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EXPRESSION PROFILING OF INTERINDIVIDUAL VARIABILITY FOLLOWING XENOBIOTIC EXPOSURES IN PRIMARY HUMAN HEPATOCYTE CULTURES

Katy M. O. Goyak¹, Mary C. Johnson¹, Stephen C. Strom², and Curtis J. Omiecinski^{1,3}

¹Dept of Veterinary and Biomedical Sciences, Penn State University, University Park, PA, 16802 USA.

²Dept of Pathology, University of Pittsburgh, Pittsburgh, PA, 15261 USA.

Abstract

In this investigation, we profiled interindividual variability of gene expression responses to three different prototypic chemical inducers in primary human hepatocyte cultures from ten independent donors. Correlation between basal expression in any two hepatocyte donors ranged from r^2 values of 0.967 to 0.857, and chemical treatment tended to negatively impact correlation between donors. Including anticipated target genes, 10812, 8373, and 7847 genes were changed in at least one donor by Aroclor 1254 (A1254), di(2-ethylhexyl) phthalate (DEHP), and phenobarbital (PB), respectively. A subset of these gene targets ($n=41$) were altered with a high level of reproducibility in at least 9 donors, gene responses that correlated well with literature-reported mechanism of action. Filtering responses to the level of gene subsets clarified the biological impact associated with the respective chemical effectors, in lieu of substantial interindividual variation among donor responses. In these respects, the use of hierarchical clustering methods successfully grouped seven of the ten donors into chemical-specific rather than donor-specific clusters. However, at the whole genome level, the magnitude of conserved gene expression changes among donors was surprisingly small, with fewer than 50% of the gene responses altered by a single chemical conserved in more than one donor. The use of higher level descriptors, such as those defined by the PANTHER classification system, may enable more consistent categorization of gene expression changes across individuals, as increased reproducibility was identified using this method.

Keywords

DNA microarray; primary human hepatocytes; interindividual variability

Introduction

Primary human hepatocyte cultures are recognized as the most appropriate *in vitro* system with which the *in vivo* liver can be modeled for studies of xenobiotic metabolism and biotransformation (Gomez-Lechon *et al.*, 2003; LeCluyse, 2001). Although advantages of the human primary hepatocyte model, such as elimination of metabolism extrapolation across

³Address correspondence to: Curtis J. Omiecinski, Center for Molecular Toxicology and Carcinogenesis, 101 Life Sciences Building, The Pennsylvania State University, University Park, PA 16802, Tel. 814-863-1625, Fax 814-863-1696, Email: cjo10@psu.edu.

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species and increased predictability potential for idiosyncratic toxicity, are attractive, interindividual variability is problematic, as widespread differences in metabolism among donors and hepatocyte preparations have been cited as a limitation for data reproducibility (Liguori *et al.*, 2005; Slatter *et al.*, 2006). Issues of genetics as well as technical factors such as the surgical, storage and transfer conditions and pathological state of the donor liver tissue may all additionally contribute to variability in response (Baccarani *et al.*, 2003; Fisher *et al.*, 2001; Lloyd *et al.*, 2004).

In studies that examine genome-wide transcriptional responses to chemical treatment, the potentially complicating nature of interindividual variability has led to difficulty in how to construe differences manifesting at the transcriptome scale. In an effort to minimize variability across samples, some studies have pooled RNA from multiple donors (Huang *et al.*, 2007; Piton *et al.*, 2005), while others have only used hepatocytes from a single donor (Keum *et al.*, 2006; Ryu *et al.*, 2006). Variability across donors complicates the determination of which genes are regulated, in that some studies report findings in each individual donor (Thum & Borlak, 2007), some follow a stringent criteria such that genes of interest must be changed in all donors (Harris *et al.*, 2004; Liguori *et al.*, 2005; Radaeva *et al.*, 2002), some apply a filtering system so that genes of interest are those changed in a particular number of donors (Richert *et al.*, 2003), while others report average expression across donors (Li *et al.*, 2007; Rae *et al.*, 2001). Overall, a limited number of donors are generally included ($n=2-6$), and surprisingly few genes survive the filtering conditions: Richert *et al.* parsed a 38 gene dataset based on the >12'000 genes on their array; Thum and Borlak, 44 of 302 possible genes; Liguori *et al.*, 142 of >22'000; Harris *et al.*, 867 of 31'000; and Radaeva *et al.*, 53 of 12'000. The apparent trend emerging from these studies is that, regardless of the nature of the chemical of interest, only small sets of responsive genes are regulated reproducibly by xenobiotic exposures among hepatocytes across individual donors, whereas large subsets of genes are uniquely regulated within individual donors. Despite this trend, there is often little consideration given to the apparent vast magnitude of genes changed in a donor-specific manner.

To broaden these analyses regarding the magnitude of human variability existing in whole-transcriptome responses to chemical challenge, we performed whole-genome expression profiling analyses following treatments with three different prototypic chemical inducers using hepatocytes from ten independent donors, maintained under primary culture techniques that largely preserve highly differentiated character endpoints associated with mature hepatocytes (Olsavsky *et al.*, 2007; Page *et al.*, 2007). Comparisons among donors were performed both at basal conditions and after treatments with Aroclor 1254 (A1254), an environmental contaminant that consists of a mixture of polychlorinated biphenyls (PCBs), di(2-ethylhexyl) phthalate (DEHP), a plasticizer agent belonging to the peroxisome proliferator class of chemicals that activate the rodent PPAR α receptor, and phenobarbital (PB), a barbiturate drug used as an anti-seizure agent. These chemicals were selected both for their diverse mechanisms of action and for the wealth of information available on the effects that these chemicals have in a variety of other model systems. Using ten human hepatocyte donors, we report findings in agreement with those from previous studies, that large subsets of genes were regulated according to individual donors, whereas a relatively small set of target genes was regulated consistently in response to individual chemical treatment across donors. However, we show that by using a PANTHER-based functional category analysis rather than a gene-level approach, more consistent identification of transcriptional patterns are detected across donors treated with common agents, patterns that are concordant with known mechanisms of action for the respective agents and indicative of biological themes identified in other model systems.

Methods

Cell culture

Primary human hepatocytes were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System, NIH Contract #N01-DK-7-0004 / HHSN267200700004C. Available donor information has been described in detail previously (Olsavsky *et al.*, 2007). Hepatocytes were plated on collagen-coated T25 flasks, and within 48 h a dilute overlay of Matrigel (225 µg/ml; BD Biosciences, San Jose, CA) was added dropwise to the cultures in William's Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 µM glutamine, 25 nM dexamethasone, 10 mM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, and 5 µg/ml transferrin. Selected cultures were treated with either DMSO, 10 µg/ml A1254, 100 µM DEHP, or 500 µM PB for 24 h after either 66 (Donors G and I) or 90 h (Donors A through F, H and J) in culture, depending on the condition of the cells. If not specified otherwise, all culturing materials were purchased from Invitrogen (Carlsbad, CA).

RNA isolation

After a 24 h treatment period, RNA was extracted from the hepatocytes with TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was removed using DNA-free™ DNase Treatment and Removal Reagents (Ambion, Inc., Austin, TX) following the manufacturer's protocol for rigorous DNase treatment. Concentration of extracted RNA was assessed by UV absorbance at 260 nm using a SmartSpec 3000 Spectrophotometer (BioRad, Hercules, CA).

Quantitative real-time PCR

A High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) was used to reverse transcribe RNA into cDNA, according to recommendations from the manufacturer. TaqMan® Assays-on-Demand™ Gene Expression assays (Applied Biosystems) were set up according to manufacturer's instructions for a 50-µl reaction volume, subsequently divided into duplicate 25-µl reactions in a 96-well plate, and read in an Applied Biosystems 7300 Real-Time PCR System. Expression of CYP2B6 (Assay ID Hs00167937_m1) was calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001), using 18S (Assay ID Hs99999901_s1) expression in the vehicle-treated sample from the appropriate donor as the reference standard. $\Delta\Delta C_t$ was transformed into fold-change using the formula, fold change = $2^{-\Delta\Delta C_t}$.

Competitive PCR

Internal standards were created for CYP1A2 and CYP4A11 by a three step method (Anderson *et al.*, 1997). Initially, an approximately 500 bp fragment was amplified from human hepatocyte cDNA using the external primers listed in Table 1 in a 50-µl reaction containing 0.25 mM dNTPs, 0.5 µM forward and reverse primers, 2.5 units of Taq polymerase, 1X reaction buffer, and 0.5 µg cDNA. Thermal cycling consisted of 30 cycles of a 1 min denaturation step at 95°C, a 45 second annealing step at 58°C, and a 2 minute extension step at 72°C. Using these amplicons as a template, two small fragments were amplified following the same reaction and thermocycler conditions; a 5' fragment was created with an overhang specific for the internal FP by using the external FP and internal RP, and a 3' fragment was created with an overhang specific for the internal RP by using the internal FP and external RP. In the final step, the 5' and 3' fragments were annealed due to the presence of these overhangs, using five annealing cycles consisting of a 2 min denaturation step at 94°C, a 3 min annealing step at 58°C, and a 4 min extension step at 72°C and 30 cycles of amplification as described above. After every step, the resulting amplicon was purified using a QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA), resulting in approximately 300 bp internal standards. CYP1A2 and CYP4A11 expression was determined in each sample using a standard curve consisting of PCR reactions.

PCR reaction and thermal cycling conditions were identical to those used to create the internal standard described above, and target gene concentration equaled the concentration of the internal standard when endogenous gene and the internal standard were present in a 1:1 ratio.

Microarray protocol

Forty RNA samples (ten donors at four treatment conditions) were hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) by Paradigm Array Labs, Inc. (Icoria, Research Triangle Park, NC) as described previously (Olsavsky *et al.*, 2007). Resulting data was analyzed using GeneChip® Operating Software (GCOS; Affymetrix) to determine detection (P, present; A, absent; or M, marginal) and signal of each probe set on each array, as well as change call (I, increase; MI, marginal increase; NC, no change; MD, marginal decrease; or D, decrease) and signal log ratio of probe sets on the treated arrays using the vehicle-treated arrays from the appropriate donor as the baseline. When the signal log ratio was a positive number, signal log ratio was transformed to fold change by the formula, fold increase = $2^{\text{signal log ratio}}$, or, when the signal log ratio was a negative number, fold decrease = $-(2^{(-\text{signal log ratio})})$. Only genes with a change call of I or D ($p < 0.002$) were considered significantly changed.

Correlation analysis

All probe sets on the arrays (54,675) were included for the correlation analysis. To obtain an estimate of similarity in global expression at the basal level, a Pearson correlation coefficient was calculated for each donor in comparison to the nine other donors, where the mean value for these nine measurements was called the mean correlation coefficient $\pm$ standard deviation (SD). A two-tailed t-test was used to determine significance between donors with high and low correlation to the other donors. Mean correlation coefficients were also calculated for each donor treated with chemical in comparison to the other donors receiving a similar treatment. One-way analysis of variance (ANOVA) in combination with Tukey's multiple comparison post-test was used to determine significance of the difference in correlation between hepatocytes treated with DMSO, A1254, DEHP, and PB. All statistical analyses were performed using GraphPad Prism v4.00 for Windows (GraphPad Software, San Diego, CA).

Hierarchical clustering

To estimate similarity between gene expression in hepatocytes from multiple donors under different treatment conditions, the 40 samples were subjected to hierarchical clustering analysis using the small reproducible response gene set ($n=41$; changed in at least 9 of 10 similarly-treated donors). Clustering was done with the publically-available program PermutMatrix version 1.8.7 (Carau & Pinloche, 2005) using a Euclidean distance measurement and an average linkage.

Transcriptome-scale reproducibility

To compare reproducibility in expression changes across human hepatocytes from different donors, differentially expressed genes in each sample were subjected to gene ontology (GO) over-representation analysis using the PANTHER classification system version 6.0. For each of 241 biological process and 254 molecular function ontology categories, PANTHER calculates the number of genes identified in that category in both the list of differentially regulated genes from a particular sample and the list of genes on the array and compares the two numbers using the binomial test to determine if there are more genes than expected in the differentially regulated list (Thomas *et al.*, 2006). Over-representation is defined as $p < 0.05$. To avoid inclusion of both larger ontology categories and the small sub-categories within the same node, larger categories determined as over-represented were excluded when a smaller sub-category within the same node was also identified. One-way ANOVA was used to

determine if there was a significant difference in the numbers of probe sets, genes and over-represented gene ontology categories found in n donors, where $n = 2$ through 9 ($p < 0.05$).

Results

Correlation between hepatocyte donors

To assess the magnitude of variation between donors across the entire transcriptome, our initial analysis consisted of obtaining correlation coefficients as a global measure of similarity between donors. A comparison of mean correlation coefficients revealed a high level of correlation between eight of the ten donors, with mean correlation values ranging from $r^2 = 0.917 \pm 0.022$ to $r^2 = 0.932 \pm 0.028$ (standard deviation; Figure 1A). The remaining two donors, D and E, had significantly lower correlation with hepatocytes from the other donors, with mean correlation coefficients of 0.887 ± 0.044 and 0.881 ± 0.043 , respectively ($p < 0.0001$). The highest correlation between any two donors was $r^2 = 0.967$ between donors G and H (Figure 1B) and the lowest was $r^2 = 0.857$ between donors D and E (Figure 1C).

In comparison to results at basal conditions, chemical treatment tended to impart a negative impact on correlation between donors, although not significantly in all cases. When all ten donors were taken into account, there was no significant difference in mean correlation between hepatocytes treated with vehicle, PB, A1254 or DEHP (Figure 2A; $p = 0.0793$). However, if hepatocytes from the two donors with poor basal correlation relative to the other donors (donors D and E) were excluded from the analysis, a significant difference in mean correlations based on treatment was detected ($p = 0.0042$), with the most significant difference noted between vehicle- and DEHP-treated hepatocytes ($p < 0.01$). The decrease noted in correlation between basal and induced conditions in hepatocytes suggests that responses to chemical challenge are more variable across individuals than are the basal profiles of global gene expression. However, the extent of interindividual variability to chemical treatment in this system did fluctuate considerably from donor to donor. For example, treatment with PB and DEHP actually increased the correlation coefficient of global gene expression in donor B when compared to basal expression of all other donors (Figure 2B and 2D; basal range: $r^2 = 0.876$ to $r^2 = 0.933$; PB range: $r^2 = 0.904$ to $r^2 = 0.951$; DEHP range: $r^2 = 0.887$ to $r^2 = 0.936$), whereas, exposures to these same chemicals decreased the correlation coefficient of global gene expression in donor E when compared to basal expression in all other donors (Figure 2C and 2E; basal range: $r^2 = 0.857$ to $r^2 = 0.941$; PB range: $r^2 = 0.802$ to $r^2 = 0.947$; DEHP range: $r^2 = 0.805$ to $r^2 = 0.919$).

Target gene validation

Transcript levels of classically responsive target genes for each chemical agent were increased in hepatocytes from most donors, providing evidence that our culturing methodology is appropriate for measures of expression changes after chemical challenge (Figure 3A–C; $p < 0.05$). For example, A1254 induced one of its target genes, CYP1A2, most consistently, as induction was apparent in all ten donors, ranging from 2.3-fold in donor F to 208-fold in donor J (Figure 3A), and, as reported previously (Olsavsky et al, 2007), PB increased a target gene, CYP2B6, in nine of ten donors, ranging from 1.4- (donor F) to 28-fold (donors G and I) (Figure 3B). The final chemical used in this study, DEHP, did not produce consistent induction patterns of its anticipated target, CYP4A11, as CYP4A11 levels were increased in hepatocytes from only five of the ten donors, ranging from 1.4-fold in donor A to 2.1-fold in donor D (Figure 3C).

Both competitive PCR and quantitative RT-PCR techniques were used to confirm the array-detected increases in target gene expression. Using the former method, CYP1A2 was increased in A1254-treated hepatocytes from five donors, ranging from a 6-fold induction in donor A to a 283-fold induction in donor H (Figure 3D), and CYP2B6 was increased in eight donors,

ranging from 2.3-fold in donor E to 27-fold in donor J (Figure 3E). Competitive PCR confirmed that CYP4A11 was inconsistently increased in the hepatocytes, as this gene was induced in four of five donors, ranging from 1.5-fold in donor H to 4.1-fold in donor B (Figure 3F). Overall, no significant difference was found in fold change measured by the two methods for any of the target genes, supporting the validity of our microarray analysis method for the quantification of mRNA levels.

Reproducible response genes

To address conserved transcriptional responses, we identified a set of genes that were consistently regulated by each chemical among hepatocytes from nearly all donors. Although 10'812, 8'373, and 7'847 genes were changed in at least one donor by A1254, DEHP, and PB, only 41 genes met the stringent criteria of being increased or decreased in at least 9 of 10 donors treated by a single chemical (Table 2). Of the three chemicals, A1254 clearly had the most distinctive, reproducible effect on transcription, as 40 genes (54 probe sets) were similarly changed in nearly all donors. Seven of the eight genes consistently regulated by PB were also regulated by A1254, which was anticipated, since A1254 acts as a broad mixed-function oxidase inducer, activating both PB-response genes and dioxin-response genes (Safe, 1993). The final chemical in our study, DEHP, consistently regulated only four genes, all of which were also regulated by A1254.

Functional analysis of reproducible response genes

All of the identified response genes sorted to at least one of eight functional categories. Although PB and DEHP consistently changed relatively few genes in common across donors, the genes that were identified as responders to these agents agreed with anticipated functional categories. For example, nearly all of the genes consistently regulated by PB in different donors were genes encoding enzymes active in biotransformation/xenobiotic metabolism, including CYP2A6, CYP2B6, CYP2C8, CYP3A7, and SULT1C1 (Figure 4A). DEHP also regulated biotransformation genes, CYP2B6 and a likely pseudogene, CYP2B7P1. Activation of THRSP, another DEHP-response gene, is more consistent with known mechanisms of peroxisome proliferators, due to its role in lipid metabolism, a biological process regulated by the activation of PPAR α . The functions of the remaining PB- and DEHP-response genes, SEC14L4 and TSKU, have not yet been fully described.

A1254 exhibited the most reproducible transcriptional effects across donors. Consistent with its role as a mixed function oxidase effector, A1254 regulated, with the exception of SEC14L4, all of the PB-response genes, as well as known dioxin-response genes also involved in biotransformation, including CYP1A1, CYP1A2, NQO1, NQO2, and UGT1A1 (Figure 4A). In our analyses, A1254 was found to inhibit proapoptotic genes (Figure 4B) and suggested tumor suppressors (Figure 4D) and to increase expression of chaperones (Figure 4C), antioxidants (Figure 4E), and damage response (Figure 4F) genes. Genes within the transport (Figure 4G) and lipid and fatty acid metabolism (Figure 4H) categories were also regulated consistently by A1254.

Hierarchical clustering of reproducible genes

When all 40 samples were subjected to hierarchical clustering, the reproducible gene set was sufficient to overcome the influence of interindividual variation for seven of the ten donors. When considering only these seven donors, chemical-specific clusters were apparent, as the majority of samples within each of the three clusters clustered according to chemical treatment, despite the evident influence of interindividual variability (Figure 5). The most distinctive results were apparent for the A1254-treated samples, as all 7 samples in this cluster were treated with this agent. DEHP and PB treatments resulted in less distinctive profiles within this sample

set, as only 6 of 9 and 4 of 5 samples within the “DEHP” and “PB” clusters, respectively, were treated with the chemical of interest.

Transcriptome-scale reproducibility

Although the identification of reproducible target genes is supportive of the use of human hepatocytes as a model system that reflects known mechanism of action despite the source of donor, our goal was ultimately to determine the magnitude of conserved gene changes in response to chemical treatment across donors. Overall, at a whole transcriptome scale, a surprising lack of conserved regulation of genes was found (Figure 6A), even for A1254 treatment, which elicits a more distinctive gene expression profile than either of the other two chemicals. In A1254-treated hepatocytes, only 11 out of 14,102 total changed genes were increased or decreased in all ten donors. In other words, less than a tenth of a percent of all changed genes were always changed. Using less stringent criteria, 1,470 genes were changed consistently in at least five donors; still, approximately only 10 percent of all changed genes. Further, only about half of the genes were changed in at least two donors, leaving half of all genes identified as changed found to be done so in only a single donor. This pattern is similar for the other two chemicals used in this study, although the percentage of genes regulated in multiple donors was lower in DEHP- and PB-treated hepatocytes than for A1254-treated hepatocytes at each number of donors (data not shown).

Because this lack of reproducibility may have important implications for studies that rely on a small number of donors to verify responses in a limited number of genes, we examined the use of higher level descriptors to attempt to increase the level of reproducibility in changes across donors. A total of 292, 341, and 324 distinct ontology categories were over-represented among transcriptional changes induced by A1254, DEHP, and PB, respectively, in at least one donor. When considering the transcriptional changes induced in at least five, six, seven or eight donors, a significantly higher level of reproducibility among individual hepatocyte donors was obtained when gene ontology (GO) categories calculated to be over-represented among the transcriptional changes induced by each chemical were compared rather than probe sets or genes ($p < 0.05$; Figure 6B). A higher level descriptor did not increase reproducibility across donors for changes induced in at least nine donors or in four or fewer donors.

Discussion

In this study, global transcriptional responses were examined in primary hepatocyte cultures from ten independent donors. Basal gene expression profiles as well as responses to three prototypic chemical inducers were assessed, with the intent of evaluating both trends in reproducibility across donors and mechanisms that may be conserved among donors, unique for each chemical class. Overall, the data obtained revealed that only small sets of genes were regulated consistently across donors, whereas relatively large gene sets were regulated uniquely according to individual. Further, the degree of interindividual variability across donors was substantial, such that, of the three chemicals studied, only A1254 effected a large enough number of consistently regulated gene responses to permit analysis of conserved biological mechanisms. PB exposures resulted in surprisingly few conserved gene responses across individuals, although PB did evoke consistent induction of the known target gene, CYP2B6, as well as other anticipated response genes. These hallmark PB responses engendered confidence in the validity of the overall donor response profile associated with this chemical (Figure 3B and E, Figure 4). On the other hand DEHP less consistently induced its anticipated target gene, CYP4A11 across individuals (Figure 3C and F), and the reproducible response genes that DEHP did perturb in hepatocytes were not all obviously consistent with reported DEHP mechanisms of action. These latter data suggest that human hepatocytes are not particularly impacted by DEHP exposures under the conditions evaluated. A similar chemical-

specific phenomenon was reported by Liguori et al (Liguori *et al.*, 2005), in which only one of six quinolone agents regulated a set of genes as defined by significant change in hepatocytes from all four donors treated with that specific quinolone but not with the remaining quinolone agents.

In the case of A1254, where the number of conserved gene sets of responsive genes was large enough to permit functional analysis, results were consistent with literature-reported functional changes ascribed to A1254, or PCB exposures in general. In agreement with the hypothesis that dioxin-like tumor promoters, such as A1254, promote carcinogenesis through inhibition of apoptosis (Park & Matsumura, 2006), two proapoptotic genes were identified as decreased by A1254, TNFSF10 (Yamanaka *et al.*, 2000) and TXNIP (Wang *et al.*, 2006). Similarly, chaperones, particularly Hsp70 family members, also contribute to inhibition of apoptosis (Bivik *et al.*, 2007). Our study revealed that two Hsp70 family members, HSPA1B and HSPA8, as well as a cofactor for Hsp90, AHSA1, were increased in nine of ten A1254-treated donors. Also relating to a role as a modulator of carcinogenesis, A1254 exposures in the hepatocyte model resulted in reduced expression of three suggested tumor suppressor genes, TXNIP (Yoshida *et al.*, 2005), HPGD (Yan *et al.*, 2004), and ANXA10 (Liu *et al.*, 2002), and in increased expression of a potential oncogene, AKR1B10 (Fukumoto *et al.*, 2005).

Possibly related to tumor promotion, PCBs have been implicated in increasing reactive oxygen species (ROS) (Saito, 1990; Srinivasan *et al.*, 2001). In concert with this scheme, multiple chaperones, often increased under stressful conditions, are consistently upregulated in the human hepatocyte donors, as were two antioxidants, SRXN1 (Findlay *et al.*, 2005) and UGT1A1 (Yueh & Tukey, 2007). Although enhanced cellular ROS damages DNA, proteins, and lipids, DNA appears particularly sensitive to damage following PCB exposures, as PCBs have been reported to increase DNA strand breaks and adduct formation (Srinivasan *et al.*, 2001). Similarly, our study identified two up-regulated genes involved in the DNA damage response. TIPARP is a DNA-binding protein activated by ROS-generated DNA strand breaks, including those produced by PCB exposure, that appears to play a role in the decision of the cell to undergo apoptosis or necrosis (Cole & Perez-Polo, 2004). One mode of TIPARP signaling may involve the initiation of apoptosis by mediating the translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, where it initiates DNA fragmentation (Yu *et al.*, 2002). Interestingly, our results identified AIFM2, a closely related homolog to AIF that triggers a functionally similar caspase-independent apoptotic pathway (Wu *et al.*, 2002), as consistently upregulated by A1254. The goal of our functional analysis was not to force every identified response gene into a single role, but to create a view of possible conserved mechanisms based on the genes changed upon chemical treatment and direction of those changes. To this end, we found that conserved sets of genes exhibiting change across individuals sorted well into known functional categories defined by PANTHER, therefore supporting the use of the primary human hepatocyte culture system as a biologically relevant model for capturing responses occurring *in vivo*.

Despite these considerations, interindividual variability remains problematic for toxicological assessments. Throughout the course of our study, hepatocytes from two of the ten human donors were repeatedly marked as outliers, as correlation analysis identified donors D and E as exhibiting significantly lower correlation to all other samples than did the remaining donors (Figure 1A). Additionally, Donor E appeared to regulate a particularly unique subset of genes in response to PB and DEHP, as the correlation of gene expression in this donor to expression in all other donors decreased when treated with PB and DEHP when compared to basal level expression patterns (Figure 2C and E). Donor D was the only donor to not significantly increase the PB target gene, CYP2B6 (Figure 3B). Consequently, donors D and E, as well as F, were omitted from the hierarchical clustering analysis, since the PB- and DEHP-treated samples from these donors clustered uniquely by donor, rather than by treatment. Although the factors

contributing to this outlier status cannot be defined, the finding that some hepatocytes are of sufficiently sub-optimal quality as to mark these donors as significantly different from other donors has implications for studies in which genetic material is pooled from multiple donors.

Further, studies that compare gene expression even in a limited number of donors is problematic, as it has been reported that mean and variance data are misleading due to the fact that gene expression, particularly of those genes associated with metabolic processes, does not follow a normal distribution across donors (Slatter *et al.*, 2006). Our study provides further evidence of this contention, in that the standard deviations in fold induction of both CYP1A2 and CYP2B6 are nearly as large as the “average” fold induction itself (data not shown). Our results are consistent with other reports suggesting that the use of biological theme as a comparator, rather than reliance on probe set or individual gene responses, increases reproducibility in results across laboratories and microarray platforms (Bammler *et al.*, 2005; Currie *et al.*, 2005). The analyses presented here further support the concept that the use of higher level descriptors, such as those engendered in the PANTHER classification used here are valuable approaches, as these methods enable focus on biological processes and functions, and provide a viable method for managing the otherwise large degree of interindividual variability data that are encountered when focusing on individual gene descriptors as the primary analysis tool. Expanding on previous ontology structures, the PANTHER system queries extended database content and was built using a combination of scientific curation and computational algorithms that sort genes into groups based on the function of the gene product (Thomas *et al.*, 2003). These attributes provide the system with an enhanced ability to extract biologically meaningful information.

Despite the many factors contributing to extensive variability in basal and inducer-related gene expression profiles across hepatocytes from individual donors, the data reported here, and in our earlier studies (Olsavsky *et al.*, 2007; Page *et al.*, 2007), validate the use of primary human hepatocytes, cultured under highly defined conditions, as a valuable and biologically relevant *in vitro* model system that largely reproduces conserved profiles of gene expression changes that are comparable to results obtained with *in vivo* model systems.

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Figure 1.

Overall correlation in basal gene expression between donors is significantly higher in eight of ten donors compared to the remaining two donors. For each donor, expression of all genes on the array was compared to each of the nine other donors in order to obtain a mean correlation coefficient for each donor pair. Significance between high and low correlation donors was determined by a two-tailed t-test (A; $p < 0.0001$). Examples of the scatter plots of the donor pairs with the highest and lowest correlation are shown in B and C, respectively.

Figure 2.

Effect of chemical treatment on correlation of gene expression between donors is highly donor-specific. For each donor, expression of all genes on the vehicle-, PB-, A1254-, and DEHP-treated arrays was compared to the expression of all genes on the appropriate array corresponding to the nine other donors to obtain mean correlation coefficients between each donor pair at each treatment condition. Significance between treatment conditions was determined using one-way ANOVA in combination with Tukey's multiple-comparison post-test (A; $p<0.01$). Significance between correlation of donor B and all other donors at basal and PB (B) or DEHP (D) conditions and between correlation of donor E and all other donors at basal and PB (C) or DEHP (E) conditions was determined using a two-tailed t-test ($p<0.05$).

Figure 3.

Hallmark target genes are increased in human hepatocytes treated in vitro with A1254 (A, D), PB (B, E), or DEHP (C, F). Panels A–C show signal intensity values for individual arrays representing the ten donors in which CYP1A2 was increased, the nine donors in which CYP2B6 was increased, and the five donors in which CYP4A11 was increased. Significance in expression difference between vehicle and treatment conditions was determined by a paired t-test ($p < 0.05$). Panels D–F show the fold change of CYP1A2, CYP2B6, and CYP4A11 in the donors mentioned above, as measured by microarray analysis and by either quantitative RT-PCR (QRT-PCR) or competitive PCR (C-PCR).

Figure 4.

Target genes that were increased or decreased in at least nine hepatocyte donors by a single chemical reflect known functional effects of chemical treatment *in vivo*. The 40 genes regulated by A1254, 4 by DEHP (italicized), and 8 by PB (underlined) were categorized according to function. A single gene, SEC14L4, was regulated by PB but not Aroclor (indicated by *). Genes decreased by chemical treatment are enclosed in a box; otherwise genes are increased.

Figure 5. Hierarchical clustering groups hepatocyte donors by chemical treatment when the dataset consists of genes regulated similarly by one chemical in at least 9 donors. Numbered circles indicate chemical-specific clusters: 1, A1254; 2, DEHP; and 3, PB. Dataset consisted of 41 genes. Clustering was done using a Euclidean distance measure and average linkage.

Figure 6.

Globally, transcriptional changes in response to chemical treatment are not highly conserved between individual human hepatocyte donors. The percentage of total changed genes found in at least n number of donors ($n=1$ through 10) is shown in Panel A. Error bars reflect results from each of the three different chemicals used to treat human hepatocytes. The mean number of probe sets, genes, and overrepresented GO categories identified in n number of donors is shown in Panel B. Results from $n = 2, 5, 8$, and 9 donors are shown. Significant difference in the means is considered as $p < 0.05$ (one-way ANOVA).

Figure 7.

Table 1

Competitive PCR primer sequences.

Gene	Primer Name	Sequence
CYP1A2	External FP	TGCGGCAGGGCGACGATTTC
	External RP	GCAGGGCAGGGTTAGGCAGGTA
	Internal FP	TGATGGCCAGAGCTTGACGAGCATGTGAGCAAGGAG
	Internal RP	CTCCTTGCTCACATGCTCGTCAAGCTCTGGCCATCA
CYP4A11	External FP	AGGCAAAGTTCGTGTCCAGCTCTA
	External RP	TGGATCACTTGGTCTGTGTGCTGA
	Internal FP	TCCTGGCTCCATGGATTGCTGGACACCATCATGAAG
	Internal RP	CTTCATGATGGTGTCCAGCAATCCATGGAGCCAGGA

Table 2

Probe sets identified as either increased or decreased in at least 9 human hepatocyte donors treated with either A1254, DEHP, or PB.⁴

Chemical ⁵	Probe Set Name	Gene Title	Gene Symbol	Change ⁶
A, D, P	206755_at; 217133_x_at	cytochrome P450, family 2, subfamily B, polypeptide 6	CYP2B6	Inc
A, D, P	206754_s_at	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	CYP2B7P1	Inc
A, D, P	218245_at	tsukushin	TSKU	Inc
A, D	229477_at; 1553583_a_at	thyroid hormone responsive (SPOT14 homolog, rat)	THRSP	Dec
A, P	1494_f_at; 207244_x_at; 211295_x_at; 214320_x_at; 207718_x_at	cytochrome P450, family 2, subfamily A, polypeptide 6	CYP2A6	Inc
A, P	208147_s_at	cytochrome P450, family 2, subfamily C, polypeptide 8	CYP2C8	Inc
A, P	205939_at	cytochrome P450, family 3, subfamily A, polypeptide 7	CYP3A7	Inc
A, P	205342_s_at; 211470_s_at	sulfotransferase family, cytosolic, 1C, member 1	SULT1C1	Inc
A	222592_s_at	acyl-CoA synthetase long-chain family member 5	ACSL5	Inc
A	201491_at	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)	AHSA1	Inc
A	206561_s_at	aldo-keto reductase family 1, member B10 (aldose reductase)	AKR1B10	Inc
A	205633_s_at	aminolevulinatase, delta-, synthase 1	ALAS1	Inc
A	224461_s_at	apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	Inc
A	205749_at	cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	Inc
A	207608_x_at; 207609_s_at	cytochrome P450, family 1, subfamily A, polypeptide 2	CYP1A2	Inc
A	216025_x_at	cytochrome P450, family 2, subfamily C, polypeptide 19 /// cytochrome P450, family 2, subfamily C, polypeptide 9	CYP2C19 /// CYP2C9	Inc
A	214421_x_at	cytochrome P450, family 2, subfamily C, polypeptide 9	CYP2C9	Inc
A	202581_at	heat shock 70kDa protein 1B	HSPA1B	Inc
A	210338_s_at	heat shock 70kDa protein 8	HSPA8	Inc
A	204679_at	potassium channel, subfamily K, member 1	KCNK1	Inc
A	227055_at	methyltransferase like 7B	METTL7B	Inc
A	201468_s_at	NAD(P)H dehydrogenase, quinone 1	NQO1	Inc
A	203814_s_at	NAD(P)H dehydrogenase, quinone 2	NQO2	Inc
A	225252_at	sulfiredoxin 1 homolog (S. cerevisiae)	SRXN1	Inc
A	212665_at	TCDD-inducible poly(ADP-ribose) polymerase	TIPARP	Inc
A	230830_at	Transmembrane protein 177	TMEM177	Inc
A	208596_s_at; 215125_s_at	UDP glucuronosyltransferase 1 family, polypeptide A10 /// UDP glucuronosyltransferase 1 family, polypeptide A8 /// UDP glucuronosyltransferase 1 family, polypeptide A7 /// UDP glucuronosyltransferase 1 family, polypeptide A6	UGT1A10 /// UGT1A8 /// UGT1A7 /// UGT1A6 /// UGT1A5 /// UGT1A9 /// UGT1A4 /// UGT1A1 /// UGT1A3	Inc
A	204719_at	ATP-binding cassette, sub-family A (ABC1), member 8	ABCA8	Dec
A	219803_at	angiopoietin-like 3	ANGPTL3	Dec
A	210143_at	annexin A10	ANXA10	Dec
A	1552389_at; 1552390_a_at	chromosome 8 open reading frame 47	C8orf47	Dec
A	205776_at	flavin containing monooxygenase 5	FMO5	Dec
A	205498_at	growth hormone receptor	GHR	Dec
A	225424_at	glycerol-3-phosphate acyltransferase, mitochondrial	GPAM	Dec
A	219313_at	GRAM domain containing 1C	GRAMD1C	Dec
A	203914_x_at; 211548_s_at	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	Dec
A	1558603_at	plasminogen-like B2 /// plasminogen-like B1	PLGLB2 /// PLGLB1	Dec
A	220786_s_at	solute carrier family 38, member 4	SLC38A4	Dec
A	202687_s_at; 202688_at	tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	Dec
A	201009_s_at; 201010_s_at	thioredoxin interacting protein	TXNIP	Dec
P	239492_at	SEC14-like 4 (S. cerevisiae)	SEC14L4	Inc

Chemical ⁵	Probe Set Name	Gene Title	Gene Symbol	Change ⁶
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⁴ When more than one probe set is listed as changed by more than one chemical, all probe sets were changed by each chemical listed unless underlined or italicized. Underlining signifies that that probe set was changed only by A1254, and italicizing signifies that that probe set was changed only by DEHP.

⁵ A, Aroclor 1254; D, DEHP; P, PB.

⁶ Inc, Increased; Dec, Decreased; Change call was determined by GCOS; $p < 0.002$.