250429	4.8	0.05
Il1r2		2.1	0.05
Wnt4		1.9	0.05
Hba-X	M13125	−3	0.03
Sox11		−2	0.05
Hoxd13	X99291	−5	0.04
	Skin differentiation genes		
Sprr1b	X91825	21	0.02
Krt2-6a	K02108	10.5	0.01
Lgals7	AF038562	10.5	0.01
Krt2-6c		8	0.05
Plxnb2		8.7	0.04
Lor	U09189	6.3	0.04
Ivl		6.7	0.03
Sprr1a	AF057156	5.3	0.04
Krt1-13	X03492	3.8	0.03
Krt1-19	M36120	3.8	0.03
Krt1-17	M13805	4	0.03
Krt2-16	X65505	2.1	0.04
Krtap13	AF031485	2.9	0.04
Krt2-8		2.4	0.03
Scel		2.5	0.05
	Cell cycle genes		
Cdkn1a (p21)		3.4	0.05
Ndr1	U60593	2.5	0.05
Nmyc1	M12731	−2	0.05
	Cell proliferation genes		
S100a6	X66449	4.3	0.04
Fos	V00727	2.8	0.05
Tcfap2c	X94694	2.1	0.05
	Apoptosis genes		
Casp14	AF092997	4	0.04
Anxa1	M69260	3.8	0.03
Perp (Pmp22)		2.4	0.05
Bcl6		1.9	0.05
	Differentiation genes		
Mal (T cell)	Y07812	7	0.02
Crabp2	M35523	−2.3	0.04
Rbp1	X60367	−2	0.05
	Ion channel/transporter genes		
Clic3		3.4	0.04
Kcnj15		5.7	0.03
Slc2a1		2	0.05
Lgals3		4	0.03

TABLE 1—*Continued*

Gene name	Accession number	Fold change	*p* value (RMA analysis)
	Immune system/defense genes		
Ly6d	X63782	6	0.03
Ly6g6c		8.7	0.01
Ly57		2.8	0.04
Pp1	AF013715	2.7	0.05
F3	M26071	2.3	0.05
Mab21l2		2.6	0.05
	Signal transduction/transcription factor/coactivator genes		
Map3k6	AB021861	4	0.03
Cebpb	M61007	3.4	0.04
Calm4		14.5	0.02
Ahrr	AB015140	4.5	0.03
Klf3		2.3	0.05
Bhlhb2		2.5	0.05
Klf13		2.1	0.05
Hipk2		2	0.05
Elf5	AF0497702	3	0.04
Itpr5		2.9	0.05
Ell2		1.8	0.05
Acp5		2.3	0.05
	Protease/inhibitor genes		
Serpinb2	X16490	6.7	0.01
Spi12		3	0.02
Spint1		2.4	0.04
Klk8	D30785	2.3	0.05
Serpinb5	U54705	13	0.001
Pcsk6		2.1	0.05
	Stress response genes		
Orm1	M27008	20	0.04
Selenbp1		4.3	0.02
Hsp70-3	M12571	2.8	0.04
Hsp70-1	AF109906	2.1	0.001
Fkbp5	U16959	1.9	0.02
Glrx1	AB013137	1.8	0.04
	Endogenous metabolism genes		
Alox12b	Y14334	21	0.01
Fac12	U15977	3.2	0.03
Ptgs1		2.3	0.03
Ech1		1.6	0.05
Hsd11b1		3.2	0.04
Obph1		1.6	0.03
Hdc	X57437	2.8	0.04
Hk2		1.6	0.03
Ch25h		−2.3	0.04
	Cell-cell/matrix interaction genes		
Degs		2.1	0.05
Plec1		2.1	0.05
Capg		1.9	0.05
Cldn1	AF072127	3.5	0.02
Jup	M90365	2.1	0.05
Dsc1	X97986	3	0.04
Pkp3		2.5	0.05
Pkp1	Y07941	2.8	0.05
Map17		3.2	0.04
Gsn	J04953	2.1	0.05

TABLE 2
Probe and Primer Sequences for Quantitative Real-Time Polymerase Chain Reaction Validation

Genes of interest	Genbank accession #	AOD number
Fgfbp1	AF065441	Mm00456064_s1
P450-1	K02588	Mm00487218_m1
Ahrr	AB015140	Mm00477445_m1
Nmyc-1	M12731	Mm00476449_m1
Klf-3		Mm00492957_g1
Anxa1	M69260	Mm00440225_m1
Cdkn1a(p21)		Mm00432448_m1
Map3k6	AB021861	Mm00522244_g1
Krt2-6a(KER)	K02108	Mm00833464_g1

activity was observed in the developing paws are also sites undergoing considerable cell differentiation and programmed cell death (Fernandez-Teran *et al.*, 2006; Ganan *et al.*, 1996). Whether the coincidence of these patterns may be related to the demonstrated, but undefined, role of the AhR in the regulation of cell cycle events (Huang and Elferink, 2005) requires additional work to determine.

Reciprocal signaling, originating in the epithelial cells may also impact the underlying mesenchymal cell layers. Some cytokines involved in mediating these interactions include interleukin (IL)-1, IL-6, IL-8, transforming growth factor (TGF)β, (bone morphogenic protein), platelet-derived growth factor, and the fibroblast growth factor family (Bei and Maas, 1998; Schroder, 1995). A large number of AP-1 (fos/jun) target genes in the epidermis are suggested to play a significant role in the interactions between mesenchyme and epithelium (Angel and Szabowski, 2002). Interestingly, many of the effects of TCDD are similar to those of epidermal growth factor and TGF, i.e., early tooth eruption, changes in keratinocyte differentiation, and hepatocyte proliferation (Sewall *et al.*, 1993). Furthermore, several studies indicate that altered mesenchymal-epithelial interactions contribute to TCDD-induced abnormalities in keratinocyte differentiation (Loertscher *et al.*, 2001), as well as in the developing palate (Abbott and Birnbaum, 1990), molar teeth (Lukinmaa *et al.*, 2001), and prostate (Ko *et al.*, 2004). In particular, the data by Ko *et al.* (2004) indicated that AhR present in the mouse urogenital sinus mesenchyme mediates the inhibition of prostate epithelial bud formation by TCDD. Together these data suggest that mesenchymal cells may be primary sites of action for TCDD via the AhR.

Other genes altered in developing paws following TCDD treatment are worthy of discussion. Several genes involved in xenobiotic metabolism, such as Cyp1A1, Cyp1B1, Ugt, Sult, Adh, and Gpx3 were strongly upregulated following TCDD exposure (Table 1). In addition, the gene for the aryl hydrocarbon receptor repressor (Ahrr) was strongly induced. These genes have DREs in their upstream promoter regions and serve as an *ad hoc* validation of the exposure of the developing mouse paw to TCDD. The gene for prostate stem cell antigen (Psca) was upregulated 11.5-fold (Table 1). This protein is suspected to play a role in prostate cancer (Tricoli *et al.*, 2004), and TCDD is known to have effects on prostate development (Roman *et al.*, 1998). However, no DRE was found in the 5′ regulatory region of Psca. Of the other genes that were very highly upregulated ($\geq$ 13-fold induction), only serine peptidase inhibitor clade B member 5 (serpinb5) and arachidonate 12-lipoxygenase (Alox12b) were found to have potential DREs located in their promoter regions (analyses done using Genomatix Gene2Promotor/MatInspector Software and AhR-based Position-Weighted Matrices). Notably, 12-lipoxygenase is suspected to be a key regulator for the development of human prostate cancer as well as for cancers of the breast, kidney, and pancreas (Gao *et al.*, 1995; Nie *et al.*, 2006; Yoshimura *et al.*, 2004). Furthermore, increased expression of 12-lipoxygenase has been shown to promote angiogenesis (Nie *et al.*, 2006), and this is of particular interest given recent data suggesting a role of the AhR in regulation of vascular development (Walisser *et al.*, 2004). No putative DRE sites were identified on Calmodulin 4 (Calm4), orosomucoid 1 (Orm1) and Sprr1b.

Several factors may influence the results and interpretation from these microarray studies. First, paws were pooled from

TABLE 3
Gene Expression Data Summary for Validated Genes

			Microarray data		QRT-PCR	
Gene of interest	Affy. probe ID	Genbank accession #	Fold change	*p* value	Fold change	*p* value
Fgfbp1	103995_at	AF065441	2.5	0.06	3.2	0.01
P450-1	94715_at	K02588	28	0.02	14.5	0.04
Ahrr	101709_at	AB015140	4.5	0.03	2.6	0.02
Nmyc-1	103048_at	M12731	−2	0.05	−4.3	0.01
Klf-3	100011_at		2.3	0.05	6.7	0.001
Anxa1	161703_f_at	M69260	3.8	0.03	1.8	0.01
Cdkn1a(p21)	94881_at		3.4	0.05	4.5	0.02
Map3k6	92276_at	AB021861	4	0.03	6.7	0.05
Krt2-6a(KER)	104370_s_at	K02108	10.5	0.01	15.4	0.03

two fetuses, thus each fetus was not examined individually. In addition, the sex of fetuses was not determined, hence gene expression may be representative of two males, two females, or both male and female fetuses. Evidence suggests that TCDD and other members of the dioxin class of compounds can produce their deleterious affects by disrupting endogenous sex-driven hormone systems. Secondly, homogenates of paws were examined and not individual cells. Multiple tissue types in the paw are developing simultaneously, thus the response of complex tissues, as opposed to individual cells, to TCDD was measured. It would be important to examine the spatial and temporal expression of these genes and their protein products. In addition, fetal tissues were not excised until 24 h following treatment of the dam with TCDD. It is possible, indeed likely, that some of these elicited changes may be secondary or tertiary events subsequent to the alteration of genes that are directly AhR responsive. Finally, litter size and uterine position of the fetuses may influence the toxicokinetics of TCDD. However, analysis, albeit qualitative, of the genes modulated by TCDD exposure indicated no striking differences that could logically be attributed to either paw or uterine position.

Previous investigations utilizing this TG model were the first to show a direct transcriptional effect of TCDD in the limbs along with other tissues during development (Willey *et al.*, 1998). TCDD-induced LacZ activity was blocked by the AhR antagonist 3′-methoxy-4′-nitroflavone in both adult and embryonic tissues supporting the AhR dependence of the reporter system (Nazarenko *et al.*, 2001b). Despite the profound increase in reporter gene activity and the numerous and substantially altered gene expression patterns observed in the paw, this study, along with others (Birnbaum, 1998), did not find that TCDD exposure induced obvious limb defects, independent of exposure period. There may be several reasons for this. During this period, rapid changes in cell death, differentiation, and proliferation patterns are taking place. As such, rapid responses to molecular insults may trigger compensatory mechanisms so that morphologic changes, that are quickly repaired, may be difficult to temporally define. In this respect, within some cell/tissue types, the AhR signaling pathway may also be protective, inducing the appropriate mechanisms to balance the response. Notably, while genes associated with skin differentiation were induced (Table 1), several genes, e.g., Casp14 and Anxa1, associated with cell death pathways (Casp14 and Anx1) and stress response (Orm1 and Selenbp1) were also increased. On the other hand, and despite this lack of a gross morphological effect, it is possible that TCDD may influence other, more complex, processes in paw development such as nerve formation/conduction and subtle functional aspects of the paw and footpad that cannot be easily detected by gross morphologic or even histologic examination. This would need further evaluation. From another perspective, the regio-, cellular-, and developmental period-specificity of the AhR activation observed might suggest a normal role of the AhR-Arnt complex in paw/digit development.

Taken together, these observations demonstrate that the developing mouse paw is a target of TCDD in terms of being able to have significant effects on the AhR signaling pathway. Furthermore, the prevalence of TCDD-induced AhR activity in mesenchymal cells suggests a cellular selectivity that, although may not significantly disrupt paw development, at least in the mouse, may play an important role in other developing tissues to cause alterations in TCDD-sensitive–dependent pathways and/or an endogenous function of the AhR during development resulting in toxicity. Identifying AhR-mediated disruptions in the balance of regulatory processes determining cell proliferation, differentiation or apoptosis, and specifically, how these affect epithelial-mesenchymal interactions, may significantly contribute to the current knowledge of the ability of TCDD to produce toxicity as well as how the AhR may normally function.

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