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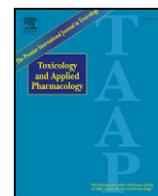
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Transcriptional profiling of rat white adipose tissue response to 2,3,7,8-tetrachlorodibenzo- ρ -dioxin



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ABSTRACT

Polychlorinated dibenzodioxins are environmental contaminants commonly produced as a by-product of industrial processes. The most potent of these, 2,3,7,8-tetrachlorodibenzo- ρ -dioxin (TCDD), is highly lipophilic, leading to bioaccumulation. White adipose tissue (WAT) is a major site for energy storage, and is one of the organs in which TCDD accumulates. In laboratory animals, exposure to TCDD causes numerous metabolic abnormalities, including a wasting syndrome. We therefore investigated the molecular effects of TCDD exposure on WAT by profiling the transcriptomic response of WAT to 100 $\mu\text{g}/\text{kg}$ of TCDD at 1 or 4 days in TCDD-sensitive Long-Evans (*Turku/AB*; L-E) rats. A comparative analysis was conducted simultaneously in identically treated TCDD-resistant Han/Wistar (*Kuopio*; H/W) rats one day after exposure to the same dose. We sought to identify transcriptomic changes coinciding with the onset of toxicity, while gaining additional insight into later responses. More transcriptional responses to TCDD were observed at 4 days than at 1 day post-exposure, suggesting WAT shows mostly secondary responses. Two classic AHR-regulated genes, *Cyp1a1* and *Nqo1*, were significantly induced by TCDD in both strains, while several genes involved in the immune response, including *Ms4a7* and *F13a1* were altered in L-E rats alone. We compared genes affected by TCDD in rat WAT and human adipose cells, and observed little overlap. Interestingly, very few genes involved in lipid metabolism exhibited altered expression levels despite the pronounced lipid mobilization from peripheral fat pads by TCDD in L-E rats. Of these genes, the lipolysis-associated *Lpin1* was induced slightly over 2-fold in L-E rat WAT on day 4.

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Background

2,3,7,8-tetrachlorodibenzo- ρ -dioxin (TCDD) is an organic toxicant introduced to the ecosystem as a by-product of industrial processes, such as low-temperature incineration of polyvinyl chlorides and pesticide production. A concerning biological property of TCDD lies in its

highly stable and lipophilic nature. Once in the body, TCDD primarily localizes to liver and adipose tissues (Gasiewicz et al., 1983), resulting in an average half-life of 3 weeks in rats and 8 years in humans (Pohjanvirta et al., 1990; Geyer et al., 2002).

TCDD is a particularly potent ligand of the aryl hydrocarbon receptor (AHR) (Safe, 1990), a basic helix-loop-helix/PAS (bHLH/PAS) transcription factor highly conserved throughout evolution (Hahn et al., 1997). Normally bound to chaperone proteins situated in the cytosol, the AHR translocates to the nucleus upon ligand binding and activation, where it dimerizes with the AHR nuclear translocator (ARNT). The AHR/ARNT dimer binds to specific DNA response elements (AHRE-I and AHRE-II) and regulates transcription in a gene-specific manner (Dolwick et al., 1993; Denison and Whitlock, 1995; Sogawa et al., 2004). Substantial evidence indicates a primary role for the AHR in mediating TCDD toxicities: AHR-knockout mice (Fernandez-Salguero et al., 1996; Mimura et al., 1997), mice with ARNT-null hepatic tissue (Nukaya

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo- ρ -dioxin; WAT, white adipose tissue; L-E, Long-Evans rat; H/W, Han/Wistar rat; AHR, aryl hydrocarbon receptor.

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et al., 2010) or mice with AHRs that have a defective AHRE-binding domain (Bunger et al., 2003) all display significantly diminished phenotypic effects of TCDD insult relative to wild-type mice.

Exposure to TCDD elicits a wide range of toxicities that vary substantially in both nature and degree between (and even within) species. In humans, the hallmark of high TCDD exposure is a dermal condition known as chloracne, whereas laboratory animals demonstrate a diverse range of toxicological endpoints and sensitivities to toxic effects (Pohjanvirta et al., 1993; Krasler et al., 2007). One of the best-documented effects of TCDD exposure is a wasting syndrome characterized by rapid weight loss and subsequent lethality (Seefeld et al., 1984). This wasting syndrome is dose-dependent. Its severity can be reduced by a high-calorie diet, although lethality persists (Courtney et al., 1978). Although wasting syndrome occurs in most laboratory rodent species, not all animals are affected equally. A well-established model of inter-strain variation comprises the TCDD-sensitive Long-Evans (Turku/AB) rats (L-E; LD₅₀ 9.8–17.7 µg/kg) and the TCDD-resistant Han/Wistar (Kuopio) rats (H/W; LD₅₀ > 9600 µg/kg) (Pohjanvirta et al., 1993). The exceptional resistance of the H/W strain, especially to wasting syndrome and lethality, is attributed to a point mutation in the transactivation domain of the AHR (Pohjanvirta et al., 1998, 1999).

White adipose tissue (WAT) is potentially a very important interface between TCDD toxicokinetics and wasting syndrome physiology since WAT plays many roles: an endocrine organ involved in the regulation of food intake and energy metabolism (Ahima and Flier, 2000), an immune organ (Exley et al., 2014) and a major location of sequestered xenobiotics (Mullerova and Kopecky, 2007), including TCDD (Pohjanvirta et al., 1990). Considerable amounts of TCDD are shown to accumulate in WAT of both L-E and H/W as early as 1 day after exposure (Pohjanvirta et al., 1990), with a significant body weight loss ($p < 0.01$) observed 4 days following exposure in L-E but not in H/W (Lensu et al., 2011; Linden et al., 2014). Therefore, to investigate the role of WAT in TCDD-induced toxicities, particularly pertaining to wasting syndrome, we isolated WAT from dioxin-sensitive L-E rats and dioxin-resistant H/W rats 1 and 4 days following exposure to TCDD or vehicle control. WAT was additionally isolated from L-E rats treated with vehicle control and subjected to feed restriction for 4 days. The inclusion of feed-restricted animals allows for the differentiation of transcriptomic changes directly associated with exposure to TCDD from those resulting from secondary effects pertaining to the reduction in feed intake experienced by dioxin-sensitive rats. As a similar weight loss was not observed in H/W rats, this comparison was not replicated for this strain.

Materials and methods

Animal handling

Inbred, male, Long-Evans (L-E) and Han/Wistar (H/W) rats were obtained from the breeding colonies of the National Public Health Institute (Kuopio, Finland). Animals were housed individually in suspended stainless-steel, wire-mesh cages with pelleted R36 feed and tap water available *ad libitum*, with one exception: a subset of L-E rats was feed-restricted in which feed was reduced to amounts ingested by animals that had wasting syndrome (as described in (Pohjanvirta et al., 2008)). The housing environment was maintained at a temperature of 21 ± 1 °C and relative humidity of $50\% \pm 10\%$, with a 12 hour light/dark cycle. H/W rats were 15–16 weeks of age at time of treatment, while L-E rats were 18–19 weeks of age to ensure comparable body weights due to more rapid growth of H/W rats.

Experimental design

The experimental design is outlined in Fig. 1. Eight L-E and eight H/W rats were equally divided into treatment and control groups. Animals were treated by oral gavage with either 100 µg/kg of TCDD

dissolved in corn oil or corn oil vehicle alone and euthanized 1 day after treatment. An additional cohort of twelve L-E rats was similarly divided into three groups, with two groups treated as above and the final group subjected to corn oil treatment accompanied by feed-restriction to mimic the reduced feed intake observed in TCDD-treated L-E rats (Pohjanvirta et al., 2008). This cohort was followed for 4 days, the point at which significant loss of body weight ($p < 0.01$) is observed in L-E but not H/W rats (Lensu et al., 2011; Linden et al., 2014). A similar experimental procedure has been described previously in studies of hepatic tissue (Linden et al., 2014). This treatment dose is essentially lethal to the TCDD-sensitive L-E rats, while being readily tolerated by the TCDD-resistant H/W strain. All animal treatment information is provided in Supplementary Table 1. At the end of the observation period, all animals were euthanized by decapitation; tissue was rapidly extracted and frozen in liquid nitrogen. All study plans were approved by the Animal Experiment Committee of the University of Kuopio and the Provincial Government of Eastern Finland. All animal handling and reporting comply with ARRIVE guidelines (Kilkenny et al., 2010).

Qiagen RNeasy kits were used to isolate total RNA from WAT according to the manufacturer's instructions (Qiagen, Mississauga, Canada). UV spectrophotometry was used to quantify total RNA yield and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was assayed on Affymetrix RAE230-2.0 arrays at The Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Canada), following the standard protocols.

Statistical analysis

Raw data were loaded into the R statistical environment (v.3.1.2) using the affy package (v.1.44.0) of the BioConductor open-source project (Gautier et al., 2004; Gentleman et al., 2004). Probes were mapped and summarized using the custom rat2302rntrezgdcdf (v.19.0.0) package (Dai et al., 2005). Raw data were pre-processed using the RMA algorithm (Irizarry et al., 2003) and tested for spatial and distributional homogeneity (Supplementary Fig. 1). Unsupervised pattern recognition employed the complete linkage hierarchical clustering algorithm in the cluster package (v1.15.3) using Pearson's correlation as a similarity metric. The distribution of coefficients of variation for each experimental group was analyzed to quantify inter-replicate variation (Supplementary Fig. 2).

All statistical analyses were performed using the limma package (v3.22.1). Linear modeling was performed for each probeset to contrast TCDD-treated and control animals at each time-point (i.e. HWT–HWC and LET–LEC for the 24 h groups and LET–LER for the 96 h groups). An empirical Bayes method was used to reduce standard error amongst probes (Smyth, 2004) and moderated *t*-tests were used to compare each coefficient to zero. All experimental *p*-values were adjusted for multiple testing using a 5% false discovery rate (Storey and Tibshirani, 2003). Following *q*-value analysis, significance was defined as a *q*-value threshold <0.05. Results of the linear model are available in Supplementary Table 2. Raw and pre-processed data are available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO ID: GSE18301).

Data visualization

Visualizations were generated using the lattice (v.0.20–29) and latticeExtra (v.0.6–26) packages. Normalized intensity values for the most variable probes across all samples (variance >0.1) were clustered using DIANA agglomerative hierarchical clustering, again using Pearson's correlation as a similarity metric. To visualize differences in tissue sensitivities following TCDD exposure, volcano plots were generated to compare results from multiple studies (Supplementary Fig. 3). Current array results from the 24 h time point were compared to results from similar studies of hypothalamus (Houlihan et al., 2014) and liver (Yao et al., 2012) that employed similar treatments. A Venn diagram

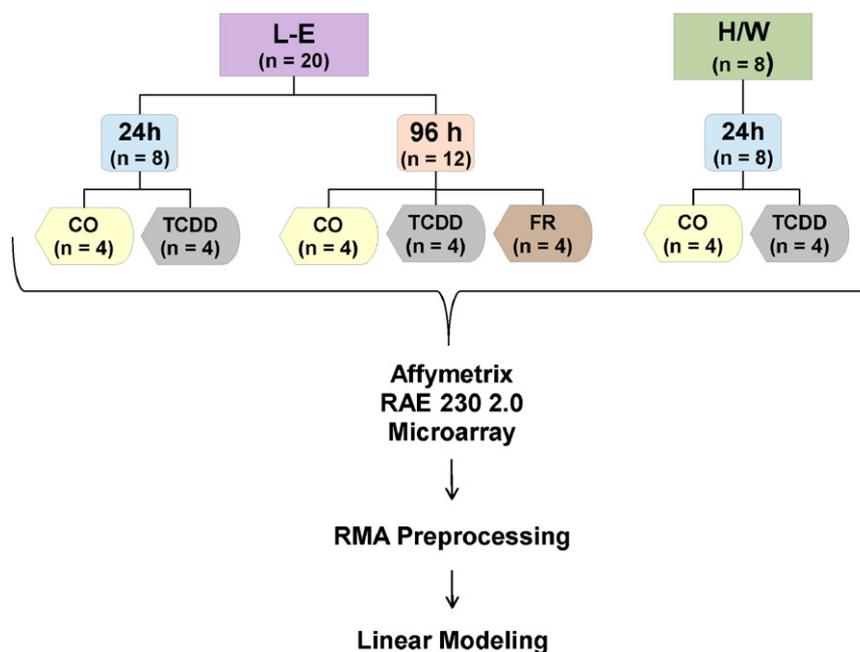


Fig. 1. Experimental design. Twenty-eight male rats were employed to evaluate TCDD-induced transcriptomic effects on adipose tissue at early (1 day) and late (4 days) time points following TCDD (100 µg/kg) exposure. Control animals at each time point were treated with either corn oil vehicle or a feed-restrictive diet (low-calorie diet) to mimic food intake in animals that had wasting syndrome. mRNA extraction, data preparation and analyses are described in the methods.

was generated to visualize overlapping genes significantly altered in each experimental group using the VennDiagram (v.1.6.9) package (Chen and Boutros, 2011). For each group, significantly altered genes were evaluated for chromosomal bias using hypergeometric testing (Supplementary Table 3). Target genes were identified using a dual threshold ($|\log_2\text{fold-change}| > 1$ and $q < 0.05$) and visualized using dotmaps.

Transcription-factor binding analysis

Since the AHR is known to associate with conserved response elements during transcriptional regulation, the presence of these elements within genes of interest was examined. The occurrence (count) and conservation (score) of three motifs, AHRE-I (core), AHRE-I (full) and AHRE-II with the sequences GCGTG, [T]G[NGCGTG[A]C][G]C[A and CATG{N6}C[T]A]TG, respectively were audited (Denison and Whitlock, 1995; Sogawa et al., 2004). Using REFLINK and REFFLAT tables from UCSC genome browser data (rn4, downloaded on May 9, 2012) (Karolchik et al., 2003), transcription start sites were determined. For each gene, a PhyloHMM conservation score was calculated to assess conservation across species, zero indicates low conservation and one represents high conservation. Genes were categorized based on the number of experimental groups in which they achieved statistical significance and the distribution of occurrence and conservation scores for each motif were visualized (Supplementary Fig. 4).

Human–rat comparison

Comparison with human cell lines was carried out using data from human multipotent adipose-derived stem (hMADS) cell lines treated with persistent organic pollutants, including TCDD, for 48 h (Kim et al., 2012). Raw data for all available treatments were obtained (GSE32026). The data contained two subtypes of hMADS cells: undifferentiated and differentiated. Data were pre-processed in the R statistical environment (v3.0.2) and modeled using the limma package (v3.16.7). Each subtype was normalized and modeled independently. Data were \log_2 -transformed and normalized using global loess smoothing in an attempt to mirror the original procedure conducted by the authors. Linear

modeling was performed as described above to identify differences between treated and control groups. All genes were annotated with Homologene IDs (HIDs) where applicable, matched using Entrez Gene IDs (Supplementary Table 4). Homologene data was obtained from the National Center for Biotechnology Information Homologene database (downloaded on December 10, 2013). Hypergeometric testing was used to assess chromosomal enrichment of significantly altered genes (Supplementary Table 5). A Venn diagram was used to compare significantly altered genes in the rat and hMADS cell line datasets (Supplementary Fig. 5), as described above.

Pathway analysis

Gene Ontology analysis was performed using the GOMiner web interface (v.2011-01) (Zeeberg et al., 2005). For rat data, genes that were significantly altered ($q < 0.1$) by TCDD were compared with all genes available on the array to identify enriched functional pathways. Each experimental group was assessed independently. Analysis was performed using all rat databases, look-up options and gene ontologies. Similarly, for hMADS cells, significantly altered genes ($q < 0.05$) were assessed using all human databases, look-up options and gene ontologies. All analyses utilized a null distribution generated using 1000 permutations and a false discovery rate threshold of 0.1. The minimum category size in all incidences was set to five. For rat data, all GO terms and enrichment scores are provided in Supplementary Table 6, while GO terms and enrichment scores for hMADS cells are available in Supplementary Tables 7–8.

NanoString validation

Nine “AHR-core” genes, as well as a subset of 14 genes determined to be significantly altered ($|\log_2\text{fold-change}| > 1$ and $q < 0.05$) in at least one experimental group were selected for validation using NanoString custom gene expression assays. The target list was submitted in advance and the required CodeSet was developed by NanoString. Total RNA (>100 ng) was shipped on dry ice to the Princess Margaret Genomics Centre (Toronto, ON) for analysis. Once complete, data were analyzed as described previously (Houlihan et al., 2014). Briefly, raw data were

collected and normalized in the R statistical environment (v3.1.2) using the NanoStringNorm (v1.1.18) package (Waggott et al., 2012). Endogenous probes were adjusted first using the positive controls followed by normalization for sample content (using the ‘sum’ and ‘housekeeping.geo.mean’ methods respectively) using housekeeping genes (*Hprt1*, *Pgk1* and *Sdha*) suggested previously (Pohjanvirta et al., 2006). Normalized data were log₂-transformed and visualizations generated as above.

Results

Experimental approach

To identify whether changes in abundance of specific mRNAs in WAT are associated with TCDD-induced toxicities (wasting syndrome in particular), the transcriptomic profiles of WAT from L-E and H/W rats were compared (Fig. 1). L-E rats are highly sensitive to TCDD-induced lethality and demonstrate a rapid and irreversible reduction in feed intake. H/W rats are exceptionally refractory to these effects and experience only a temporary reduction in feed intake before returning to near normal levels (Lensu et al., 2011). Transcriptomic changes specific to the TCDD-sensitive L-E rats identified 1 day after treatment coincide with the onset of toxicity. At the 4-day time point, L-E rats demonstrate a significant reduction in body weight relative to control animals (Linden et al., 2014). To allow for identification of TCDD-dependent/feed-independent transcriptomic changes in L-E rats treated for 4 days, feed restricted animals were used for comparison.

Transcriptomic responses to TCDD in WAT

To provide a general overview of differences in the transcriptomic profiles of WAT from each experimental condition, normalized intensity values from the most variant genes (variance > 0.1; $n_{\text{genes}} = 1859$) were visualized and subjected to unsupervised pattern recognition (Fig. 2A). Clusters were largely associated with strain differences and time of collection. To estimate the relative sensitivity of WAT to transcriptomic changes caused by TCDD, the 1-day experiments were compared to transcriptomic analyses of liver (Yao et al., 2012) and hypothalamus (Houlihan et al., 2014) collected from H/W and L-E rats following similar treatments. Liver was confirmed to be the most responsive amongst these tissues, with more genes revealing altered expression by TCDD and showing changes of greater magnitude and significance than non-hepatic tissues in both strains (Supplementary Fig. 3). Both hypothalamus and WAT exhibit relatively few genes with altered mRNA abundance following treatment with TCDD at the 1-day time point.

Following linear modeling, substantially more genes displayed altered abundance in L-E at 4 days than in any other condition (Fig. 2B). At a significance threshold of $q < 0.05$, expression patterns of 136 genes were significantly altered 4 days after TCDD treatment in L-E rats (relative to corn oil treated, feed-restricted controls), while only nine and five genes showed altered expression 1 day after exposure in either L-E or H/W WAT respectively (Fig. 2C). Only three genes had altered mRNA abundance following treatment in all three groups (i.e. *Cyp1a1*, *Nqo1* and *Stab1*), while an additional three genes (i.e. *Ahrr*, *F13a1* and *Ms4a7*) showed differential transcription in L-E animals at both time points.

A subset of genes has been termed “AHR-core”, and has been shown to have significantly altered abundance resulting from TCDD-activation of the AHR in multiple species and tissue types (Nebert et al., 1993, 2000; Yeager et al., 2009; Watson et al., 2014). Most of these were measured in our experiment, although *Cyp1b1* was not represented on the array. The magnitude and significance of TCDD-induced transcriptional changes to “AHR-core” gene mRNAs in WAT were examined (Fig. 3A). As noted above, levels of *Cyp1a1* (>30-fold induction) and *Nqo1* (>2-fold induction) mRNAs were markedly induced in all treatment groups, consistent with AHR activation by TCDD in this tissue.

Furthermore, *Ahrr* was significantly up-regulated in L-E rats at both time points. Comparison with additional datasets (Boutros et al., 2009; Kim et al., 2012; Yao et al., 2012; Houlihan et al., 2014) confirms that these genes are altered by TCDD in a wide variety of species and tissues.

A set of 19 genes demonstrated significantly altered expression ($|\log_2 \text{fold-change}| > 1$ and $q < 0.05$) in at least one group and was evaluated further (Fig. 3B). As with the “AHR-core” genes, this subset was contrasted with results from additional species and tissue types (Boutros et al., 2009; Kim et al., 2012; Yao et al., 2012; Houlihan et al., 2014). Abundance of *Stab1* mRNA was altered in WAT from H/W rats at 1 day and L-E rats at both time points, as well as in hypothalamic tissue from both strains. Only one gene demonstrated altered abundance in H/W but not L-E rats (*Gatm*).

Validation of TCDD-altered genes

To supplement the array experiments, a subset of genes that were found to be TCDD-responsive, including nine “AHR-core” and 14 genes of interest, were assessed using the NanoString system. The TCDD-responsiveness of AHR-core genes in WAT was confirmed as *Cyp1a1*, *Cyp1b1* (which had not been tested on arrays) and *Nqo1* all displayed significant induction in all three experimental groups (Fig. 4, top panel). In addition, the enhanced sensitivity of the NanoString technology (Geiss et al., 2008; Prokopec et al., 2013) detected significant changes in abundance of *Tiparp* in all three groups, as well as *Nfe2l2* in H/W and *Aldh3a1* in L-E rats 4 days post exposure. Of the genes of interest, 8/14 were fully validated (in all experimental groups), while 6/14 were validated in L-E rats and showed moderate, previously-undetected alterations in H/W rats using the more sensitive NanoString measurements (Fig. 4, bottom panel).

Functional analyses

To determine whether observed transcriptional changes might be attributed to direct transcriptional regulation by the AHR, the rat reference genome was searched and genes were examined for the presence/absence of known response elements. Specifically, an area of 3 kbp to either side of the transcription start site was examined for AHRE-I (core), AHRE-I (full) and AHRE-II motifs (see Materials and methods) and conservation across experimental groups was assessed (Supplementary Fig. 4). The AHRE-I (core) motif (Supplementary Fig. 4A) existed most often within the set of responsive genes and was highly conserved across species. AHRE-I (full) was highly conserved amongst those genes whose abundance was significantly altered ($q < 0.05$) in all three experimental groups while AHRE-II was not (Supplementary Fig. 4B–C). *Gatm* contained both the highly conserved AHRE-I (core) motifs and a poorly conserved AHRE-II motif.

To relate TCDD-dysregulated genes to putative functional consequences, pathway analysis was performed using Gene Ontology enrichment analysis (Zeeberg et al., 2005). Three terms were identified as significantly enriched ($q < 0.1$) in L-E rats only, 1 day after treatment – all of which related to S phase of cell cycle control. Many more terms were enriched in L-E rats, 4 days after exposure, including many lipid metabolic processes, while none were significantly enriched in H/W rats (Supplementary Table 6).

Rat WAT–hMADS comparison

Finally, to compare our analyses in rat WAT to what is known about transcriptional responses to TCDD in human WAT, we analyzed published data on hMADS cells (Kim et al., 2012). We first examined the “AHR-core” genes (Nebert et al., 1993, 2000; Yeager et al., 2009; Watson et al., 2014) as previously. Of these, only *Ahrr* and *Cyp1b1* were significantly responsive to TCDD in both differentiated and undifferentiated hMADS cells. Expression of *Nqo1* was altered only in differentiated hMADS cells while *Tiparp* responded only in undifferentiated

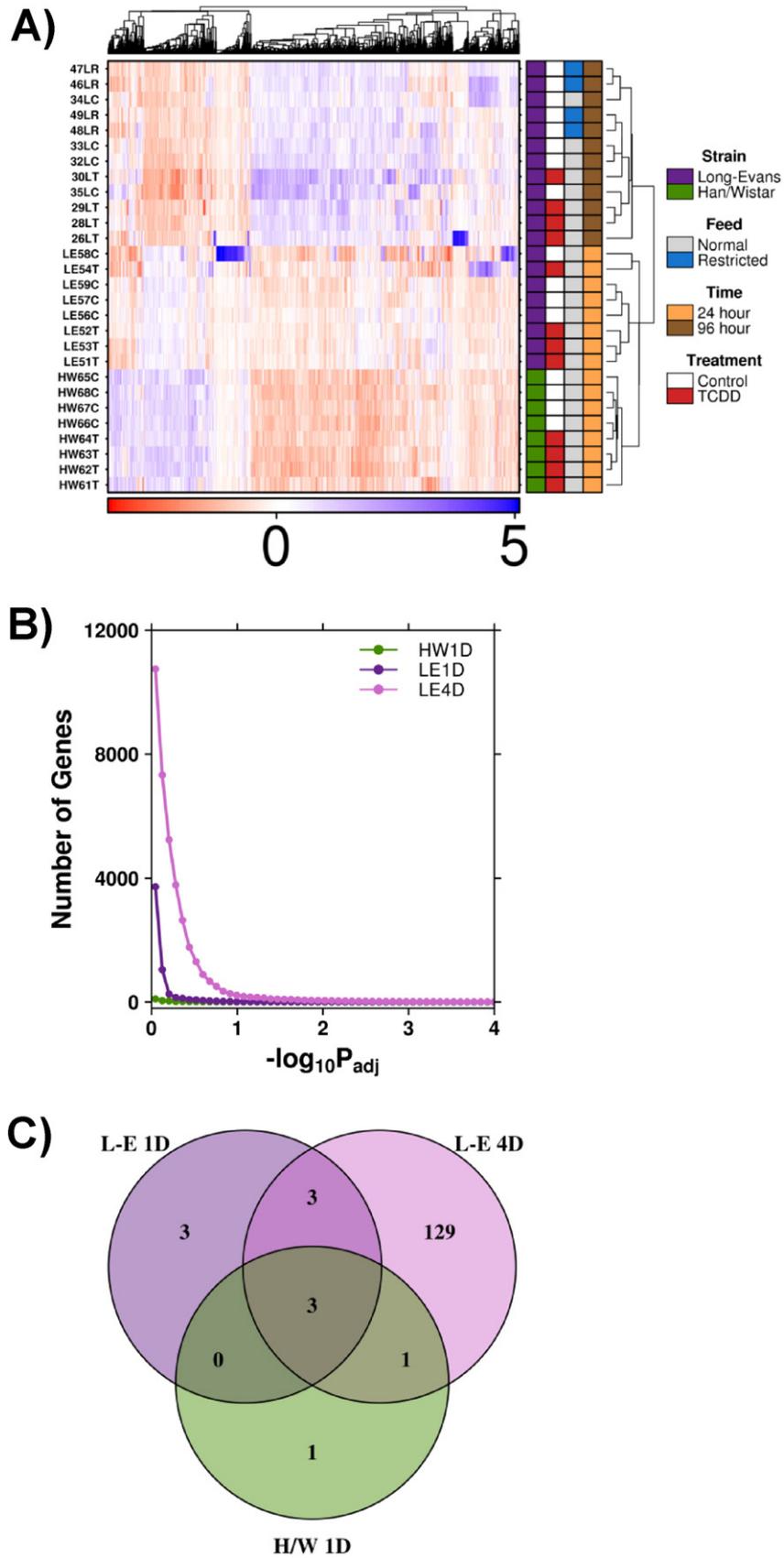


Fig. 2. Overall transcriptomic profile. (A) Following pre-processing, the most variable genes across all samples (variance >0.1) were chosen for unsupervised hierarchical clustering using the DIANA algorithm to identify abundance patterns. (B) Linear modeling was used to identify differences between treatment groups and results were subjected to *q*-value analysis. The number of genes determined to be differentially altered in each group is shown at various *q*-value thresholds. (C) Significantly altered genes (*q*-value < 0.05) were compared between each experimental group.

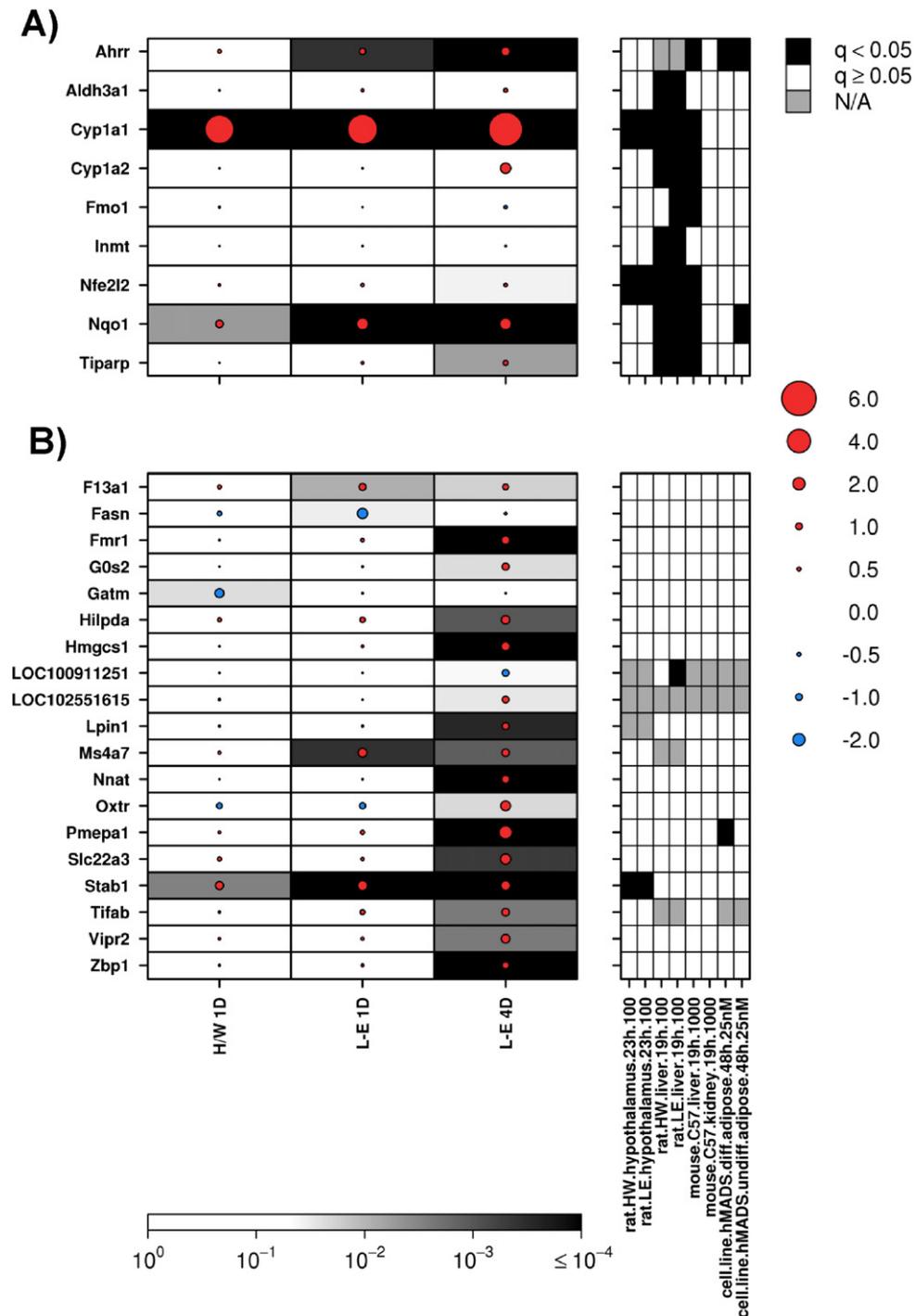


Fig. 3. TCDD-mediated response. A subset of genes including (A) “AHR-core” response genes and (B) those genes determined to be significantly altered (absolute \log_2 (fold-change) > 1 and $q < 0.05$) in at least one experimental group were further examined. Dot size represents magnitude of change (in \log_2 space), dot color indicates direction of change (red = increased abundance; blue = decreased abundance) and background shading indicates significance of change (q -value). Covariates reflect significance status (white = q -value ≥ 0.05 ; black = q -value < 0.05; gray = data unavailable) in additional species and tissue types.

hMADS cells. Surprisingly, expression of *Cyp1a1* was not affected by TCDD in either type of hMADS cells (Supplementary Table 4). Next, we compared our rat data directly with the human data, using only homologous genes and a threshold of $q < 0.05$. No overlap was observed in the transcriptomic response between hMADS cells (either differentiated or undifferentiated) and H/W rat WATs (Supplementary Fig. 5A). By contrast, abundance of one gene (*Ahrr*) was significantly altered following TCDD exposure in differentiated as well as undifferentiated hMADS cells and in L-E rats at both time points. Four genes (*Atp13a1*, *Cep41*, *Nmt2* and *Pmepa1*) demonstrated altered mRNA abundance in

both the L-E (4 days) and differentiated hMADS cells. Of these, *Atp13a1* expression was altered to a similar magnitude, however in opposite directions, being induced in L-E rat and showing decreased abundance differentiated hMADS cells. Three genes (*Plce1*, *Plin4* and *Tiparp*) displayed changed abundance in L-E WAT (4 days) and undifferentiated hMADS cells with *Plce1* and *Plin4* altered in opposite directions (reduced in undifferentiated hMADS, induced in L-E rat) (Supplementary Fig. 5A). Hypergeometric testing indicates that the degree of overlap between L-E (4-day) rats and either group of hMADS cells is not significantly more than expected by chance alone.

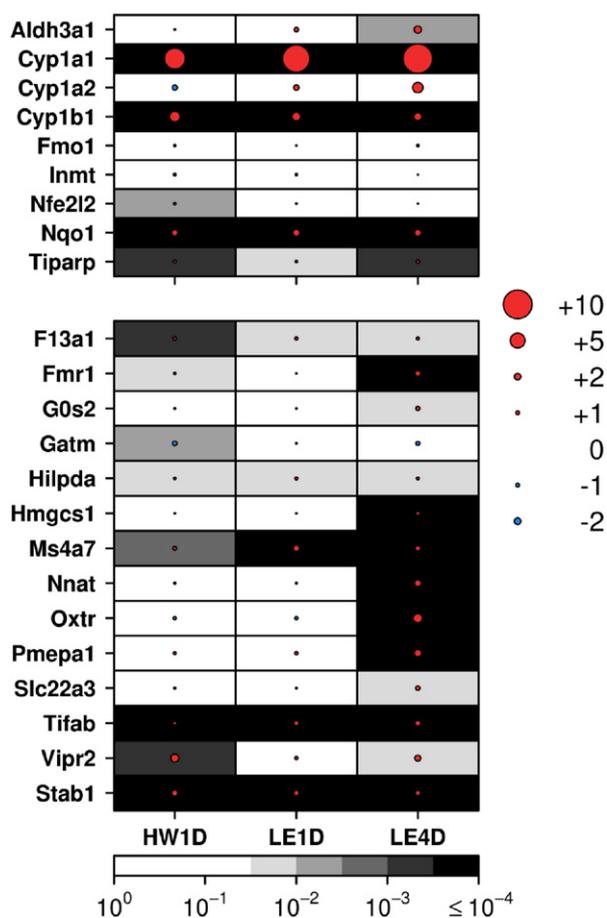


Fig. 4. NanoString validation of genes of interest. A subset of 9 “AHR-core” genes and 14 genes of interest were selected for validation by NanoString. Dot size depicts magnitude of the change (\log_2 fold change) while color depicts direction – up (red) or down (blue). Background shading reflects the q -value.

On the whole, differentiated hMADS cells appeared more transcriptionally responsive to TCDD treatment than undifferentiated hMADS, with a total of 611 genes (418 human–rat orthologs) and 307 genes (212 homologous genes) demonstrating altered expression. Pathway analysis using GOMiner indicated numerous significantly enriched gene ontologies in both sets of hMADS cells: 347 and 679 pathways enriched in differentiated and undifferentiated hMADS cells respectively (Supplementary Tables 7–8). Of these, 194 altered pathways were common to both cell subtypes. The top ten pathways affected in both differentiated and undifferentiated cells includes those involved in anatomical structure morphogenesis, cell development, cell migration, vasculature development, response to external stimulus and cell motility. As above, hypergeometric testing indicated that no particular chromosomes were enriched for genes responsive to TCDD (Supplementary Table 5).

Discussion

White adipose tissue (WAT) is the main site for long-term storage of high energy lipids, as well as one key location in which TCDD accumulates. For this reason, we hypothesized that WAT would play a significant role in the pathogenesis of TCDD-induced toxicity. To investigate this potential link, the transcriptomes of WAT from TCDD-sensitive Long-Evans (L-E) and TCDD-resistant Han/Wistar (H/W) rats were analyzed 1 day following TCDD treatment. In addition, WAT from L-E rats at 4 days following treatment with TCDD or feed restriction was also examined. The early time point coincides with the onset of wasting syndrome, as food consumption is depressed as early as 24 h following

TCDD exposure in L-E rats and – to a lesser degree – by 48 h in H/W rats (Lensu et al., 2011). The later time point allowed detection of distinct TCDD-mediated transcriptional changes – those not strictly resulting from metabolic changes due to reduced feed intake – when compared with animals in the feed-restriction cohort (Pohjanvirta et al., 2008).

Our analysis revealed that the largest number of transcriptomic changes occurred in L-E rats 4 days after treatment. In rats, TCDD has a half-life of ~3 weeks (Pohjanvirta et al., 1990; Geyer et al., 2002). Additionally, previous studies on the toxicokinetics, distribution and metabolism of TCDD indicated a large accumulation in L-E WAT as early as 4 h post-exposure and gradually increasing to maximum levels at the 8-day mark (Pohjanvirta et al., 1990). The prolonged presence of TCDD may continue to activate the AHR long after the initial exposure. In response, L-E WAT shows increased abundance of mRNAs for both *Ahr* and *Tiparp* (MacPherson et al., 2014), potentially leading to the observed decreased abundance of *Ahr* mRNA at 4 days. However, these actions are not entirely sufficient to halt transcriptional regulation by AHR, as shown by the increasing expression of the prototypical “AHR-core” gene, *Cyp1a1*. In addition, the presence of AHREs in the majority of TCDD-responsive genes in L-E at 4 days again indicates the probable involvement of the AHR. These results suggest a combination of both primary TCDD-mediated effects and secondary, adaptive or maladaptive effects in the onset and progression of toxicities.

Expression of two genes (*Ms4a7* and *F13a1*) was induced approximately 2-fold in L-E rats at both time points, but not in H/W rats. *Ms4a7* and *F13a1* were each found to contain a single AHRE-II motif. *F13a1* encodes coagulation factor XIII, a subunit involved in fibrin stabilization during blood clotting (Barry and Mosher, 1990). *F13a1* expression is inversely correlated with HDL levels, and its mRNA abundance decreases following insulin treatment (Laurila et al., 2013), suggesting a role in lipid metabolism. In L-E rats, serum HDL cholesterol is elevated from day 4 onward (Pohjanvirta et al., 1989), whereas circulating insulin levels drop significantly as early as day 1 and show a progressive downward trend thereafter (Linden et al., 2014). Thus, our findings are consistent with a view that a major regulator of *F13a1* expression is insulin. *Ms4a7* encodes membrane spanning 4-domains subfamily A member 7, a transmembrane protein primarily expressed in lymphoid tissues (Ishibashi et al., 2001; Liang and Tedder, 2001) and hypothesized to be involved in signal transduction.

The oxytocin receptor gene (*Oxtr*) has previously been shown to be drastically repressed in WAT of feed-restricted rats, 4 days after the onset of the feeding regimen (Pohjanvirta et al., 2008). Consistent with these results, *Oxtr* was repressed in WAT of both TCDD-treated rats and feed-restricted rats, when compared with rats given corn oil alone (data not shown). Interestingly however, mRNA abundance of *Oxtr* was increased following TCDD treatment as compared with feed-restriction alone (Fig. 3B). Thus, TCDD-mediated changes in abundance of *Oxtr* may warrant further investigation.

Stab1 (transmembrane protein Stabilin 1) was significantly up-regulated in both WAT and hypothalamic tissue of both L-E and H/W rats (Houlahan et al., 2014). This gene encodes a receptor protein typically expressed by macrophages which aids in the removal of apoptotic cells and is therefore involved in the immune response (Park et al., 2009). Increased abundance of *Stab1* mRNA is likely a response to TCDD-induced cell death. Interestingly, lipolysis rapidly induces macrophage infiltration in adipose tissue (Kosteli et al., 2010) and the dose of TCDD used in the present study (100 $\mu\text{g}/\text{kg}$) results in a marked reduction of adipose triglycerides, diacylglycerols and the bulk of phospholipids in L-E rats already on day 1 (our unpublished data). On the other hand, lipid accumulation, as occurs in obesity, causes macrophage numbers to increase in adipose tissue (Red Eagle and Chawla, 2010), and H/W rats display a mirror image of lipid changes to those of L-E rats on day 1 after exposure to 100 $\mu\text{g}/\text{kg}$ TCDD (our unpublished data). Thus, an identical outcome in the expression level of *Stab1* in the two rat strains might emanate from two opposite effects of TCDD.

Wasting syndrome is one of the hallmark toxicities of TCDD and is particularly obvious in L-E rats, with significant body weight loss observed by day 4 in this strain that is not observed in H/W rats (Linden et al., 2014). The extent of weight loss experienced by TCDD-treated L-E rats is mimicked by rats subjected to feed-restriction (Linden et al., 2014). Expression of *Lpin1* (lipin 1) is known to be affected by nutritional status, as well as by serum leptin and adiponectin levels (Gonzalez et al., 2012). As these factors are not significantly different between TCDD-treated L-E rats and feed-restricted controls by day 4 (Linden et al., 2014), increased abundance of *Lpin1* in TCDD-treated animals relative to feed-restricted controls may be associated with increased lipolysis and thus with wasting syndrome. In addition, lipin 1 is a known transcriptional coactivator of other lipid metabolism-associated genes (Finck et al., 2006). The ability of H/W rats to maintain body weight may be related to the reduced expression of *Gatm* following TCDD exposure. *Gatm* encodes mitochondrial glycine amidinotransferase – a key enzyme in the creatine biosynthetic pathway. Reduced abundance of *Gatm* mRNA may be part of the adaptive mechanism for energy conservation.

We also compared transcriptomic responses to TCDD in rat WAT *in vivo* with previously published data (Kim et al., 2012) on the effects of TCDD on human adipocytes in culture. In general, there was a low degree of similarity in the transcriptomic responses of TCDD-treated hMADS cells versus those of rat WAT. *Ahrr* demonstrated the greatest interspecies overlap, significant in both differentiated and undifferentiated hMADS cells as well as in L-E WAT at both 1 and 4 days. Contrary to expectation, *Cyp1a1*, which was markedly upregulated in both rat strains, was not noticeably changed in hMADS cells, whereas *Cyp1b1*, which was found to be upregulated by TCDD in rat WAT (NanoString assay), was significantly up-regulated in both types of hMADS cells. *Pmepa1*, which had the highest fold change in L-E WAT at 4 days, was also altered in differentiated hMADS cells. Over-expression of *Pmepa1*, prostate transmembrane protein, androgen induced 1, has been implicated in several types of cancer, including lung (Hu et al., 2013; Vo Nguyen et al., 2014) and prostate (Liu et al., 2011). The product of this gene modulates TGF- β -mediated signal transduction (Watanabe et al., 2010). The induction of *Pmepa1* following TCDD exposure may have a role in the development of toxic outcomes. Although the similarities appear sparse between rat WAT and human adipocytes, it is important to note that there are several barriers in performing such comparisons. Numerous factors, including variations in experimental parameters (dosage of TCDD, concentration at the cellular level, length of exposure) present major challenges for this comparison. There also are clearly challenges in comparing cell lines to intact organisms. The complexity of molecular processes that occur within organisms is far greater than that of single-cell systems. The fact that most changes occurring in hMADS cells were relatively modest could be partially attributed to this.

In summary, TCDD treatment was found to have only a modest impact on the transcriptomic profile of WAT from L-E rat by 4 days following exposure, especially in comparison with the extensive, and well-studied, responses to TCDD in both intact liver and hepatocytes in culture. Although several “AHR-core” response genes were found to have altered expression, the overall WAT transcriptome was only marginally affected by TCDD treatment, relative to hepatic tissue and regardless of strain. The paucity of transcriptomic responses to TCDD after 1 day in WAT suggests that WAT is not the site of initial insult that triggers TCDD toxicity even though WAT reservoirs are profoundly affected during subsequent wasting. However, expression of several genes was found to be significantly altered and may provide insight into the mechanisms of TCDD-induced toxicities. Increased expression of *Ms4a7*, *F13a1* and *Pmepa1* may play a role in the pro-inflammatory outcomes which have been observed in the TCDD-sensitive L-E rats following TCDD exposure. Alternatively, suppression of *Gatm* in the TCDD-resistant H/W rats following treatment may encourage energy conservation during the period of feed-reduction. Minimal overlap was detected between rat models and human adipocytes and further

studies are necessary to outline the mechanisms involved in TCDD-induced toxicities within these species.

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Conflict of interest statement

All other authors declare that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found online.

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