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Hepatic transcriptional networks induced by exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin¹

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Abstract

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) serves as a prototype for a range of environmental toxicants and as a pharmacologic probe to study signal transduction by the aryl hydrocarbon receptor (AHR). Despite a detailed understanding of how TCDD exposure leads to the transcriptional up-regulation of cytochrome P450-dependent monooxygenases, we know little about how compounds like TCDD lead to a variety of AHR-dependent toxic endpoints such as liver pathology, terata, thymic involution and cancer. Using an acute exposure protocol and the toxic response of the mouse liver as a model system, we have begun a detailed microarray analysis to describe the transcriptional changes that occur after various TCDD doses and treatment times. Through correlation analysis of time- and dose-dependent toxicological endpoints, we are able to identify coordinately-responsive transcriptional events that can be defined as primary transcriptional events and downstream events that may represent mechanistically linked sequelae or that have potential as biomarkers of toxicity.

INTRODUCTION

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) has long been a focus of public concern. Formed by industrial processes such as combustion, bleaching of wood pulp, and chlorination of phenols, TCDD and structurally-related compounds are widely-distributed in the environment and have been shown to elicit a broad spectrum of biological responses in mammalian systems (1). At doses in the ng/kg range, TCDD leads to the up-regulation of genes important for the metabolism of xenobiotics and endogenous hormones (2). At doses in the µg/kg range, TCDD up-regulates the xenobiotic response while also causing a number of toxic endpoints such as hepatotoxicity, thymic involution, birth defects, cancer and lethality (1, 3-5).

It is widely accepted that TCDD acts through the aryl hydrocarbon receptor (AHR), a member of the Per-Arnt-Sim (PAS) family of environmental sensors (6-10). In its unliganded state, the AHR is maintained in the cytosol in a complex with chaperones such as HSP90 and ARA9 (also known as AIP1 or XAP2) (11-13). Upon binding TCDD, the AHR translocates to the nucleus where it dimerizes with another PAS protein known as ARNT (13). The AHR-ARNT complex binds to dioxin-responsive enhancers (DREs)², modulating the expression of a battery of regulated genes (2). Interaction of AHR-ARNT dimers with DREs appears to explain the

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expression of many TCDD-responsive genes such as those encoding xenobiotic metabolizing enzymes (XMEs) (e.g., *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Ugt1a6* and members of the GST family) (2,15,16).

Despite our detailed understanding of AHR-regulated xenobiotic metabolism, the mechanistic links between DRE-driven gene expression and toxic endpoints such as hepatotoxicity are still unclear. A number of observations support the idea that DRE-regulated gene expression within the hepatocyte is a fundamental aspect of TCDD-induced liver toxicity. For example, recombinant mouse models with mutations in the DNA binding domain of the AHR, hepatocyte specific deletions of the AHR, or hypomorphic expression of ARNT are all resistant to TCDD-induced hepatotoxicity(17-19). While this genetic evidence points to the importance of AHR-ARNT-DRE interactions in TCDD-hepatotoxicity, it does not identify the causally related DRE-driven genes. In addition, we still do not know the number of steps that exist between the DRE-regulated battery and any given pathological endpoint.

Based upon the above ideas, we are left with a model of TCDD toxicity where a subset of DRE-regulated gene products within the hepatocyte leads directly to liver-specific pathology. This subset of DRE-regulated genes could lead directly to cell death, perhaps through stimulation of oxidative or apoptotic mechanisms (20). Alternatively the DRE-regulated subset could stimulate a multi-step sequence of physiological or developmental events, perhaps stimulation of an inappropriate differentiation pathway that results in tissue pathology. In an effort to provide evidence for these models of toxicity, we have begun an extensive transcriptomic effort to describe the batteries of genes that are deregulated by TCDD exposure. Our hypothesis is that sequences of TCDD-inducible events can be defined by monitoring coordinate transcriptional changes in gene sets. To this end, we characterized the transcriptional changes that occur over time in response to various dosages of TCDD. We then used functional and statistical analyses as methods to identify coordinately regulated gene batteries, as well as batteries that are coregulated with pathology. Through this global gene expression analysis over time and dose, we have generated a training set that can be used to estimate the number of steps in toxicity, as well as to identify those genes or gene batteries that can serve as mechanistically linked markers of specific pathological endpoints.

EXPERIMENTAL PROCEDURES

Animals and Treatments

All animal treatments were reviewed and approved by the Animal Care and Use Committee at the University of Wisconsin. TCDD dissolved in corn oil or corn oil alone was administered by oral gavage. In addition to TCDD-treated animals, a corn oil cohort was harvested at each time point. At the indicated times, the liver was removed, examined and weighed, and a section placed in 10% buffered formalin for histological preparation. The remainder of the liver was placed in "RNA Later" (Qiagen, Valencia, CA) and frozen at -80°C for subsequent preparation of RNA. In some cases, blood was withdrawn by cardiac puncture and sera sent to the Clinical Pathology Laboratory at the University of Wisconsin, School of Veterinary Medicine (Madison, WI) for quantification of alanine aminotransferase (ALT). The *Cyp1a1*^{-/-} and *Cyp1a2*^{-/-} animals were a generous gift from Dr. Daniel Nebert (University of Cincinnati, OH).

²Here we define DRE-regulated (a.k.a. AHRE or XRE) genes to be any gene driven by the classical DRE (TNGCGTG), the recently reported DRE-II element (CATGNNNNNC(T/A)TG) or any other undiscovered AHR-ARNT-target sequence (14).

RNA Isolation and Quantification

Total RNA was prepared using the RNA Protect system (Qiagen, Valencia, CA). Quality and quantity of RNAs were assessed on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) using the RNA Nano Labchip.

cDNA Microarrays

All experiments were performed using the EDGE cDNA microarray, Liver Version 5 (<http://edge.oncology.wisc.edu>) (21). This microarray is composed of liver-derived cDNA clones fortified with cDNAs representing toxicologically relevant genes such as those encoding drug metabolizing enzymes, inflammatory responsive genes, and genes that we previously identified as useful for classifying chemicals (22).

cDNA Microarray Hybridization

When the amount of available total RNA exceeded 20 µg, labeling and hybridizations were carried out using the Genisphere cDNA50 system (Genisphere, Hatfield, PA). When the available total RNA was less than 20 µg, the Genisphere cDNA350 system was used. Briefly, total RNA was reverse-transcribed into cDNA using specific primers containing a Cy-3 or Cy-5 “capture sequence.” The cDNAs from both a treatment group and a pool of the corresponding control were then mixed and hybridized onto a microarray for eighteen hours. The arrays were washed according to the manufacturer’s specifications. The Cy-3 or Cy-5 fluor was included in a second hybridization where DNA complementary to the capture sequences was covalently linked to a dendrimer, containing up to 350 dye molecules. All experiments were performed in a “dye flop” experimental design where replicate hybridizations of the same sample and control are performed in each dye direction. That is, Cy-3-labeled cDNA from control sample was hybridized against Cy-5-labeled cDNA from the treated sample in dye direction #1, and Cy-5-labeled control RNA was hybridized against Cy-3-labeled treated RNA in dye direction #2.

cDNA Microarray Image Acquisition and Analysis

After hybridization, slides were scanned on a DNA Microarray Scanner, Model G2565BA (Agilent, Palo Alto, CA). Images were processed and the data was extracted using Agilent Feature Extractor software version 7.1 (Agilent, Palo Alto, CA). Further data processing were then performed by custom Perl scripts and imported into MySQL databases. Normalization was performed by the LOWESS method using the Agilent Feature Extraction Software (Agilent, Palo Alto, CA). After removing the maximum and minimum spot ratios to generate a single value, all cDNA replicates were averaged, and the values from the “dye flop” were then averaged. All microarray experiments discussed adhere to the Minimum Information About a Microarray Experiment (MIAME) guidelines. All microarray data are publicly available through the EDGE database (<http://edge.oncology.wisc.edu/>).

Northern Analysis

When microarray analysis identified a gene of interest, results were confirmed by Northern blot analyses. 10 µg of total RNA was electrophoresed in a 0.8% agarose gel containing 18% formaldehyde and was transferred to Hyband™-N+ membrane (Amersham Bioscience). cDNA fragments of Cyp1a2, Hctd2, Hsd17b5, Gstm1, Ugt1a6, Cyp2a5 and rGAPDH genes were randomly primed (Amersham) with ³²P-dCTP and used as probes in Northern blot analysis of liver mRNA from mice treated with various doses of TCDD. Values obtained from phosphor autoradiography (STORM, Molecular Dynamics, Sunnyvale, CA) of blots probed with test and GAPDH control probes allowed for normalization of expression to eliminate mRNA loading variability.

Histology

Sections of mouse liver from equivalent regions were fixed in 10% buffered formalin, embedded in paraffin, and 5 μ m sections were cut for Hematoxylin-eosin (H&E) staining. Two experienced personnel, who were blinded to specimen genotype and treatment, independently performed interpretation of histology. Hydropic degeneration, inflammatory cell infiltration, and steatosis were scored in a scale of 0 - 4 with 0 representing no change and 4 representing the most severe change observed. The values presented represent the average of the two interpreters.

Correlation Analysis

Probes that changed with a greater than two-fold response in at least one dose/time-point were selected for further correlation analyses. These included 373 probes from both the long-term and short-term time courses. Pearson correlation coefficients were generated, comparing the values of the time series for each probe to that of every pathological endpoint. Significance of Pearson values were assessed by generating 10,000 false time series for each probe, by randomly sampling from that probe's true values with replacement. The actual "true" value was then ranked among the false values, creating a probability of that Pearson score arising randomly from the values generated.

RESULTS AND DISCUSSION

Over the last few years, experiments with mutant alleles of the *Ahr* and *Arnt* loci have provided support for the idea that binding of the AHR-ARNT complex to the DRE is required for many aspects of TCDD toxicity (17-19,23,24). Moreover, related studies have shown that TCDD-induced toxicity is often cell-autonomous, e.g., hepatotoxicity is dependent upon AHR signal transduction within the hepatocyte (17-19,23,24). Despite this insight into TCDD toxicity, we still have little understanding of how signaling progresses from AHR-ARNT-DRE binding to any specific toxic endpoint. It seems clear that we have not identified all of the toxicologically-relevant DRE-regulated genes and that the genes most important in TCDD toxicity are still undefined.

In our effort to define TCDD-responsive gene batteries that might be related to hepatotoxicity, we set out to accomplish three bioinformatic tasks. The first task was to expand the number of liver transcripts that are dysregulated in response to TCDD. We hypothesize that the genes up-regulated with temporal and dose-response patterns similar to known DRE-regulated genes will have a high probability of being DRE regulated genes themselves. The second task was to identify batteries of genes that respond secondarily to the primary DRE-mediated transcriptional response. We hypothesize that these secondary or downstream genes will respond TCDD exposure with patterns that are temporally delayed from the primary battery. Our third task was to identify genes or batteries of genes that can act as surrogate markers of various pathological states. Identification of these gene batteries may prove valuable in defining biomarkers of TCDD-induced pathology.

Range finding and method validation

In an effort to define an exposure range for these studies, microarray analyses were first performed using mouse liver RNA after a 48 hour treatment with 0.1, 1.0, 10, 25, or 64 μ g/kg TCDD. Data analysis revealed that 71 target genes displayed a significant change as compared to the untreated control (Figure 1). To validate the microarray approach, select transcriptional responses were assayed via northern blot analysis (Figure 2). This experiment led us to conclude that these microarray data provided a robust description of dioxin-induced gene expression, and that even small (1.5-fold) transcriptional changes can readily be detected by this approach. In all cases examined, data from the northern blots agreed with both the direction

and the magnitude of the changes observed in the microarray experiments (Figure 2 and data not shown). Moreover, known DRE-driven gene products including *Cyp1a1*, *Cyp1a2* and *Ugt1a* were shown to follow a classical dose-response relationship over the range employed (2).

A preliminary analysis of the dose-response data revealed that a number of genes previously not thought of as “DRE-driven” were found to follow a dose-response pattern similar to *Cyp1a1*, *Cyp1a2* and *Ugt1a*. These genes included *Hectd2*, *Hsd17b2* and *Cyp2a5* (NM_172637, NM_008290, NM_007812, respectively). Recently, similar studies have shown evidence supporting *Hectd2* and *Cyp2a5* as early dioxin responsive genes (25,26). Although a dose-response relationship was less clear, at 64 ug/kg, TCDD upregulated three Serum Amyloid A loci (i.e., *Saa1*, *Saa2*, and *Saa3*). These loci have previously been reported to be indicative of the acute phase response arm of inflammation (27) The up-regulation of these genes provides preliminary data to support the idea that some hepatic damage/inflammation is occurring at higher doses of TCDD exposure.

Long term study

In an effort to describe the relationships between dose, time, transcriptional response, and toxic response, we next performed a time-course study at three doses of TCDD over a period of sixty-four days. Based on the dose-response study, three doses were chosen: a) sub-maximal induction of transcriptional targets (1 µg/kg or “low dose”), b) a dose that gave rise to maximal induction of known target genes (10 µg/kg or “medium dose”) and c) a dose that exceeded the maximal induction dose and that was predicted to lead to pathological endpoints such as hepatomegaly, hydropic degeneration, inflammatory cell infiltration of the liver and thymic involution (64 µg/kg or “high dose”). At 1, 4, 8, 16, 32 and 64 days post-treatment, three animals from each dose were sacrificed, and the RNA and tissues were prepared from each group. For normalization of microarray data, each pooled group was compared against a control pool comprised of eight animals that had undergone treatment with vehicle alone for the same time period.

The known DRE-driven genes, *Cyp1a1* and *Cyp1a2*, displayed marked induction at all doses and times examined (Figure 3). The response profiles of these genes differed as a function of both dose and time. For example, induction in the high dose group remained high throughout the course while induction following the low dose returned to near-baseline levels at 64 days, most probably due to redistribution and excretion. The greatest number of transcriptional changes appeared in the high dose cohort at eight and sixteen days post treatment.

We then examined histological changes in the livers of animals from the long-term TCDD exposure study (Figure 4). We quantified levels of hydropic degeneration in the hepatocytes, fatty changes (steatosis) in the hepatocytes, evidence of hepatocellular necrosis and apoptosis, as well as the level of inflammatory cells infiltrating the liver (“inflammation”). Hydropic degeneration was the earliest pathology noted in all groups and its severity was dependent on dose and time. Significant pathology began to appear four days after treatment in all dose groups. In correlation with the substantial transcriptional changes observed at eight and sixteen days, histological examination of animals treated with the high dose of TCDD revealed severe hydropic changes, massive inflammatory cell infiltration and some evidence of apoptosis and necrosis (Figures 4 and 5). At sixteen days, steatosis was related to the presence of both microvesicular and macrovesicular fat deposits in the high dose animals (data not shown). Steatosis was only observed in the high dose group at 16, 32 and 64 days.

Short Term Study

The observation from the long-term study that histological changes were observed as early as four days led us to perform a more detailed analysis of earlier time points in a short-term study. Mice were again treated with the 1, 10, and 64 µg/kg doses of TCDD and pathology and liver gene expression were examined after 6, 12, 24, 36, 48, 60, 72 and 96 hours. In order to assess biological variance of hepatic response to TCDD, we assayed individual animals at each time-dose point. In most cases, the data were generated from three animals per individual time-dose point.

Microarray analyses were performed to describe the difference in global gene expression in response to the three doses of TCDD. Induction of *Cyp1a1* and *Cyp1a2* occurred within six hours, with average peak induction occurring twenty-four hours after treatment (Figure 6). During the time course, 103 targets, or 6.4% of those genes assayed were either up-regulated or down-regulated at least 2-fold. Assessment of pathology from the short-term time course experiment revealed distinct differences in the dose-response. As with the long-term study, histological examination of animals from all three doses displayed hydropic degeneration, with severity being dose-responsive (Figure 7). At 96 hours, we observed inflammatory cell infiltration in the high dose cohort. In this experimental series, we added a direct measure of hepatocyte damage, with the quantification of serum levels of alanine aminotransferase (ALT). The ALT levels did not significantly vary from the control values at any time point except for 72 and 96 hours in the high dose. This rise corresponded in time to the infiltration of inflammatory cells (Figure 7).

Looking for batteries of coregulated genes

Our approach to identify batteries of coregulated genes is to identify transcripts that respond to TCDD with similar dose- and time dependent profiles. In our approach, we incorporated all of the available expression data from the “dose response”, “short-term” and “long-term” experiments in a correlation analysis with specific model transcripts or with the presentation of various pathological endpoints (Table 1 and Figure 8). After running a 100,000 bootstrap-permutation test, we selected those response pairs where a statistically significant correlation was observed ($p < 0.001$). These correlations, which ranged from 1 to -0.885, were then organized using a hierarchical cluster analysis (Figure 8)(28). This analysis revealed four major groups that were then annotated by using the DAVID analysis tool from the National Cancer Institute (29). We postulate that genes with high positive correlations values over various treatments and times are candidates for coregulation and that transcripts with negative correlations represent regulation of transcripts that are moving in the opposite direction to the model transcript. Negatively correlated transcripts may represent a linked, yet opposing mechanism of gene regulation and they may be as valuable as positively correlated transcripts as biomarkers of pathology or for insight into mechanism.

Primary targets and potential DRE-driven genes

Our approach to identify primary targets of TCDD was to identify transcripts that respond similarly to the known DRE-driven genes *Cyp1a1*, *Cyp1a2*, *GSTm1* and *Ugt1a* (Table 1). Using this approach, we identified twenty-seven genes as being co-regulated with known DRE-driven genes (Fig 8). That is, over the dose-response, long-term and short-term experiments, these 27 genes responded similarly (i.e., with a positive correlation) to *Cyp1a1*, *Cyp1a2*, *Gstm1* and/or *Ugt1a* (Table 1). Given that our model predicts that the initial induction of DRE-driven genes lies at the heart of dioxin hepatotoxicity; this list may include those DRE-driven transcripts that underlie some of the toxic effects of dioxin. Given that these microarrays are only sampling about one quarter of the mouse transcriptome it is also possible that a number of primary response genes will only be detected by a similar study using whole genome arrays.

Secondary response genes

As a first attempt to identify downstream (e.g., secondary and tertiary) transcriptional responses to TCDD, the correlation analysis was interrogated to identify transcripts that correlated with two prototype transcripts that were selected because they provided robust transcriptional changes that were temporally distinct from known DRE-driven gene products. The *Car3* gene was selected because its TCDD response was down-regulated at high doses and temporally distinct from classical DRE-driven responses (Figure 3). The *Saa1* gene was selected because its TCDD response was upregulated at high doses and temporally distinct from classical DRE-driven responses (Figure 3). Correlation analysis with these transcripts revealed the potential for the existence of two large TCDD-responsive gene batteries that can be described by the prototypes, *Car3* and *Saa1* (Figure 8). The gene batteries that correlate with these model transcripts appear to represent downstream responses to TCDD as their expression is markedly delayed in comparison to classical DRE regulated genes and occurs only at higher doses of TCDD exposure where toxicity is significant.

Potential biomarkers

In our attempt to identify biomarkers of TCDD hepatotoxicity, transcriptional responses were correlated with three pathological endpoints and those pairs of transcriptional and pathological changes where a statistically significant correlation or anti-correlation was observed were selected ($p < 0.001$). The underlying idea of this experiment was that transcripts that correlated with pathological events that were temporally distinct and later than DRE driven events are later steps in toxic signaling and represent potential biomarkers of toxicity. Hierarchical clustering revealed that three large classes of transcripts showed a relationship with the toxic endpoints studied. Moreover, two of these groups of genes showed a relationship with the expression of our model genes *Saa1* and *Car3*.

Our prior work profiling the liver's transcriptional response to many hepatotoxic agents leads us to predict that many of the transcripts that are coregulated with *Car3* are related to a state of general hepatic stress (<http://genome.oncology.wisc.edu/edge2/edge.php>) (21). In our earlier work, we found that hepatotoxins commonly downregulated many of the transcripts in this group, including the transcripts for Mup1-5 and Temt. Both these and related genes are down-regulated by TCDD as pathological endpoints worsen (i.e., they are anticorrelated). This observation leads us to suggest that common pathological states within the liver are the cause or consequence of this battery of down-regulated genes. It follows that transcripts from this group may provide molecular biomarkers for the correlated pathological endpoints induced by TCDD or a wide spectrum of hepatotoxic agents.

In our previous arrays work, we also observed that treatment of animals with hepatotoxic chemicals often results in a transient response from inflammatory cytokines such as IL1 α and tumor necrosis factor alpha (TNF α) (21). The action of these cytokines in downstream steps of TCDD hepatotoxicity is evident in the current data set. In this regard, the third largest group of correlated genes was found to contain a number of acute-phase genes, such as *Saa1*. As TCDD is not known to directly stimulate the IL-1 α pathway, and the battery is temporally delayed from the DRE-cluster, we conclude that the acute phase is a secondary response battery. The observation that the acute phase response is inversely related to induction of cytochrome P450s (Figure 8 and EDGE website) is probably due to the well-known activity of cytokines to repress the expression of particular P450s (30).

Testing of Downstream Batteries

Armed with an objective list of primary TCDD targets, we inquired as to how these genes might be responsible for the regulation of secondary gene batteries by using null alleles for candidates. We hypothesized that downstream transcriptional responses were secondary to a

DRE-regulated transcript. To test this idea we examined two well understood direct targets, *Cyp1a1* and *Cyp1a2*, using null animal models (Figure 9a). These results supported the existence of downstream targets of the primary DRE-battery implicating apolipoprotein-A4 and cytochrome P450 2a5 as downstream targets of *Cyp1a2* (Figure 9a and data not shown). Cytochrome P450 2a5 and *Apoa4* were selected as prototype downstream targets of *Cyp1a2* and confirmed in independent biological samples (Figure 9b, 9c and data not shown). The probe interrogated on the cDNA microarray shows greater than ninety percent homology to both *Cyp2a4* and *Cyp2a5*, but since only *Cyp2a5* is expressed in male animals, we conclude that the *Cyp2a5* is the affected gene (31). We suggest that these data show that *Cyp2a5*, previously labeled as AHR-responsive, is better described as *Cyp1a2* responsive (32). The mechanism by which *Cyp1a2* regulates *Cyp2a5* expression is unclear. Given that the *Cyp1a1* and *Cyp1a2* null genotypes had no effect on our defined histopathological endpoints, we suggest that *Cyp1a1* and *Cyp1a2*, as well as their downstream targets, might play a lesser role in the causality of TCDD toxicity.

In this study, we have characterized both the breadth and magnitude of the response of hepatic gene expression to TCDD. Through correlation analysis with prototype DRE-driven genes, we defined a list of primary target genes that directly respond to TCDD. Secondary and downstream targets were identified by a similar approach to genes and pathologies that were temporally distinct from primary events. We have further begun to separate the list of primary response genes into those that we believe to be direct (e.g. *HctD2*) from those that are dependent (e.g. *Cyp2a5*). Moreover, we have identified a series of transcriptional surrogates for hepatotoxicity that will aid in the quantitation of toxicity in future studies. This approach also allows for prioritization of candidate genes for future mechanistic studies.

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Figure 1.

Dose-response results: The heat map displays differential expression at 48 hours after five different (0.1, 1, 10, 25, and 64 µg/kg) doses of TCDD. At each dose, liver RNA was obtained from five treated mice, pooled, labeled and hybridized on the EDGE2 microarray containing 4,608 liver derived cDNAs. Ten vehicle treated animals were used to create a control pool that was then labeled and co-hybridized against labeled samples from each of the doses. Each comparison was performed as a dye-swap experiment, performing the hybridization in both color directions. Data from the two dye-swap hybridizations were then trimmed and averaged to produce a final fold ratio. Seventy-one targets that responded significantly were retained for K-means cluster analysis.

Figure 2.

Northern blot validation: Confirmation of targets identified by microarray analyses by Northern blot. Targets identified as dose-responsive were isolated, sequence-verified and used as radiolabeled probes against a Northern blot of total RNA isolated from livers used in the dose-response microarray analysis. All Northern blot results were normalized to levels of beta-actin (bottom). Results of Northern and microarray analyses were then compared, indicating high correlation between microarray and Northern analyses.

Figure 3.

Long-term TCDD toxicity study: microarray results. Heat map displays differential expression of genes across 64 days in response to TCDD. Sixty-three transcripts showed either 2-fold up-regulation or the same fold down-regulation in any one of the treatments. These changes are clustered in 5 groups using K-means analysis.

Figure 4.

Long-term TCDD toxicity study: Histology scoring showing dose-dependent increase in toxicity. The graphs show quantitative results of histological examination conducted using the Hematoxylin and Eosin (H&E) stained slides from respective doses and times. Histology observed after (A) data from 1 µg/kg TCDD dose (B) data from 10 µg/kg TCDD dose and (C) data from 64 µg/kg TCDD dose. Histological sections were scored blindly by two experienced technicians and assessed scores for hydropic degeneration, inflammatory cell infiltration and steatosis. The open squares denote hydropic degeneration, the closed triangles denote inflammatory cell infiltration, and the open triangles denote steatosis.

Figure 5. Long-term TCDD toxicity study: Dose dependent induction of hepatomegaly by TCDD. Representative H&E stained sections displaying varying grades of pathology. Histological photographs were taken from H&E stained slides at 400x magnification.

Figure 6.

Short-term TCDD toxicity study: microarray results. Heat map displays differential expression of genes from three doses of TCDD across eight time points. Animals were dosed with the indicated dose and sacrificed and the specified time. RNA from individual treated livers were labeled and hybridized on the EDGE2 microarray. At least two individual replicates were used for each dose-time point. A control cohort for each time point was used to create a RNA pool that was co-hybridized against the time-matched treated samples. Each comparison was performed as a dye-swap experiment, with the hybridization in both color directions. Data from the dye-swap hybridizations were then trimmed and averaged to produce a final ratio. Seventy-

seven targets that responded with a 2.4-fold or greater change were retained for K-means cluster analysis.

Figure 7.

Short-term TCDD toxicity study: Histology scores showing dose-dependent increase in toxicity. The graphs show quantitative results of histological examination conducted using the Hematoxylin and Eosin (H&E) stained slides from respective doses and times. Data from (A) 1 $\mu\text{g}/\text{kg}$ TCDD dose, (B) data from 10 $\mu\text{g}/\text{kg}$ TCDD dose and (C) data from 64 $\mu\text{g}/\text{kg}$ TCDD dose. Histological sections were scored blindly by two experienced technicians and scored for hydropic degeneration and inflammatory cell infiltration. The black lines denote hydropic degeneration, the blue lines denote inflammatory cell infiltration, and the red lines denote serum ALT leakage.

Figure 8.

Results of correlation-permutation test. All transcripts that changed significantly at least one time point across both short-term and long-term experiments were individually correlated with pathological endpoints and prototype marker genes. Positive Pearson correlation coefficients are displayed in yellow, showing that those transcripts followed a pattern of both up-regulation and down-regulation that was similar to the condition/gene. Negative correlation coefficients, shown in blue, denote transcripts with anti-correlation, that is responded in an opposite direction from the condition/gene. Gene Ontology enrichment was then run on the major four clades to look for overrepresented functions within those batteries of transcripts.

Figure 9.

Examination of the effects of *Cyp1A1*^{-/-} and *Cyp1A2*^{-/-} loci on TCDD-mediated toxicity. **A.** Hierarchical cluster of 19 targets that responded with a greater than 3-fold change in response to 64 μ g/kg TCDD over four days. Results indicate that the null alleles are not induced by TCDD along with downstream effectors such as CBG and *Cyp2A4*. **B.** Northern Blot analysis of *Cyp2a5* levels in WT, *Cyp1a1*^{-/-} and *Cyp1a2*^{-/-} mice. **C.** Quantitative analysis of northern blot shown in b, showing reduced induction of *Cyp2a5* in *Cyp1a2*^{-/-} animals.

Table 1 Novel target transcripts consistent with DRE-mediation. Transcripts that showed a positive Pearson correlation coefficient with known DRE-driven genes were subjected to permutation significance analysis. All genes included are considered hypothetical DRE-regulated, having both positive correlation (Pearson > 0.5) and low p-value (P < 0.001)

Gene	refseq	Cyp1A1	Cyp1A2	UGT1A6	GSTm1
CYP1a1	NM 009992.3	1.000	0.785	0.283	0.088
Gstm1	NM 010358.2	0.088	0.317	0.573	1.000
Ugt1a2	NM 013701.1	0.283	0.536	1.000	0.573
Cyp1a2	NM 009993.2	0.744	0.940	0.563	0.304
Tm4sf1	NM 008536.2	0.356	0.527	0.884	0.546
Elov15	NM 134255.2	0.278	0.472	0.742	0.344
Sep2	NM 011327.1	0.317	0.631	0.738	0.646
Por	NM 008898.1	0.172	0.427	0.691	0.186
Ugdh	NM 009466.2	0.656	0.660	0.537	0.255
Rps20	NM 026147.3	0.179	0.400	0.653	0.354
Htatip2	NM 016865.2	0.493	0.505	0.642	0.317
Es1	NM 007954.1	0.445	0.633	0.401	0.445
Cyp2c37	NM 010001.1	0.274	0.543	0.570	0.625
Akr1a4	NM 021473.2	0.259	0.502	0.624	0.235
Cyp1c29	NM 007815.2	0.279	0.424	0.522	0.619
4930542G03Rik	NM 146116.1	0.284	0.267	0.613	0.237
Slc35d1	NM 177732.4	0.529	0.500	0.609	0.116
Hsd17b2	NM 008290.1	0.581	0.580	0.579	0.336
creg1	NM 011804.2	0.454	0.499	0.514	0.566
MGI:1930666	NM 019814.2	0.282	0.566	0.533	0.354
Cyp2e50	NM 134144.1	0.042	0.220	0.513	0.561
Rpl38	NM 023372.1	0.393	0.503	0.554	0.127
Selenbp2	NM 019414.1	0.541	0.296	-0.124	-0.206
Selenbp1	NM 009150.2	0.527	0.338	0.022	-0.203
Ugt1a2	NM 013701.1	0.208	0.524	0.460	0.495
RPI41	NM 018860.2	0.173	0.386	0.523	0.014
Hax1	NM 011826.1	0.313	0.285	0.512	0.092