Dioxin-Dependent and Dioxin-Independent Gene Batteries: Comparison of Liver and Kidney in AHR-Null Mice

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The aryl hydrocarbon receptor (AHR) is a widely expressed ligand-dependent transcription factor that mediates cellular responses to dioxins and other planar aromatic hydrocarbons. Ahr-null mice are refractory to the toxic effects of dioxin exposure. Although some mechanistic aspects of AHR activity are well understood, the tissue specificity of AHR effects remains unclear, both during development and following administration of exogenous ligands. To address the latter issue, we defined and compared transcriptional responses to dioxin exposure in the liver and kidney of wild-type and Ahr-null adult C57BL/6J mice treated with either 2,3,7,8-tetrachlorodibenzo-p-dioxin or corn-oil vehicle. In both tissues, essentially all effects of dioxin on hepatic mRNA levels were mediated by the AHR. Although 297 genes were altered by dioxin exposure in the liver, only 17 were changed in the kidney, including a number of well-established AHR target genes. Ahr genotype had a large effect in both tissues, profoundly remodeling both the renal and hepatic transcriptomes. Surprisingly, a large number of genes were affected by Ahr genotype in both tissues, suggesting the presence of a basal AHR gene battery. Alterations of the renal transcriptome in Ahr-null animals were associated with perturbation of specific functional pathways and enrichment of specific DNA motifs. Our results demonstrate the importance of intertissue comparisons, highlight the basal role of the AHR in liver and kidney, and support a role in development or normal physiology.

Key Words: aryl hydrocarbon receptor; dioxin; TCDD; microarray; kidney; liver.

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor, widely expressed in vertebrate tissues, that mediates cellular responses to dioxins and related planar aromatic hydrocarbons (Okey, 2007). The AHR resides quiescent in a cytoplasmic complex containing HSP90 and several other components (Hankinson, 1995). Upon ligand-binding the AHR translocates into the nucleus where it exists as a dimer with the AHR nuclear translocator (ARNT, also known as HIF1B) (Hankinson, 2005; Matsushita et al., 1993). The AHR:ARNT:ligand complex associates directly with DNA in a sequence-specific fashion, modulating the expression of target genes through the recruitment or interference of other regulatory proteins, such as transcription factors (Kobayashi et al. 1996; Ohtake et al. 2003; Sogawa et al. 2004), coactivators (Beischlag et al., 2002; Nguyen et al., 1999), and components of the basal transcriptional machinery (Rowlands et al., 1996).

The transcriptional effects of AHR activation are of profound importance in several biological systems. Mice whose Ahr locus has been genetically ablated are viable, but suffer several developmental defects (Schmidt et al., 1996; Walisser et al., 2004). Further, they are essentially immune to most toxicities induced by AHR ligands, and in particular the prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Lin et al., 2008; Schmidt et al., 1996; Walisser et al., 2004). AHR activity has recently been shown to be essential for the development of regulatory T cells (Quintana et al., 2008; Veldhoen et al., 2008).

Several gaps prevent us from linking our mechanistic knowledge of AHR function to the many and varied phenotypic consequences of AHR activity. One of these is a limited understanding of the tissue specificity of AHR target genes, both during development and following stimulation by exogenous ligands. Although the transcriptional effects of AHR activity in liver are relatively well characterized (Boverhof et al., 2006; Fletcher et al., 2005; Franc et al., 2008; Frueh et al., 2001; Moennikes et al., 2004; Puga et al., 2000), other organs have received less attention. As a result, the degree of intertissue conservation of AHR target genes, binding motifs, and functional pathways is unknown.

Although the liver is well characterized as a site of major TCDD-induced toxicities, the kidney also plays an important role. During fetal development in mice, kidney is one of the most susceptible target tissues for TCDD, displaying hydropnephrosis at doses that are too low to cause cleft palate (Couture et al., 1990). The kidney change is secondary to induced proliferation of ureteral epithelium by TCDD.
associated with an increase in EGF receptor density (Abbott and Birnbaum, 1990; Abbott et al., 1987). Overall, rats are more resistant to this impact of TCDD, but there are marked interstrain differences (Huuskonen et al., 1994).

We previously demonstrated that adult mice whose AHR locus has been genetically ablated (Ahr$^{-/-}$ mice) exhibit profoundly altered hepatic transcriptomes, even in the absence of TCDD exposure (Tijet et al., 2006). Here we describe, for the first time, the transcriptional effects of Ahr knockout on adult kidney, both in the presence and absence of TCDD treatment. Although the kidney transcriptome is mostly refractory to TCDD exposure, genetic ablation of AHR expression profoundly alters mRNA levels.

**MATERIALS AND METHODS**

2,3,7,8-Tetrachlorodibenzo-p-dioxin. TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was > 99% pure as determined by gas chromatography-mass spectrometry.

**Animals and in vivo treatment.** Male, Ahr-null (Ahr$^{-/-}$) mice 10 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Male, wild-type (Ahr$^{+/+}$) C57BL/6 mice 15 weeks of age were bred at the National Public Health Institute, Kuopio, Finland, from stock originally obtained from The Jackson Laboratory. Mice were handled as described previously (Tijet et al., 2006). Briefly, a single dose of 1000 μg/kg TCDD or corn-oil vehicle was given by gavage, and liver and kidney were harvested 19 h after treatment, sliced, snap frozen in liquid nitrogen and stored at −80 °C until homogenization. All animal study plans were approved by the Animal Experiment Committee of the University of Kuopio and the Provincial Government of Eastern Finland. Twelve wild-type Ahr$^{+/+}$ mice were used; six treated with TCDD and six treated with corn-oil vehicle. Six Ahr-null mice were used: three treated with TCDD and three treated with corn-oil vehicle.

**RNA isolation.** Total RNA was extracted using RNeasy kits (Qiagen, CA) according to the manufacturer’s instructions. DNase (Qiagen) was added to the RNeasy elution column as recommended by the manufacturer. RNA yield was quantified by UV spectrophotometry and RNA integrity was verified using an Agilent 2100 BioAnalyzer. The isolated RNA was then assayed on Affymetrix MOE430-2 arrays at The Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Canada) following standard manufacturer’s protocols. Each tissue was hybridized to a separate array, but one wild-type vehicle-treated liver sample was not run, leading to 35 total arrays.

**Preprocessing of microarray data.** Array data were loaded into the R statistical environment (v2.8.1; R Development Core Team, 2008) using the affy package (v1.20.2) of the BioConductor open-source library (Gautier et al., 2004). Array data were investigated for spatial and distributional homogeneity; all arrays were included in subsequent analyses. The liver and kidney data were separately preprocessed using the GCRMA algorithm (Wu et al., 2004), as implemented in the gcma package (v2.14.1) for the R statistical environment (v2.8.1) (R Development Core Team, Vienna, Austria). The array data were associated with updated sequence annotation using the mouse 4302m2m genetarget package (v1.11.0), which re-maps each 25-bp probe to a specific Entrez Gene ID (Dai et al., 2005). Raw and preprocessed microarray data are available in the GEO repository (accession GSE15859).

**Statistical analysis of microarray data.** Within each tissue the experiment has a two-factor, two-level design with AHR status (knockout or wild-type) and TCDD treatment (vehicle or TCDD) as the factors. Accordingly we fit a general-linear model (GLM) using the limma package (v2.16.4) in BioConductor, as previously (Tijet et al., 2006). The following linear model was fit to each ProbeSet:

\[
Y = \text{Basal} + \text{AHR} + \text{TCDD} + \text{AHR:TCDD}
\]

Here Y refers to the expression level of a single ProbeSet; Basal refers to the underlying basal expression level across all animals; AHR captures “AHR-dependent, TCDD-independent” expression changes; TCDD captures “TCDD-dependent, AHR-independent” expression changes; and AHR:TCDD captures “AHR-dependent, TCDD-dependent” expression changes. This linear model was fit separately to the preprocessed liver and the kidney data and the results subject to an empirical Bayes moderation of the standard error (Smyth, 2003), followed by a false-discovery rate correction for multiple testing (Efron and Tibshirani, 2002). The significance threshold was set at $p_{\text{adjust}} < 0.05$ for all comparisons.

**Clustering of microarray data.** Unsupervised machine-learning employed the DIANA divisive hierarchical clustering algorithm, as implemented in the cluster package (v1.11.12) of the R statistical environment (v2.8.1). Pearson’s correlation coefficient was used as the distance metric, and scaling was performed before clustering as previously (Boutros et al., 2008).

**Functional enrichment analyses.** To analyze the functional pathways associated with Ahr genotype and the AHR-dependent response to TCDD in liver and kidney, we employed Gene Ontology (GO) enrichment analysis. Six gene lists were created: the liver-specific, kidney-specific, and common genes displaying responses to either Ahr genotype or to the AHR:TCDD interaction. For each gene on this list GO terms were identified, and for each GO term statistical significance was assessed using Fisher’s Exact test, as implemented in the GOMiner tool (Zeeberg et al., 2003). All mouse GO databases available from GOMiner were used in this analysis. All three GO ontologies and all evidence codes were included. We used 1000 permutations to estimate the false-discovery rate. The resulting list of p values was log$_{10}$ transformed and genes with $p_{\text{cumulative}} < 10^{-12}$ (i.e., a sum of the log$_{10}$p values) across the six lists smaller than 12 were extracted. This matrix was subjected to unsupervised hierarchical clustering using the DIANA algorithm with Pearson’s correlation as the distance metric, as described above.

**Transcription factor binding-site analyses.** We performed a targeted analysis of three transcription factor binding-site motifs known to be associated with transcriptional regulation by the AHR: the aryl hydrocarbon response elements I and II (AHRE-I and AHRE-II) and the antioxidant response element (ARE). We employed established methods to identify these motifs in the same 6001 bp of regulatory DNA as assessed in the library-based search (Boutros et al., 2004). Evolutionary conservation was assessed using the 30-vertebrate species PhastCons alignment scores from the UCSC Genome Browser Database (Siepel and Haussler, 2004), as described previously (Moffat et al., 2007). The results were visualized using violin plots with the lattice package (v0.17-22) for the R statistical environment (v2.8.1) and default parameters.

To identify transcription factors putatively enriched in the regulatory regions of genes whose mRNA abundances were altered by TCDD exposure (AHR:TCDD) or by Ahr genotype (AHR) we employed a library-based analysis using the CLOVER motif-finder algorithm (Frith et al., 2004) and the JASPAR open-source motif library (Sandelin et al., 2004). We searched 6001 bp of regulatory sequence, centered on the transcriptional start-site and extracted from the UCSC genome assembly (build mm9) using the RefSeq annotation in the UCSC genome browser (Karolchik et al., 2003) as downloaded on 2009-03-24. CLOVER was configured to return all motifs with p values less than 0.5 and motif-scores greater than three, and three separate statistical tests were performed. First, standard dinucleotide randomization was employed. Second, a background file containing the regulatory regions of all mouse genes was used. Third, a background file containing the regulatory regions of all mouse genes contained on our microarray platform was used. Only transcription-factor binding sites (TFBS) motifs that were enriched at $p < 0.05$ in all three statistical tests were considered. This analysis was repeated separately for the promoter regions of genes whose abundances were altered by TCDD exposure or Ahr genotype in the kidney alone, the liver...
RESULTS

Experimental Design

We used mRNA transcript microarrays to study the transcriptional response to 1000 μg/kg TCDD or corn-oil vehicle in adult Ahr⁻/⁻ and Ahr⁺/+ mice (Fig. 1). Animals were sacrificed nineteen hours after exposure, a time point around or prior to early manifestations of hepatic toxicity in mice (Boverhof et al., 2006). Kidneys and livers were excised and subjected to mRNA transcriptional profiling on oligonucleotide microarrays. The dose chosen corresponds to approximately three-fold the individual LD₅₀ for male Ahr⁻/⁺ C57/BL6 animals (Pohjanvirta, 2009). Taken together, then, we expect this choice of dose and time point to allow identification of the early and acute results of dioxin exposure. The hepatic transcriptional response was reported previously (Tijet et al., 2006).

To ensure that transcriptional profiles from our new kidney data and older liver data are directly comparable we employed the same microarray platform and hybridization protocols for the two studies (Affymetrix MOE430-2 arrays) and analyzed the datasets using identical algorithms and software versions. All microarray data were carefully assessed for quality; none were excluded. Raw and preprocessed microarray data have been deposited in the GEO archive (accession GSE15859).

Transcriptional Profiling of Kidney

Using a GLM, we identified genes whose mRNA abundances were altered by Ahr genotype alone, by TCDD exposure alone, or by TCDD exposure in an AHR-dependent manner. To make accurate comparisons with our previous liver data, this same analysis was repeated for samples from that tissue.
In the kidney (Fig. 2A) only five genes were altered by TCDD exposure in Ahr<sup>−/−</sup> mice, and the magnitudes of change were modest, ranging from 0.52- to 2.5-fold. Similarly, only seven genes are affected by TCDD in an AHR-independent manner in the liver (Fig. 2). Again, these changes are modest in magnitude, ranging from 0.52- to 2.22-fold. Thus a functional AHR is required for almost all TCDD-mediated alterations of transcript levels in mouse kidney and liver.

The two tissues were also similar in the number of genes exhibiting basal differences in mRNA levels between Ahr<sup>−/−</sup> and Ahr<sup>+/+</sup> animals, even in the absence of TCDD exposure. In the kidney 379 genes exhibited AHR-dependent, TCDD-independent changes in mRNA levels—similar to the 471 genes altered in the liver.

Where the tissues differed significantly was the response to TCDD exposure. TCDD altered the mRNA levels of far fewer genes in mouse kidney than in mouse liver. Only 17 genes exhibited AHR-dependent mRNA alterations in response to TCDD exposure in mouse kidney (Table 1). By contrast 297 genes were altered in liver of these animals, and fully 22% (64/297) were also basally altered by Ahr genotype. A listing of all genes and their alterations in either tissue is in Supplementary Table 1. We confirmed that these conclusions were independent of our statistical threshold of \( p_{\text{adjusted}} < 0.05 \) with a threshold-analysis (Supplementary Fig. 1). To validate these findings, we employed real-time PCR. Five genes displaying AHR-dependent responses to TCDD and six genes responsive to Ahr status alone were
Comparison of Kidney and Liver

Previous studies by ourselves and others have demonstrated that the majority of TCDD-induced alterations in hepatic mRNA levels are inductive (Boutros et al., 2008; Boverhof et al., 2006; Fletcher et al., 2005). By contrast we previously showed that the majority of Ahr-associated changes in hepatic mRNA levels were repressive (Tijet et al., 2006). We studied the fraction of mRNA alterations that were inductive in each tissue (Fig. 3A). Intriguingly, our previous finding that Ahr-associated changes in hepatic mRNA abundances tend to be repressive is the outlier (purple curve), as the majority of TCDD-induced alterations in both tissues are inductive, as are the majority of genotypetype-dependent changes in kidney. Thus, although Ahr status alters mRNA abundances of large numbers of genes in both tissues, these alterations are different in character.

To compare the trends between kidney and liver in an unbiased manner we again applied unsupervised machine-learning. Clustering of data from diverse tissues or datasets is a challenging problem because technical factors can obviate other trends. To solve this problem we applied our recently developed co-clustering method (Boutros et al., 2008) to integrate the liver and kidney datasets (Fig. 3B). Three large clusters are evident. The first cluster (at the bottom of the heatmap) contains 15 samples, including all Ahr−/− animals (blue labels), which are subdivided by tissue (liver in green, kidney in yellow). Three wild-type (Ahr+/+) animals are also in this cluster. A second cluster, just above the first, comprises all tested. Nine of these eleven genes were validated (Supplementary Table 2).

Taken together these data indicate that Ahr genotype substantially affects the transcriptomes of liver and kidney, but that adult kidney is largely unaffected by TCDD exposure, at least at this time point and dose. To confirm this observation we took an unbiased approach and employed unsupervised machine learning (clustering) to identify the strongest trends in our data (Boutros and Okey, 2005). We selected the genes with the highest variance across samples and employed divisive hierarchical clustering. In both kidney (Fig. 2C) and liver (Fig. 2D) three clusters are observed. The first cluster (at the bottom of each heatmap with blue labels) contains all six Ahr−/− animals, with separation between TCDD-treated animals (with blue and red labels). The second cluster (in the centre of each heatmap) contains the wild-type, vehicle-treated animals (white labels). The final cluster (at the top of each heatmap) contains the TCDD-treated Ahr+/+ animals.

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#### TABLE 1
mRNAs Altered by TCDD in Kidney and Liver

| Entrez gene ID | Symbol          | M (liver) | M (kidney) | p (liver) | p (kidney) | Name                                                                 |
|---------------|-----------------|-----------|------------|-----------|------------|----------------------------------------------------------------------|
| 13076         | Cyp1a1          | 7.78      | 11.33      | 2.22 × 10−2 | 3.40 × 10−10 | Cytochrome P450, family 1, subfamily a, polypeptide 1                |
| 192113        | Atp12a          | 0.00      | 3.05       | 1.00 × 100  | 4.67 × 10−2  | ATPase, H+/K+ transporting, nongastric, alpha polypeptide           |
| 234593        | Ndrg4           | 0.00      | 2.12       | 1.00 × 100  | 3.63 × 10−4  | N-myc downstream regulated gene 4                                    |
| 56794         | Hac1            | 0.75      | 1.96       | 7.66 × 10−2 | 5.03 × 10−5  | 2-Hydroxyacyl-CoA lyase 1                                            |
| 99929         | Tiparp          | 5.98      | 1.25       | 1.14 × 10−7 | 3.63 × 10−4  | TCDD-inducible poly(ADP-ribose) polymerase                           |
| 76884         | Cyp4f2          | 4.23      | 1.04       | 1.14 × 10−7 | 1.10 × 10−3  | Cytoplasmic FMR1 interacting protein 2                               |
| 22217         | Usp12           | −0.02     | 0.88       | 6.02 × 10−1 | 7.41 × 10−3  | Ubiquitin specific peptidase 12                                     |
| 53415         | Htatip2         | 3.01      | 0.64       | 4.42 × 10−6 | 1.04 × 10−2  | HIV-1 tat interactive protein 2, homolog (human)                    |
| 18024         | Nfe2l2          | 1.99      | 0.55       | 4.87 × 10−5 | 3.46 × 10−2  | Nuclear factor, erythroid derived 2, like 2                         |
| 73353         | Actr12          | 0.01      | −0.03      | 1.00 × 100  | 1.95 × 10−2  | Actin-related protein T2                                             |
| 269704        | Zfp664          | 0.00      | −0.18      | 1.00 × 100  | 8.43 × 10−3  | Zinc finger protein 664                                             |
| 12484         | Cd24a           | −0.19     | −0.37      | 8.46 × 10−1 | 3.68 × 10−3  | CD24a antigen                                                       |
| 12177         | Bnip3l          | −0.36     | −0.38      | 3.04 × 10−1 | 3.46 × 10−2  | BCL2/adenovirus E1B interacting protein 3-like                      |
| 13645         | Esgf            | 0.00      | −0.40      | 1.00 × 100  | 1.07 × 10−2  | Epidermal growth factor                                             |
| 20377         | Sfp1            | 0.00      | −0.44      | 1.00 × 100  | 7.64 × 10−3  | Secreted frizzled-related protein 1                                 |
| 12192         | Zfp362l         | −0.23     | −0.65      | 1.00 × 100  | 4.67 × 10−2  | Zinc finger protein 36, C3H type-like 1                              |
| 13370         | Dio1            | −0.55     | −1.36      | 6.89 × 10−1 | 1.95 × 10−2  | Dehydrogenase, idoiothryonine type I                                |

Note. A listing of 17 genes whose mRNA abundances was altered by TCDD exposure in an AHR-dependent manner in kidney. The Entrez Gene ID is a stable gene identifier. The M values give the fold-changes of differential abundance in log 2 space. For example, the M (kidney) of 3.05 for Atp12a indicates a 8.3-fold increase in abundance in Ahr+/+ animals exposed to TCDD. p Values have been subjected to a false-discovery rate adjustment for multiple testing.
TCDD-exposed animals, again subdivided by tissue. The final cluster (at the top of the heatmap) includes the remaining wild-type liver and kidney samples. Thus the strongest signal in the dataset is Ahr status: TCDD exposure and tissue play lesser roles. This suggests substantial overlap in genes affected by Ahr status in the two tissues.

To explore this hypothesis we constructed Venn diagrams of genes displaying altered mRNA abundances based on Ahr status (Fig. 4A). In accord with the unsupervised machine-learning results, approximately 10% of the genes altered in either tissue are altered in both (9.3% in kidney, 11.6% in liver). This is far more than expected by chance alone \((p < 10^{-64})\). A similar analysis of dioxin-responsive genes (Fig. 4B) highlights the paucity of dioxin-responsive genes in the kidney. Five genes are common to both tissues, a significant enrichment over chance expectations \((p = 3.99 \times 10^{-18})\).

A subset of the 44 genes altered by Ahr genotype in both kidney and liver is shown in Table 2. The magnitudes of change vary dramatically, both within and between tissues. For example, metallothionein 1 mRNA levels are reduced by 99% in liver, but only by 40% in kidney. Some genes are altered in divergent directions: NADPH oxidase 4 (Nox4) and retinol saturase (Retsat) are induced in liver but repressed in kidney.

The 17 genes whose mRNA renal abundances were altered by TCDD exposure in an Ahr-dependent manner are given in Table 1. The five altered in both liver and kidney include several well-characterized members of the AHR gene battery (Cyp1a1, Tiparp, Nfe2l2) along with two genes not well-established as AHR targets: cytoplasmic FMR1 interacting protein 2 (Cyfip2) and HIV-1 tat interactive protein 2 (Htatip2). Interestingly, the three AHR gene battery members are also altered in rat liver, whereas Cyfip2 and Htatip2 are not (Boutros et al., 2008). Htatip2 is, however, altered in the livers of mice with a constitutively active AHR (Moennikes et al., 2004). These results demonstrate that species-specific but tissue-independent AHR targets exist.

We then wondered if genes that were altered by either Ahr status or TCDD exposure in one tissue were predisposed to show weak, but statistically insignificant changes in the other. This would represent “leaky control,” where AHR regulation was not completely tissue specific. To address this hypothesis we generated scatter plots of the fold changes (in log2 space) of liver and kidney both for genes altered by Ahr status (Fig. 4C) and by TCDD exposure (Fig. 4D). In both cases we observe a “cloud” of points near the origin, with many genes found directly on either the x-axis (indicating kidney-specific changes) or the y-axis (indicating liver-specific changes). Because only a few genes lie on the diagonal, we can discount the idea of “leaky control” and conclude that the majority of genes have strongly tissue-specific effects. The outlier in the top right corner of Figure 4C is acireductone dioxygenase 1 (Adi1); the outlier in the top right corner of Figure 4D is Cyp1a1.

Pathways Responsive to Ahr Genotype and TCDD

Although 44 genes respond to Ahr genotype in both liver and kidney (Fig. 4A) and five respond to TCDD in both tissues (Fig. 4B), far more genes display tissue-specific responses. There are 762 tissue-specific responses to Ahr genotype and 309 to TCDD exposure. However it remains possible that the AHR has similar functions in each tissue, but executes them through regulation of different genes. To test this hypothesis, as well as to determine the specific pathways perturbed by Ahr.

**FIG. 3.** Characteristics of transcriptional response in kidney and liver. Following microarray preprocessing and general-linear modeling, we explored transcriptome-wide characteristics of genes altered in kidney and liver. Because very few genes displayed AHR-independent responses to TCDD these were not included in our analyses. First, we looked at the fraction of genes that were induced (A). Genes that were responsive to TCDD in either liver or kidney, as well as those altered by Ahr genotype in kidney, are much more likely to be inductive than repressive. By contrast genes responsive to Ahr genotype in liver were balanced slightly enriched for repressive responses. Next, to study intertissue responses to TCDD, we performed a co-clustering analysis (B). The strongest trend was Ahr genotype, with both TCDD exposure and tissue having effects of similar magnitude. The color bar on the right side of the heatmap indicates tissue (yellow, kidney; green, liver), Ahr genotype (blue, AHR\(^{-/-}\); white, AHR\(^{+/+}\)), and TCDD exposure (red, TCDD treated; white, vehicle treated).
Following preprocessing and general-linear modeling of the microarray data, we compared the specific genes altered by Ahr genotype (A) and TCDD exposure (B) in liver and kidney. Large numbers of genes were altered by Ahr genotype in each tissue, and a significant fraction ($p < 10^{-64}$) was common to both (A). By contrast, only five few genes were TCDD responsive in both tissues, which were again more than expected by chance ($p = 3.99 \times 10^{-18}$).

To determine if these differences were related to genes just below our selected thresholds we plotted the fold-changes in liver against those in kidney for both genotype (A) and TCDD exposure (B). We compared the specific genes altered by Ahr genotype or TCDD exposure was extracted and searched for each TFBS, and the evolutionary conservation of all motifs was determined using PhastCons scores (Boutros et al., 2004; Siepel and Haussler, 2004). PhastCons scores range from 0 (no conservation across 30 vertebrate species) to 1 (perfect conservation across 30 vertebrate species). This analysis was repeated for genes altered by Ahr genotype in kidney only, liver only, or both tissues and for genes altered by TCDD exposure in kidney only, liver only, or both tissues.

The resulting six lists were visualized using violin plots for AHRE-I (Figs. 6A and 6B), AHRE-II (Figs. 6C and 6D), and ARE (Figs. 6E and 6F). Violin plots are a compact method of representing the distribution of a data set. Each column represents a specific condition, and the thickness of a column represents the fraction of data points with that value (i.e., the density of the distribution). Thus violin plots allow the simultaneous visualization of multiple distributions. For example, Figure 6A shows that AHRE-I motifs present in genes responsive to TCDD in both liver and kidney are more likely to be evolutionarily conserved than those in the other gene lists. Similarly, Figure 6B shows that genes responsive to TCDD in kidney contain more AHRE-I motifs than those in the other gene lists. Complete data on the number and conservation of motifs for each gene is in Supplementary Table 1.

This analysis raises several intriguing points. Many genes whose mRNA abundances are altered by TCDD display AHRE-I motifs (Fig. 6B) which are often conserved, especially in genes altered in both tissues (Fig. 6A). Genes altered in both tissues also tend to have more (Fig. 6D) and better-conserved (Fig. 6C) AHRE-II motifs. This suggests that known tissue-independent responses to TCDD may occur through well-characterized mechanisms of AHR activity. Genes altered by Ahr genotype, on the other hand, are slightly enriched for AREs (Figs. 6E and 6F), whereas TCDD-dependent responses are not.

These results suggest that other transcription factors may collaborate with the AHR in a combinatorial fashion to regulate...
TABLE 2
mRNAs Altered by Ahr Genotype

| Entrez gene ID | Symbol                          | M (liver) | M (kidney) | p (liver)   | p (kidney)   | Name                                                                 |
|----------------|--------------------------------|-----------|------------|-------------|--------------|----------------------------------------------------------------------|
| 104923         | Adi1                           | 3.74      | 5.18       | $3.52 \times 10^{-4}$ | $1.70 \times 10^{-3}$ | Acireductone dioxygenase 1                                            |
| 13370          | Dio1                           | 1.63      | 2.06       | $7.28 \times 10^{-4}$ | $1.92 \times 10^{-5}$ | Deiodinase, iodothyronine, type I                                     |
| 105892         | 9030619P08Rik                   | 0.42      | 1.10       | $1.39 \times 10^{-2}$ | $3.17 \times 10^{-3}$ | RIKEN cDNA 9030619P08 gene                                           |
| 71670          | Acy3                           | 0.79      | 0.62       | $2.61 \times 10^{-3}$ | $2.28 \times 10^{-3}$ | Aspartoacylase (aminoacylase) 3                                      |
| 108114         | Slc22a7                         | 3.15      | 0.53       | $7.93 \times 10^{-6}$ | $9.38 \times 10^{-3}$ | Solute carrier family 22 (organic anion transporter), member 7       |
| 70337          | Iyd                            | $-0.33$   | 0.44       | $1.80 \times 10^{-2}$ | $2.82 \times 10^{-3}$ | Idotyrosine deiodinase                                               |
| 13476          | Reep5                          | 0.57      | 0.38       | $8.55 \times 10^{-3}$ | $8.45 \times 10^{-3}$ | Receptor accessory protein 5                                          |
| 68347          | 0610011F06Rik                   | 0.49      | 0.37       | $3.76 \times 10^{-3}$ | $3.17 \times 10^{-3}$ | RIKEN cDNA 0610011F06 gene                                           |
| 19703          | Renbp                          | $-0.40$   | $-0.43$    | $3.31 \times 10^{-2}$ | $3.52 \times 10^{-3}$ | Renin binding protein                                                 |
| 50490          | Nox4                           | 0.90      | $-0.44$    | $2.22 \times 10^{-2}$ | $8.40 \times 10^{-3}$ | NADPH oxidase 4                                                       |
| 15211          | Hexa                           | $-0.60$   | $-0.46$    | $2.84 \times 10^{-4}$ | $1.70 \times 10^{-3}$ | Hexosaminidase A                                                     |
| 109900         | Asl                            | $-0.76$   | $-0.56$    | $6.85 \times 10^{-3}$ | $3.46 \times 10^{-4}$ | Argininosuccinate lyase                                               |
| 67442          | Retsat                         | 0.62      | $-0.60$    | $4.00 \times 10^{-2}$ | $4.81 \times 10^{-3}$ | Retinol saturase (all trans retinol 13,14 reductase)                 |
| 14873          | Gstol                          | $-0.75$   | $-0.66$    | $3.52 \times 10^{-4}$ | $1.70 \times 10^{-3}$ | Glutathione S-transferase omega 1                                    |
| 17748          | Mr1                            | $-6.74$   | $-0.74$    | $3.83 \times 10^{-6}$ | $1.70 \times 10^{-3}$ | Metallothionein 1                                                    |

Note. A listing of a subset of genes whose mRNA abundances were altered by Ahr genotype in both kidney and liver. The Entrez Gene ID is a stable gene identifier. The M values give the fold-changes of differential abundance in log₂ space. For example, the M (Liver) of 3.74 for Adi1 indicates a 13.4-fold increase in abundance in the liver of animals with a wild-type Ahr relative to Ahr−/− animals. p Values have been subjected to a false-discovery rate adjustment for multiple testing.

tissue-specific mRNA regulation. To assess this effect we performed a library-based TFBS analysis, searching the regulatory regions of each gene list for 130 known TFBS motifs. Only motifs enriched ($p < 0.05$) in three separate statistical tests were considered. In total 60 TFBSs met this criterion in one or more gene lists (Supplementary Table 4). Unsupervised clustering was used on these motifs to determine which gene lists shared the most regulatory structure (Fig. 6G). The six lists divided into two clusters. The first contained genes responsive to Ahr genotype in a tissue-specific manner (i.e., liver only and kidney only) and genes responsive to TCDD in the liver. The second cluster contained tissue-independent responses to Ahr genotype or TCDD activity and the genes responsive to TCDD in the kidney alone. This division further supports the concept that tissue-specific and tissue-independent responses occur through distinct mechanisms.

DISCUSSION

Dioxin-induced toxicities affect many tissues (McConnell et al., 1978; Pohjanvirta and Tuomisto, 1994; Pohjanvirta et al., 1989). Although it is becoming well-established that the vast majority of these toxicities are mediated through the AHR, the pathways leading from AHR activation to most specific toxicities remain unknown (Okey, 2007). There is also accumulating evidence that the AHR plays a critical role in normal development (McMillan and Bradfield, 2007), perhaps through activation by endogenous ligands (Bittinger et al., 2003; Nguyen and Bradfield, 2008; Song et al., 2002).

Because the AHR is a transcription factor, one way to assess its activity is to profile the transcriptional response to TCDD exposure in wild-type (Ahr+/+) or AHR-null (Ahr−/−) mice. When we studied the response of adult liver to TCDD exposure in this way three trends emerged (Tijet et al., 2006). First, the transcriptional effects of TCDD required the presence of functional AHR protein. That is, Ahr−/− mice are not only phenotypically refractory to TCDD exposure, but also show virtually no cellular response. Second, large numbers of genes in specific functional pathways are perturbed by dioxin exposure in Ahr+/+ mice. Third, surprisingly large numbers of genes are perturbed by genetic ablation of the Ahr, even in absence of exogenous ligand. These genes lie in specific functional pathways and are associated with specific TFBS, suggesting an important role for AHR activity in basal hepatic physiology.

Here, we replicate our hepatic study in kidney, performing transcriptional profiling of adult Ahr−/− and Ahr+/+ mice with or without exposure to TCDD. We extensively compared the effects of dioxin exposure and Ahr genotype in kidney and liver. We took numerous steps to ensure that this comparison could be made robustly and with minimal noise. First, tissues were excised from the same animals at the same time, thereby minimizing interanimal or interbatch variation. Second, RNA
were calculated for each GO term and the 17 terms with p
enrichments.

TCDD exposure in kidney only, liver only, or both tissues. The log 10(
mine relationships amongst GO terms and tissues. The color-bar indicates
are shown here. Unsupervised hierarchical clustering was used to deter-
log10(p values), so dark squares indicate highly statistically significant
preparations were performed in an identical manner. Third, microarray hybridizations were performed using identical platforms and protocols. Fourth, computational and statistical analyses were performed identically, using the same software versions and tests for each dataset. We note that we selected a high dose, equivalent to roughly three times the LD50 and an early time point so that we could capture acute and early effects of dioxin exposure.

The renal and hepatic effects of dioxin exposure and Ahr genotype differ substantially. Although large numbers of genes are affected by each factor in the liver, kidney was mostly refractory to dioxin exposure. The exceptions (Table 1, Fig. 4D) include well-characterized AHR-responsive genes, such as Cyp1a1 and Tiparp. These data emphasize the tissue-specific regulatory effects of the AHR. Indeed our analysis of TFBS identified motifs associated with AHR activity in a tissue-specific manner.

Surprisingly, the kidney transcriptome was substantially remodeled in the absence of a functional AHR. This might indicate a developmental role for the AHR in this tissue, or may indicate a role for the AHR in adult renal physiology. There are at least two known renal phenotypes in Ahr−/− mice. First, kidney weight is higher in Ahr−/− males than in wild-type males, at least at three months of age (Lin et al., 2001). Second, neonatal Ahr−/− mice exhibit significant vascular abnormalities in the kidney (Lahvis et al., 2000; McMillan and Bradfield, 2007). The underlying mechanism has not been investigated but could contribute to the age-related hypertension observed in adult Ahr−/− mice (Lund et al., 2008).

Several studies have considered the effects of Ahr activation on fetal mouse kidney (Johnson et al., 2004). In such studies, as well as in our own work (Choi et al., 2006) there is substantial evidence of CYP1B1 induction by AHR ligands. Surprisingly, CYP1B1 induction has not been observed in adult mouse kidney (Kopf et al., 2008; Shimada et al., 2003), although it has in adult rat kidney (Badawi et al., 2000). We confirm these results by observing no upregulation of CYP1B1 mRNA in adult mouse kidney. However we do not observe constitutive overexpression of CYP1B1 mRNA in Ahr−/− kidneys, which has been reported by others (Shimada et al., 2003). Taken together, these results suggest substantial differences in the renal transcriptional response to TCDD between adult and fetal mice, as well as mice and rats.

Although the adult liver is susceptible to acute TCDD-induced toxicity, the adult kidney is not. To the best of our knowledge, the lack of impact of TCDD on adult mouse kidney has not specifically studied in detail (Birnbaum and Tuomisto, 2000). However a very recent paper (Lu et al., 2009) has studied the response of rat kidney to a cumulative dose of 120 μg/kg TCDD in a 12-day experiment. The authors detected several signs of nephrotoxicity, including increased serum creatinine and blood urea nitrogen, histopathological changes, and increased renal oxidative stress. It is not clear, however, if these are primary effects of TCDD as these animals exhibited reduced activity, a 27% decline in food intake and a 28% decline in body-weight (feed-restricted controls were not included). On balance, then, it appears that adult kidney is not acutely responsive to TCDD, perhaps in part because TCDD is sequestered in liver and does not distribute to the kidney (Santostefano et al., 1996). It is known, however, that chronic low doses result in hypertension which may be due, in part, to effects on kidney (Kopf et al., 2008). In this context, the small number of TCDD-induced changes observed in kidney is reasonable. We hypothesize that significantly more genes will be altered by TCDD in fetal kidney.

Our conclusions about tissue specificity must be taken with at least one caveat, however. A tissue is not a homogeneous entity, but rather is a complex mixture of different cell-types. About 80% of liver volume is comprised of hepatocytes, whereas endothelial, Kupffer, stellate, and intrahepatic lymphocytes make up the remaining 20% (Lieberman et al., 2009). By contrast, kidney consists of distinct structural and functional components which, together with the supporting vasculature, comprise a large number of cell types. At least twelve cell types have been identified in the nephron, ranging in function from maintenance of the filtration barrier (glomerular epithelium) to selective reabsorption of glucose and amino acids (proximal

**FIG. 5.** Functional analysis of perturbed genes. GO enrichment analysis was performed on the sets of genes altered in response to Ahr genotype or TCDD exposure in kidney only, liver only, or both tissues. The log_{10}(p values) were calculated for each GO term and the 17 terms with p_{cumulative} < 10^{-12} are shown here. Unsupervised hierarchical clustering was used to determine relationships amongst GO terms and tissues. The color-bar indicates log_{10}(p values), so dark squares indicate highly statistically significant enrichments.
A large transcriptional effect observed in a relatively rare cell-type might be difficult to detect in our experiments. Further complications would arise if different cell-types have divergent responses, as these may cancel one another out. These factors might inflate our false-negative rate and our results represent a lower-bound on the number of genes altered by dioxin exposure and Ahr genotype.

FIG. 6. Analysis of TFBS. TFBSs were analyzed in regulatory sequence from −3000 to +3000 bp relative to the transcriptional start-site of each gene altered in one or both tissues by either Ahr genotype (AHR) or TCDD exposure (AHR:TCDD). Three TFBS motifs known to be associated with AHR activity were considered: AHRE-I (A and B), AHRE-II (C and D), and ARE (E and F). For each gene, the phylogenetic conservation of motifs was calculated on a scale of zero (low conservation) to one (high conservation) (parts A, C, and E). Additionally, the actual number of motifs was calculated (parts B, D, and F). The visualizations used for parts A–F are called “violin plots”. Each column represents a specific gene list, and the thickness and shape of the column represents the distribution of values for that gene list. A library-based TFBS-enrichment analysis was performed on the same sequence and the log10|p| from this analysis subjected to unsupervised clustering (G). The color bar indicates log10|p| values. The permutation test employed had a minimum resolution of 10−4, hence the large number of ties at this level. The color bars on the right side reflect tissue of origin (liver, green; kidney, yellow; both tissues, blue) and contrast (AHR genotype, purple; TCDD-induced changes, red).

tubular epithelium) (Gilbert, 2000; Rosenblum, 2008). A large transcriptional effect observed in a relatively rare cell-type might be difficult to detect in our experiments. Further complications would arise if different cell-types have divergent
SUPPLEMENTARY DATA

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

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