The Pennsylvania State University

The Graduate School

Department of Veterinary and Biomedical Sciences

EXPRESSION PROFILING OF INTERINDIVIDUAL AND INTERSPECIES VARIABILITY IN HEPATIC MODELS

A Dissertation in
Pathobiology
by
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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2009
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ABSTRACT

Although in vivo rodent models are a necessary part of the risk assessment process, incorporation of human models is necessary in order to evaluate whether effects in rodents are applicable to humans. Studies with in vitro hepatocytes have been hindered by the dramatic loss of differentiation status and liver-specific functions after isolation. In this study, expression profiling analyses demonstrate that hepatocytes cultured in a sandwich configuration and in the presence of physiological levels of dexamethasone maintain expression levels of liver-enriched transcription factors, drug-metabolizing enzymes, and transporters similar to that in human liver tissues. Additionally, hepatocytes in these conditions exhibit a complex biological response after xenobiotic challenge, namely, induction of cytochrome P450 mRNA and protein, marking these cells as a robust model system for studies of drug metabolism. An additional complicating factor of using primary hepatocytes in risk assessment is the magnitude of interindividual variability in hepatocytes from different donors. This study presents a detailed analysis of the comparability of response genes in hepatocytes from ten donors after challenge with xenobiotics that exhibit distinct mechanisms of action. A gene to gene comparison shows that hepatocytes exhibit limited reproducibility in response genes across donors, although a comparison of functional categories significantly increases reproducibility. Using this functional category approach, profiling results in primary human hepatocytes were compared to profiling results in mice after treatment with the same xenobiotics. Conserved cross-species responses were consistent with literature-reported mechanisms for each chemical. Interestingly, mitochondrial oxidative metabolism genes, specifically those genes in the tricarboxylic acid cycle and the electron transport chain, were consistently decreased in mice but were often increased in primary human hepatocytes. Finally, expression profiling of phenobarbital response genes identified a novel gene, Tsukushin (TSKU) that was reproducibly increased after treatment in nine of the ten human hepatocyte donors. TSKU knockdown in primary human hepatocytes resulted in a diminished cytochrome P450 induction response through an indirect mechanism.
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This research was funded by a Toxicogenomics Research Consortium grant from the National Institutes of Environmental Health Sciences, U19 ES11387 and a grant from the National Institute of General Medical Sciences, GM66411.

On a more personal note, I’d like to thank my parents, Joe and Cindy Olsavsky, for their constant support and love. Thank you for giving me the confidence to dream big and go after those dreams. Dave, Mike, Joanne, Jacob, and Emma, thank you for always listening to my stories, my complaints, and my successes; you all remind me how good it is to come home.

Also, I’d like to recognize a few people who have had such immense influence on my academic career and who I can’t thank enough: my Connellsville Area High School teachers, Mr. Zundel, for suggesting Gettysburg College and for providing such a solid chemistry background, Mr. Mimnaugh, for inspiring my love of biology (and dissections); my undergraduate advisor, Dr. Kazuo Hiraizumai, for introducing me to research and, of course, fruit flies; and to my graduate advisor, Dr. Curt Omiecinski, who has a great talent for identifying my strengths and interests before I do.

Finally, to my husband Ken, thank you for always building up my confidence, for giving me pep talks whenever I need them, and, perhaps most importantly, for introducing me to the wonderful world of databases!
Chapter 1

Applying genomics to investigate the role of primary human hepatocytes in toxicology research.

1.1 Hepatocytes in toxicology research

Of the available in vitro hepatic models, primary hepatocytes offer substantial advantages, including conserved uptake and excretion functions, the integration of phase I and phase II metabolic pathways, and the presence of cofactors necessary for enzyme activity. Although in practice since the 1950’s, early methods, involving perfusion of rodent livers under pressure, resulted in grossly damaged hepatocytes. Isolation methods were vastly improved by Berry and Friend [1] through the introduction of collagenase as a means to enzymatically disperse cells and by Seglen’s introduction of the two-step method [2]. This two-step method, now considered the standard isolation method, consists of an initial perfusion with a calcium-free buffer to disrupt desmosomes that make up the tight junctions between cells followed by a second perfusion with a calcium-rich buffer containing collagenase to further digest cell junctions. Another breakthrough in hepatocyte isolation methods was the modification of the procedure to use only segments of the liver, rather than the entire organ, allowing cost-efficient scale up of the procedure to use larger livers, such as human [3-5]. Despite the improvement in methods, hepatocytes from these early isolation experiments dedifferentiated quickly in culture, losing hallmark features of in vivo liver function, such as albumin secretion and biotransformation activity within a few hours [6-8].

This dedifferentiation has sparked investigation both of the culture conditions that preserve the differentiated phenotype and of the mechanisms responsible for differentiation status. In general, an inverse relationship has been described between a well-differentiated, growth-arrested phenotype and a proliferative one, marked by a G0/G1 transition that is triggered by the isolation process itself as defined by upregulated
proto-oncogenes such as *c-fos*, *c-jun*, and *c-myc* [9,10]. This proliferative state *in vitro* has been further characterized by activation of cell cycle-stimulating proteins such as AP-1 [10-12] and NFκB [11,13] and by loss of liver-enriched nuclear factors such as C/EBPα and HNF family members [10,11,14,15]. While the induction of a proliferative state is advantageous for investigations of liver regeneration mechanisms, studies of xenobiotic metabolism require hepatocytes that respond with fidelity to the *in vivo* liver. Thus, considerable effort has been put forth to identify conditions in which hepatocytes remain well-differentiated.

### 1.2 Markers of a differentiated hepatocyte

The identification of such conditions has established typical markers that define differentiation status, the most standard of which include expression of plasma proteins like albumin, transferrin, and transthyretin [16-21] and cytochrome P450 (CYP450) monooxygenase activity [17,20,22-24]. Both of these markers are hallmark features of the liver, in that this organ is the dominant site of plasma protein synthesis [25,26] and biotransformation activity. Morphology provides additional assessment of differentiation status, as the cuboidal networks of cells often flatten and lose expression of specialized structures such as bile canaliculi as well as distinct cell-cell contacts as dedifferentiation occurs (Figure 1) [18,19,27-29].

Although albumin secretion and monooxygenase activity are the most common endpoints evaluated, an array of additional functional endpoints can offer insight into the degree of differentiation, due to the wealth of physiological functions in which the *in vivo* liver plays a role, including the synthesis of urea, clotting factors, and acute phase proteins [20,30-32], synthesis of glucose and subsequent glycogen storage [21,30,32], excretion of bilirubin [33], and lipid and cholesterol transport [18]. Additionally, these functions are in part regulated through expression of the liver-enriched transcription factors, such as C/EBPα and HNF4. Expression of C/EBPα, in particular, has been noted to decline both as expression of proto-oncogenes increase and as normal morphology is altered [10-12,18]. HNF4, on the other hand, plays a role in liver-
specific gene expression, since targeted knockdown of this transcription factor decreased expression of the plasma proteins albumin and transthyretin [19,34].

An often overlooked aspect of the differentiated hepatocyte is the status of the plasma membrane; namely, that the membrane retains polarized domains, forms junctions between cells to facilitate cell-cell communication, and contains specialized structures like bile canaliculi. In vivo, hepatocytes are arranged in plate-like arrays, facing the sinusoids on one side and bile ductules on the other. The plasma membrane is functionally compartmentalized based on these interactions, such that the basolateral, or sinusoidal, membrane is specialized for exchange of metabolites with circulating blood (Figure 2).

Similarly, the apical, or canalicular, membrane is specialized for bile secretion, and the lateral membrane, joining adjacent hepatocytes, is specialized for intercellular communication [28,35]. Functional polarity in vitro is demonstrated by marker proteins specific for lateral domains, such as connexins 26 and 32; basolateral domains, like epidermal growth factor receptor; and apical domains, such as depeptidyl peptidase IV

Figure 1: Hepatocytes in sandwich culture exhibit a more cuboidal, three-dimensional shape in sandwich culture vs. monolayer culture. Hepatocytes were cultured for six days either on a collagen substratum layer alone (A) or in combination with a dilute overlay of Matrigel (B).
Alternatively, hepatobiliary transport, demonstrated by the appropriate accumulation and excretion of bile acids and other organic anions [21,33,40-42], and gap junctional intercellular communication between adjacent hepatocytes [38,39] demonstrate the compartmentalization of these specialized functions.

1.3 Culture methodologies

1.3.1 Three-dimensional bioreactors

Although variations are abundant, two major culture methodologies exist that preserve a well-differentiated phenotype: three-dimensional bioreactors and a two-dimensional sandwich culture configuration. The former methodology embeds hepatocytes within complex three-dimensional chambers, the most common of which is the hollow fiber membrane bioreactor (Figure 3A). The hollow fibers, woven into a three-dimensional scaffold for hepatocyte attachment, act as capillaries through which defined culture medium is perfused, thus providing a continuous supply of oxygen and nutrients to the cells, efficient removal of waste

Figure 2: Hepatocytes in vivo have polarized membranes with specialized function based on location within the liver lobule. The basolateral (sinusoidal) domain is specialized for exchange with blood, the apical (canicular) domain is specialized for bile secretion, and the lateral domain is specialized for intercellular communication. The various domains are separated by tight junctions.
products, and controlled fluid dynamics to mimic \textit{in vivo} shear stress and interstitial flow [43-47]. Under these conditions, hepatocytes exhibit a differentiated phenotype over several weeks in culture, with cuboidal morphology, extensive cell-cell contacts [31,45] and specialized structures such as bile canaliculi [32,48]. Additionally, functional hallmarks are preserved, as hepatocytes in bioreactors synthesize both albumin and urea [30-32,44,45,48], excrete galactose [30,32], and exhibit various drug biotransformation activities [31,45,46].

Nonetheless, the continuous perfusion inherent to this model has some associated difficulties, as components derived from cells or present in the media can clog pores on the membranes, subsequently altering the flow and possibly resulting in gradients of nutrients or oxygen through the chamber [32,43,49]. Additionally, even though the rate of perfusion is controlled, the flow of fluid may introduce excess mechanical stress that disrupts normal hepatocyte dynamics [50-52].

\subsection*{1.3.2 Two-dimensional sandwich culture}

A simpler, but nonetheless efficient, methodology is the sandwich culture method, in which hepatocytes are embedded between a substratum of collagen and an overlay of either collagen or a commercially available extracellular matrix, Matrigel (Figure 3B). The sandwich culture method has had the best success at prolonging viability [53,54] and improving morphology such that hepatocytes remain cuboidal and in closely-associated networks [24,28,53,55]. Functional capacity is also improved, displaying appropriately polarized membrane domains [29,33,40], enhanced biotransformation activity [22,53,56,57], and long-term albumin secretion [24,29,54]. This configuration mimics the \textit{in vivo} microenvironment, where, as shown in Figure 2, hepatocytes are anchored to two opposing surfaces, even though the precise signalling pathways that this configuration preserves have not been clearly defined.

Extracellular matrix (ECM) components present in the Space of Disse, in particular laminin and collagen, are thought to not only provide anchorage for
hepatocytes *in vivo* (Figure 1), but also promote differentiation. These matrix components preserve normal cytoskeletal organization [28,58] and regulate expression of both HNF family members [19,34,59] and albumin [60,61], highlighting the importance of

Figure 3: Two major primary hepatocyte culture methodologies have emerged that preserve a differentiated phenotype. Hollow fiber membrane bioreactors generally contain the following components: a reservoir containing defined media, a pump, a carbon dioxide/oxygen exchanger, and a chamber containing a complex network of hollow fibers to which hepatocytes attach and media is perfused through (A). In the sandwich culture methodology, hepatocytes are embedded between a collagen substratum and Matrigel overlay in the presence of defined media (B).
ECM in the stabilization of differentiation. Since extracellular signals are often communicated to the cytoskeleton via the integrin family of cell surface receptors, it has been suggested that integrin signalling is crucial for maintenance of differentiation [28,62]. α3β1 integrin, in particular, facilitates hepatocyte attachment to collagen [63,64] and fibronectin [65], and overall preservation of a differentiated morphology [66]. Recently, phosphatidylinositol signalling has been identified as a potential link between integrins and cytoskeletal rearrangement, as ECM attachment caused an increase in phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) phosphatase mRNA with a subsequent decrease in PI(4,5)P2 levels and actin polymerization [34].

1.4 Defined media conditions

In addition to culture configuration, defined media conditions are crucial for the differentiated phenotype, namely the presence of nanomolar levels of glucocorticoids in the form of the synthetic hormone dexamethasone and the absence of serum. Dexamethasone is a potent activator of the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily that, prior to ligand binding, is complexed in the cytosol with HSP90, p23, and one of several tetratricopeptide repeat proteins [67-70]. Ligand binding causes a conformational change in GR, revealing nuclear localization signals that stimulate nuclear translocation of the receptor [71,72]. Once in the nucleus, GR binds to specific response elements, acting as an immunosuppressant largely through repression of NFκB and AP-1 subunits [73-75].

In primary hepatocyte culture, dexamethasone promotes a cuboidal phenotype and expression of C/EBPα, HFN-4α, and RXRα [12,24,76,77], and has even been shown to reverse the proliferative state stimulated by growth factors such as EGF [78]. Although high doses of dexamethasone can stimulate proliferation [79], low doses are often included in conditions designed to induce hepatic lineage differentiation upon embryonic stem cells derived from human [80,81], monkey [82], and mouse [83]. Although the specific signalling pathways through which differentiation have been achieved are not
clear, dexamethasone has been shown to inhibit the induction of stress signalling pathways, such as MAPK and SAPK/JNK [12].

1.5 Complex response to inducers

1.5.1 Constitutive androstane receptor and phenobarbital

Although used in humans as an anti-seizure agent without appreciable adverse effects [84], PB promotes rodent tumorigenesis through mechanisms including inhibition of apoptosis [85], activation of β-catenin [86], selective promotion of cells with low TGFβ receptor expression [87], reduction in G1 checkpoint efficiency [88], and alteration of DNA methylation [89]. Mechanistically, PB mediates these effects through activation of the constitutive androstane receptor (NR1I3, or CAR), a member of the nuclear hormone receptor superfamily of transcription factors (reviewed in [90-93]). In vivo, CAR is retained in the cytoplasm complexed with HSP90 and the tetratricopeptide repeat-containing protein cytoplasmic CAR retention protein (CCRP), until activation by xenobiotics such as PB induce nuclear translocation [94-97]. Once in the nucleus, CAR forms a dimer with RXRα [98] and recruits coactivator proteins, such as steroid receptor coactivator 1 (SRC-1) [99], GR-interacting protein 1 (GRIP-1) [100], and peroxisomal proliferator-activated receptor-γ coactivator 1α (PGC1α) [101] in order to drive transcription of genes containing PB-responsive enhancer modules (PBREMs) within their promoter regions [102,103].

1.5.2 Aryl hydrocarbon receptor and Aroclor 1254

Another common class of inducers activates the aryl hydrocarbon receptor (AhR). Following a mechanism similar to that discussed above, AhR, a ligand-activated transcription factor belonging to the basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) family, is sequestered in the cytoplasm prior to activation, in a complex containing...
HSP90 [104,105], the co-chaperone p23 [106], and tetratricopeptide-repeat containing protein HBV X-associated protein 2 (XAP2) [107]. Synthetic ligands such as A1254 [108], β-naphthoflavone [109], or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [110], as well as dietary substances like indole-3-carbinol [111] or curcumin [112], induce a conformational change in AhR [113], revealing nuclear localization signals [114] that drive translocation of the AhR complex into the nucleus [115,116]. In the nucleus, AhR dissociates from HSP90 and heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) [117], which recognizes the core motif GCGTG [118,119] in the promoters of genes such as CYP1A1 [120]. Additionally, co-activators, like NcoA4/ARA70 [121], RIP140 [122], and SRC-1 [123,124], are recruited in order to facilitate transcription of these response genes.

Physiologically, the activation of AhR influences xenobiotic biotransformation [125,126], proliferation [127-129], and immunosuppression [130]. A1254, a mixture of polychlorinated biphenyls (PCBs), is a common environmental contaminant with heavy historical use in the electrical industry [108], with noted adverse effects on liver and kidney function [131], the nervous system [132], thyroid hormone homeostasis [133], bone metabolism [134], and the immune system [135]. A1254 acts as a ‘mixed-function oxidase’, activating both PB-responsive and dioxin-responsive genes, through activation of the constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR), respectively [136].

1.5.3 Peroxisome proliferator activated receptor α and di-(2-ethylhexyl)phthalate

Di-(2-ethylhexyl)phthalate (DEHP), a common plasticizing agent found in many plastics and products such as intravenous infusion bags and plastic tubing [137], has been subject to much investigation in recent years, as humans, especially those with medical conditions which may require extensive medical care such as dialysis patients or hemophiliacs, can potentially come into contact with considerable quantities of DEHP [138-140]. This prolonged human exposure has been intensely scrutinized, as DEHP falls within the class of chemicals known as peroxisome proliferators, which are potent rodent
carcinogens [141-143]. Peroxisome proliferators activate the peroxisome proliferator activated receptor α (PPARα), that dimerizes with RXRα to drive transcription of genes containing peroxisome proliferator response elements (PPREs) (reviewed in [144,145]). PPARα/ RXRα heterodimer activation induces peroxisome proliferation and tumorigenesis through suppression of apoptosis [146,147], an increase in DNA synthesis [146-148], and inhibition of gap junctional intercellular communication [149], mechanisms thought not to be relevant in humans and other refractory species, such as guinea pigs [145,150].

Although the mechanistic basis for the species difference has not been definitively identified, these differences have been attributed to 10-20-fold lower expression levels of PPARα in refractory vs. sensitive species [151,152] or to inactive promoter sequences in human PPARα response genes that mediate cell proliferation responses [153,154]. Interestingly, knockout mice that have been “humanized” such that human PPARα is expressed exhibit alterations in lipid metabolism enzymes after peroxisome proliferator challenge, consistent with effective hypolipidemic therapeutic treatment in humans [155,156], yet hepatocellular proliferation events leading to carcinogenesis are absent [157,158], suggesting that the PPARα receptor itself may be responsible for sensitivity issues across species.

1.6 Species-specific considerations

Even though there are noted differences across species, the vast majority of validation studies have been carried out in hepatocytes of rodent origin due to limitations in the availability of human hepatocytes. Although further experiments with human hepatocytes may only confirm current culture methodologies, past experience has shown that there are inherent species-specific phenotypic differences in hepatocytes. For instance, early isolation studies reported significantly lower viability in rat and hamster hepatocytes vs. those from mouse and rabbit under the same conditions, as well as a steep decline in cytochrome P450 content in mouse and rat hepatocytes vs. nearly unchanged concentrations in those from rabbit [8]. Time-course discrepancies have also been noted
for membrane repolarization, in that co-localization of canalicular transport proteins with canalicular markers occurs faster in hepatocytes from rats compared to those from humans [36]. Further, while a sandwich culture configuration was demonstrated as critical for the induction of biotransformation enzymes in rat hepatocytes [24,27], some studies have concluded that a collagen or matrigel overlay is not vital for enzyme induction in primary human hepatocytes, despite improved morphology and cytoarchitecture in sandwich culture [159]. Considering these species-specific responses to \textit{in vitro} conditions, further evaluation of primary human hepatocytes is warranted in order to secure confidence in their role in risk assessment.

\textbf{1.7 Applications of toxicogenomics}

One method with the potential to aid this type of evaluation is toxicogenomics, which is a term used to describe gene expression profiling to study the effects of xenobiotics on biological systems. Although initial toxicogenomics studies focused on establishing that similar toxic mechanism of action resulted in similar gene expression profiles [160-163], more recent applications of this technology include the characterization of primary hepatocytes and other \textit{in vitro} hepatic models.

In comparisons of rodent hepatocytes and rodent liver tissue, the consensus is that dramatic changes occur in hepatocytes during the adaptation to \textit{in vitro} conditions, with 70-80\% concordance in basal gene expression between hepatocytes and liver tissue [164-167]. After xenobiotic treatment, the transcriptional response was diminished \textit{in vitro} [166,167], while the response of genes within certain categories, such as “cell adhesion”, “blood coagulation”, and “regulation of blood pressure” were lost altogether [168]. Nonetheless, many genes that were directly affected by xenobiotic treatment, rather than altered via secondary mechanisms, were found to be regulated \textit{in vitro} as they were \textit{in vivo} [166,168]. All of the above validation studies were carried out in rodents, and only a single study [165] evaluated hepatocytes in a sandwich culture configuration. Those studies using monolayer cultures often reported dramatic loss of genes encoding differentiation markers and biotransformation enzymes, suggesting that the hepatocytes
in these studies were at least partially dedifferentiated. Due to these potentially confounding conditions, the concordance of gene expression between primary human hepatocytes and in vivo human liver tissue remains to be fully addressed.

Experiments done with primary human hepatocytes face an additional confounding factor that cannot be directly addressed with rodent hepatocytes: variability due to the donor source. The induction and activity of individual cytochrome P450 enzymes varies widely across individuals, partly due to genetic factors such as polymorphisms but also due to environmental factors such as disease state, diet, nutrition, etc. As expected, toxicogenomics studies found that, although expression profiles clustered on the basis of xenobiotic treatment in mouse and rat hepatocytes, human hepatocytes were more influenced by donor source than treatment [169]. Under suboptimal, monolayer conditions, one study found that only a single gene was changed in hepatocytes from all three donors after xenobiotic treatment [170]. When sandwich culture conditions were used, however, transcriptional response to treatment was found to be robust and reproducible across donors, with more than 140 genes changed by a single chemical in hepatocytes from all four donors despite considerable variation in basal expression [171]. These limited toxicogenomics studies in human hepatocytes raise intriguing questions, such as whether other xenobiotics engender a robust response in human hepatocytes or whether the above observations reflect the nature of the xenobiotic, in this case, trovafloxacin, specifically. Additional consideration should be given to whether or not the culture configurations used in the above studies played a role in the robust nature observed in one study but not the other. The answers to these questions could influence the way that studies in human hepatocytes are interpreted from a risk assessment perspective.

Indeed, human hepatocytes are a tremendous asset to risk assessment evaluations, in that in vitro experiments can be performed to evaluate the metabolism or toxicity of agents in a human model. An avenue currently under investigation is the use of toxicogenomics to evaluate the biological processes and pathways regulated both in human and rodent models with the intention of discerning shared or divergent mechanisms. For example, Uehara et al [172] reported that a gene signature shared
commonly in rodent *in vivo* and *in vitro* models was diminished or unchanged in human hepatocytes, confirming the known species-specific toxicity in rodents in response to coumarin. With this goal in mind, genomics data are being combined with more traditional “reductionist” data to create the Comparative Toxicogenomics Database, consisting of toxicologically-relevant genes and gene sets to establish their expression and activity across species in order to better elucidate species-specific responses to chemical exposure [173].

Despite this effort, few genomics studies that compare response genes across species have been undertaken. Some of the complications hindering this type of comparison, such as the identification of true functional orthologs and unequal coverage on arrays from different species, are evident in a comparative study of 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD)-mediated toxicity in mouse and rat, which reported only approximately 200 mouse and rat orthologs expressed basally and even fewer of those orthologs were actually regulated after treatment [174]. In a different approach, functional categories changed in response to xenobiotic treatment are identified first and then genes within those categories reported, without attempting to identify which of those genes are orthologs [169,175]. Comparative toxicogenomics studies remain to be fully explored for their potential to identify conserved or divergent mechanism in comparison to a human model across a range of compounds.

### 1.8 Hypothesis

In this investigation, we hypothesized that, based on previous development studies using rat hepatocytes, an optimized human two-dimensional primary hepatocyte culture system could be deployed *in vitro* that robustly reflects the differentiation status and biotransformation functions of hepatocytes *in vivo*. With these questions in mind, the research in this study uses DNA microarray-based gene expression profiling and phenotypic anchoring methodologies to address three specific aims. Firstly, we aim to validate the differentiation status by comparing basal gene expression levels in primary hepatocytes from ten different donors in comparison to expression levels in human liver
tissues themselves pooled from six different donors as a comparator to previously reported rodent studies. Combining information from three xenobiotics with distinct canonical mechanisms of action with varied expression alterations and subsequent toxic effects, the second aim of this study is to evaluate the correlation in transcriptional response to xenobiotics among hepatocytes from different donors. Additionally, a novel cross-species comparison of response genes to three distinct xenobiotics was undertaken as the final aim of this study. Although technical factors such as ortholog definition and array design may limit the magnitude of comparison across species, this analysis provides a view of the functional effects of xenobiotic exposure that may be conserved across species and those that may be contribute to differential susceptibility for toxicity.

1.9 References


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8. Maslansky, C. J. and Williams, G. M. (1982). Primary cultures and the levels of cytochrome P450 in hepatocytes from mouse, rat, hamster, and rabbit liver. *In Vitro* 18, 683-693.


Chapter 2

Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissues

2.1 Abstract

Frequently, primary hepatocytes are used as an in vitro model for the liver in vivo. However, the culture conditions reported vary considerably, with associated variability in performance. In this study, we characterized the differentiation character of primary human hepatocytes cultured using a highly defined, serum-free two-dimensional sandwich culture system, one that configures hepatocytes with collagen I as the substratum together with a dilute extracellular matrix (Matrigel) overlay combined with a defined serum-free medium containing nanomolar levels of dexamethasone. Gap junctional communication, indicated by immunochemical detection of connexin 32 protein, was markedly enhanced in hepatocytes cultured in the Matrigel sandwich configuration. Whole genome expression profiling enabled direct comparison of liver tissues to hepatocytes cultured in the hepatoma-derived cell lines, HepG2 and Huh7. PANTHER database analyses were used to identify biological processes that were comparatively over-represented among probe sets expressed in the in vitro systems. The robustness of the primary hepatocyte cultures was reflected by the extent of unchanged expression character when compared directly to liver, with more than 77% of the probe sets unchanged in each of the over-represented categories, representing such genes as C/EBPa, HNF4a, CYP2D6, and ABCB1. In contrast, HepG2 and Huh7 cells were unchanged from the liver tissues for fewer than 48% and 55% of these probe sets, respectively. Further, hierarchical clustering of the hepatocytes, but not the cell lines, shifted from donor-specific to treatment-specific when the probe sets were filtered to focus on Phenobarbital-inducible genes, indicative of the highly differentiated nature of the hepatocytes when cultured in a highly defined two-dimensional sandwich system.
2.2 Introduction

Primary hepatocytes are often used as an *in vitro* system to model biological processes that occur in the liver *in vivo*. In part due to rapid loss of hepatocyte differentiation status reported for most culture conditions, a variety of culture methodologies have been explored, with varying success, to better maintain hepatic character and function. In the two-dimensional sandwich system, primary hepatocytes are plated on collagen-coated dishes and then overlaid with either collagen or Matrigel, a commercially available extracellular matrix (ECM) material derived from the Engelbreth-Holm-Swarm sarcoma. The ECM includes such components as laminin, collagen IV, heparin sulfate proteoglycans, and entactin, components that comprise the extracellular milieu of the liver *in vivo*. Some reports have indicated that the sandwich culture model facilitates the preservation of certain liver characteristics, including cuboidal morphology of hepatocytes with features such as bile canaliculi, tight junctions, and gap junctions [1-6], expression of basolateral and canalicular domains of the plasma membrane such that polarized hepatic transport is retained [1,3,7,8], as well as expression and activity of drug metabolizing enzymes [2,5,6,9,10]. In comparison to conventional monolayer cultures, the sandwich culture model appears to enhance expression of liver-selective proteins such as albumin[3,5,6,9], transthyretin [6], and transferrin [6], and contributes to decreased levels of spontaneous apoptosis [9] and oxidative stress [4].

Despite these reports, the specific conditions used by different investigators still vary considerably, and concerns remain that the sandwich culture model may not accurately represent the *in vivo* response to chemical challenge. For example, it was reported that the sandwich configuration was unable to rescue basal levels of cytochrome P450 (CYP) activity to that observed in freshly isolated hepatocytes [4] and that, while the expression of some CYP proteins, namely CYP1A1, 2B1/2, and 3A2 in the rat, increase with time in culture, others, such as CYP2E1, decrease until expression is completely lost [11]. Phase II biotransformation enzymes were reported to undergo a similar mixed fate in culture [4,10]. Overall, losses of specific biotransformation function, together with other hepatic differentiation features, have limited the utility of
these models with respect to their predictive potential. Thus, further optimization and validation of the sandwich culture model are required, especially for human models, to establish a robust in vitro system for use in mechanistic and predictive studies of toxicology and drug metabolism.

In this investigation, we hypothesized that, based on previous development studies using rat hepatocytes [5,6], an optimized human two-dimensional primary hepatocyte culture system could be deployed that robustly reflects the differentiation status and biotransformation functions of hepatocytes in vivo. To this end, we adapted the use of a dilute ECM overlay within a highly defined serum-free media, supplemented with physiological concentrations of a glucocorticoid, and used DNA microarray analysis to assess transcriptional expression patterns in culture across a series of ten independent human donors. In parallel, whole genome expression profiling was conducted directly in human liver tissue samples and in two commonly used cell lines derived from human hepatomas, HepG2 and Huh7 cells. The data from the microarrays were subjected to expression analyses using the Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification system (www.pantherdb.org; Applied Biosystems, Foster City, CA) to identify over-represented biological processes and molecular functions in each model. Using these methods, we illustrate that primary human hepatocytes cultured in a defined sandwich configuration represent an in vitro hepatic model that closely resembles an in vivo hepatic phenotype, with respect to differentiation, liver-specific markers and the ability to support functional responses to xenobiotic challenge.

2.3 Materials and Methods

2.3.1 Cell culture conditions

Primary human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTDPS) at the University of Pittsburgh, through NIH Contract #NO1-DK-9-2310. Available donor information is presented in Table 1. Donor organs
not designated for transplantation were used to isolate hepatocytes according to a three step collagenase perfusion protocol [12]. Preparations enriched for hepatocytes were received plated in collagen-coated T25 flasks. Upon arrival, the media were changed to William’s Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 nM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, 5 μg/ml transferrin. Within 4-16 h, a 10 mg/ml stock solution of Matrigel (BD Biosciences, San Jose, CA), diluted to a final concentration of 225 μg/ml, was added dropwise to the culture media and evenly distributed by gentle swirling. The media were subsequently changed every 2 days until cells were harvested for RNA extraction at 90 h (HH-G, HH-I) or 114 h (HH-A through HH-F, HH-H, HH-J), depending on the condition of the cells. Time course studies were also performed on hepatocytes from 3 donors cultured up to 258 h, however, the extended time in contact with Matrigel did not result in any substantive changes in global gene expression profiles or on hierarchical clustering results relative to the 90- or 114-h culture periods. Huh7 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 1% penicillin/streptomycin, and 0.1 mM nonessential amino

<table>
<thead>
<tr>
<th>Donor identification</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Cause of death</th>
<th>Smoker/Alcohol use</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH-A</td>
<td>0.75</td>
<td>M</td>
<td>C</td>
<td>Anoxia</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-B</td>
<td>57</td>
<td>M</td>
<td>C</td>
<td>CVA/ICH</td>
<td>Yes (15 years)/Yes (5 drinks/week)</td>
</tr>
<tr>
<td>HH-C</td>
<td>52</td>
<td>M</td>
<td>C</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HH-D</td>
<td>61</td>
<td>M</td>
<td>C</td>
<td>Gunshot wound</td>
<td>No/Yes (21 drinks/week)</td>
</tr>
<tr>
<td>HH-E</td>
<td>63</td>
<td>M</td>
<td>C</td>
<td>IVH</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-F</td>
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<td>F</td>
<td>C</td>
<td>Anoxia</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-G</td>
<td>73</td>
<td>F</td>
<td>C</td>
<td>Head injury</td>
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</tr>
<tr>
<td>HH-H</td>
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<td>F</td>
<td>C</td>
<td>N/A</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-I</td>
<td>46</td>
<td>F</td>
<td>C</td>
<td>Head injury</td>
<td>Yes (13 years)/Yes (3 drinks/week)</td>
</tr>
<tr>
<td>HH-J</td>
<td>16</td>
<td>F</td>
<td>C</td>
<td>N/A</td>
<td>No/No</td>
</tr>
</tbody>
</table>

1 Donors were obtained from Dr. Stephen Strom at the University of Pittsburgh under the regulations of the Liver Tissue Procurement and Distribution System (LTPDS).
2 Caucasian.
3 CVA/ICH; cerebrovascular accident/intracranial hemorrhage.
4 N/A; not available.
5 IVH; intraventricular hemorrhage.
acids. HepG2 cells were maintained under the same conditions as Huh7 cells with one additional supplement to the media, 1 mM sodium pyruvate. All cells were maintained at 37°C under 5% CO₂. All culturing materials were purchased from Invitrogen (Carlsbad, CA), unless otherwise noted.

2.3.2 Immunohistochemistry

Following the removal of culture media, the cells were rinsed with phosphate-buffered saline (PBS), fixed in methanol for 5 min, rinsed with PBS, and blocked with 1x blocking buffer (Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature. Anti-Connexin 32 monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA) was added to the cells diluted in blocking buffer (1:250) for 48 h at 4°C. After rinsing with PBS 3x for 5 min each, he cells were incubated with secondary antibody, AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes, Invitrogen), diluted 1:400 in blocking buffer, for 45 min. After rinsing the cells with PBS, immunofluorescence of Connexin 32 protein was observed with a Nikon inverted fluorescence microscope (Nikon USA, Melville, NY). Image capture was performed with SpotRT software and digital camera (Diagnostic Instruments, Sterling Heights, MI).

2.3.3 Phenobarbital treatment

After 66 or 90 h in culture, selected cell cultures were treated with 500 µM phenobarbital (PB) for 24 h, an exposure shown previously to provide optimal induction of typical markers of response to PB without associated toxicity [6].

2.3.4 RNA isolation

For primary hepatocytes and hepatoma-derived cell lines, the media were aspirated, 1 ml of TRIzol® Reagent (Invitrogen) was added directly to each flask, and
RNA was extracted according to the manufacturer’s instructions. Contaminating DNA was removed using DNA-free™ DNase Treatment and Removal Reagents (Ambion, Inc., Austin, TX) according to manufacturer’s instructions for rigorous DNA treatment. RNA concentrations were assessed with UV absorbance at 260 nm, using a SmartSpec 3000 spectrophotometer (BioRad, Hercules, CA). Human liver RNA was obtained from flash-frozen human livers generously contributed by Dr. Kenneth Thummel from the University of Washington.

2.3.5 Quantitative real-time PCR

RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), following the manufacturer’s instructions. Gene expression was estimated using TaqMan® Assays-on-Demand™ Gene Expression systems (Applied Biosystems). Assays were prepared according to the manufacturer’s recommendations for a 50-µl reaction volume (5 µl cDNA, 1x TaqMan® Universal PCR Master Mix, and 1x Assays-on-Demand™ Gene Expression Assay Mix containing unlabeled PCR primers and TaqMan® FAM™ dye-labeled MGB probe), divided into duplicate 25-µl reactions in a 96-well plate, and conducted using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycling consisted of a UNG activation step for 2 min at 50°C and an initial denaturation step for 10 min at 95°C followed by 40 cycles of denaturation (15 s at 95°C) and annealing/extension (1 min at 60°C). Gene products measured by QRT-PCR were albumin (ALB; Assay ID Hs00609411_m1) and α-fetoprotein (AFP; Assay ID Hs00173490_m1). Expression levels were estimated using the ΔΔCt method and normalized to 18S (Assay ID Hs99999901_s1) using pooled RNA from 6 human livers as a reference standard. ΔΔCt was transformed into fold-change with the following formula: fold change = 2^{-ΔΔCt}. 
2.3.6 Microarray protocol

A total of 27 RNA samples (5 µg) were hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) by Paradigm Array Labs, Inc. (Icoria, Research Triangle Park, NC). The samples consisted of: three identical human liver RNA samples, each pooled from six individuals (3 arrays); one pool each of untreated and PB-treated HepG2 RNA, each from two cultures (2 arrays); one pool each of untreated and PB-treated Huh7 RNA, each from two cultures (2 arrays); and RNA from untreated and PB-treated human hepatocytes from ten independent donors (20 arrays). RNA quality was assessed at Paradigm Array Labs using an Agilent 2100 Bioanalyzer and NanoDrop® ND-1000 Spectrophotometer. Paradigm Array Labs performed amplification and labeling of cRNA, hybridization of the cRNA to the arrays, and scanning of the arrays, using Affymetrix reagent kits and instrumentation platforms.

2.3.7 Differentiation marker analysis

To estimate differentiation status in the in vitro models, prototypical differentiation markers were evaluated. Detection of each probe set on each array was assessed by the Microarray Suite version 5 (MAS5) algorithm in ArrayAssist 4.0 (Affymetrix), which uses a One-sided Wilcoxon’s signed rank test to assign a p-value for each probe set. This p-value is then used to make a ‘detection call’ to determine if a transcript is measurable (present), undetectable (absent), or uncertain (marginal) (for a more detailed explanation of the algorithm, see the Affymetrix Technical Note ‘Statistical Algorithms Reference Guide’, Part No. 701137, Rev. 3 at www.affymetrix.com). Only present probe sets were included in all subsequent analyses. The PLIER algorithm in the software package ArrayAssist 4.0 (Stratagene, La Jolla, CA) was used to summarize data from individual probes within a probe set (for further explanation of the algorithm, see the Affymetrix Technical Note ‘Guide to Probe Logarithmic Intensity Error (PLIER) Estimation’, Part No. 701903, Rev. 1 at www.affymetrix.com). Data were converted to signal log ratio format using ArrayAssist 4.0. For all gene-level analysis, the probe set
determined as the most specific for the respective gene was chosen for subsequent analyses, unless otherwise noted [13]. Specificity was determined by the identifier at the end of the probe set ID; ‘_at’ indicates that that probe set is predicted to perfectly match only a single transcript, ‘_s_at’ may match multiple transcripts, and ‘_x_at’ indicates that one or more probes in this probe set are predicted as identical or highly similar to sequences other than the intended transcript (Affymetrix Technical Note ‘Design and Performance of the GeneChip® Human Genome U133 Plus 2.0 and Human Genome U133a 2.0 Arrays’, Part No. 701483, Rev. 2 at www.affymetrix.com). In this study, the probe sets used for ALB and AFP were 214837_at and 204694_at, respectively. When the signal log ratio resulted in a positive value, the fold change was calculated as: fold increase = $2^{\text{Signal log ratio}}$. When the signal log ratio resulted in a negative value, the fold change was calculated as: fold decreased = $-(2^{\text{(-Signal log ratio)}})$.

### 2.3.8 Correlation analysis

The resulting data were then filtered from 54,675 probe sets to 39,485 probe sets by removing any probe sets detected as ‘Absent’ in all 27 arrays. To obtain an estimate of similarity in global gene expression between the arrays, ArrayAssist generated scatter plots and correlation coefficients for the three comparisons between technical replicates (Liver A-Liver B; Liver A-Liver C; Liver B-Liver C), the six comparisons between the liver replicates and the two untreated hepatoma-derived cell lines (Liver A-HepG2; Liver A-Huh7; Liver B-HepG2, etc.), and the thirty comparisons between the liver replicates and the untreated hepatocytes from ten donors (Liver A-HH-A; Liver B-HH-A; Liver C-HH-A; Liver A-HH-B; Liver B-HH-B; etc.). Data were expressed as mean correlation ± SD, where SD represented deviation between the three measurements of correlation between a single in vitro sample and the three human liver replicates. One-way analysis of variance in combination with Tukey’s multiple comparison post-test was used to determine significance of the difference in correlation between Liver B (chosen as a representative liver sample) and each of the three groups of samples (technical replicates, cell lines, and hepatocytes).
2.3.9 Over-representation analysis

In order to identify biological trends, differentially expressed genes were subjected to gene ontology (GO) over-representation analysis. For each untreated array, a list was created consisting of all probe sets that were both present and expressed differentially in that array from the human liver tissues. Differential expression was defined as expression greater than a signal log ratio of 2 (4-fold increase from human liver) or less than a signal log ratio of -2 (4-fold decrease from human liver). Each list of differentially expressed probe sets was then submitted to the PANTHER Classification System to find over-represented ontology categories [14]. For each ontology category, PANTHER calculates the number of genes identified in that category in both a list of differentially regulated genes and a reference list containing all the probe sets present on the Affymetrix array and compares these results using the binomial test to determine if there are more genes than expected in the differentially regulated list [15]. Over-representation is defined by \( p < 0.05 \).

2.3.10 Regulation of individual probe sets in GO categories

To determine how the over-represented categories were regulated in each in vitro model, a GO category-specific list for each category of interest was compiled containing those probe sets identified as differentially expressed in any of the three in vitro models. For each untreated array, all of the probe sets in each GO category-specific list detected as present on that array were scored as belonging to one of three regulation groups: ‘Not changed’ \((-2<\text{signal log ratio}<2)\), ‘Increased’ \((\text{signal log ratio}>2)\), or ‘Decreased’ \((\text{signal log ratio}<-2)\). For HepG2 and Huh7 cells, data in each regulation group are expressed as the percentage of probe sets detected as present. For the human hepatocytes, data in each regulation group are expressed as the mean percentage \( \pm \) SD of probe sets detected as present, summarized from the ten individual hepatocyte donors. For the human hepatocytes, one-way analysis of variance in combination with Tukey’s multiple

\( \phantom{\text{Not changed’ \((-2<\text{signal log ratio}<2)\), ‘Increased’ \((\text{signal log ratio}>2)\), or ‘Decreased’ \((\text{signal log ratio}<-2)\). For HepG2 and Huh7 cells, data in each regulation group are expressed as the percentage of probe sets detected as present. For the human hepatocytes, data in each regulation group are expressed as the mean percentage \( \pm \) SD of probe sets detected as present, summarized from the ten individual hepatocyte donors. For the human hepatocytes, one-way analysis of variance in combination with Tukey’s multiple
comparison post-test was used to determine significance of the difference between regulation groups in each GO category.

2.3.11 Gene-level analysis of liver-specific functions

To verify the biological relevance of the over-representation analysis, genes with known biological roles in three liver-specific ontology categories were assessed. Data are expressed as fold change, as described under the Differentiation marker analysis section. The following probe sets were used: C/EBP\(\alpha\), 204039_at; HNF4\(\alpha\), 230914_at; NFE2L2, 201146_at; NR1I2, 207202_s_at; NR1I3, 207007_at; PPAR\(\alpha\), 206870_at; PPAR\(\delta\), 37152_at; RXR\(\alpha\), 202449_s_at; TCF1, 210515_at; CYP1A2, 207609_s_at; CYP2B6, 206755_at; CYP2C9, 217558_at; CYP2D6, 207498_s_at; CYP2E1, 209975_at; CYP3A4, 205999_x_at; FMO5, 205776_at; EPHX1, 202017_at; EPHX2, 209368_at; GSTA1, 203924_at; GPX1, 200736_s_at; ABCB1, 209993_at; ABCB11, 208288_at; ABCC1, 202804_at; ABCC2, 206155_at; ABCC4, 203196_at; SLC22A1, 207201_s_at; SLC22A3, 205421_at; SLC22A7, 231398_at; and SLCO2B1, 211557_x_at.

2.3.12 Gene-level analysis of a complex biological response

To assess the ability of each \emph{in vitro} hepatic model to perform a complex biological response to chemical treatment, three marker genes were analyzed for transcriptional induction in response to PB. Data are reported as fold change as described under the Differentiation marker analysis section.

2.3.13 Hierarchical clustering

To obtain a global view of the response of each \emph{in vitro} model system to PB, hierarchical clustering was done on two different datasets. Signal log ratio was obtained as described in the Differentiation marker analysis section. First, the 27 arrays were
subjected to hierarchical clustering analysis using the 39,485 probe sets found to be present or marginal in at least one array as the dataset. Second, both the 27 arrays and PB-inducible probe sets were subjected to clustering analysis using a dataset consisting only of PB-inducible probe sets. PB-inducible probe sets were defined in each pair of samples (untreated and PB-treated) by defining the baseline array as the untreated sample and identifying any probe sets increased at least 4-fold in the treated sample. The PB-inducible dataset consisted of 54 probe sets, representing 31 unique genes that were both inducible in at least two pairs of samples and present in at least half of the arrays (13 arrays). All hierarchical clustering was performed using a Euclidean distance measurement and an average linkage.

2.3.14 Protein isolation

For each human hepatocyte and cell line sample, protein was isolated from the organic phase of the TRIzol® Reagent remaining after RNA isolation according to the protocol provided by the manufacturer. Protein concentrations were determined according to the Bradford method, using a commercially available kit (BioRad Protein Assay) and bovine serum albumin as the standard. S9 fractions were derived from human liver tissue samples as describe previously [16].

2.3.15 Western blotting

Protein samples (S9 fractions, 20 µg; protein isolated from TRIzol® Reagent, 40 µg) were separated by electrophoresis on a precast Ready Gel (10% Tris-HCl, BioRad) and transferred to Immun-Blot PVDF membrane (BioRad). The PVDF membrane was subjected to a blocking procedure by incubation in 5% non-fat dry milk in TBS-Tween 20 (0.1%) for 1 h. Primary antibodies were diluted in blocking buffer as follows: albumin, 1:7500 (monoclonal antibody #A6684; Sigma-Aldrich); β actin, 1:3000 (monoclonal antibody #A5441; Sigma-Aldrich); CYP2B6, 1:650 (monoclonal antibody
clone 49-10-20); CYP2C9/18, 1:1000 (monoclonal antibody clone 592-2-5); CYP3A4, 1:1000 (monoclonal antibody 275-1-2); and, RXRα, 1:1000 (polyclonal antibody D-20 Santa Cruz Biotechnology, Santa Cruz, CA). Incubation in primary antibody was either at 2 h at room temperature (for detection of albumin, β actin, CYP2C9/18, and CYP3A4) or overnight at 4°C (for detection of CYP2B6 and RXRα). Antibodies for CYP2B6, CYP3A4, and CYP2C9/18 were obtained through Dr. Harry C. Gelboin at the NCI/NIH. The PVDF membranes were then washed 4 times for 5 min each, followed by incubation in the appropriate secondary antibody, either goat anti-rabbit IgG-HRP (Santa Cruz, #sc-2004; 1:3000 for detection of RXRα,) or goat anti-mouse IgG-HRP (Santa Cruz, #sc-2005; 1:3000 for detection of β actin and CYP2B6; 1:5000 for detection of CYP2C9/18 and CYP3A4; 1:20,000 for detection of albumin). Subsequently, the membranes were washed 4 times for 5 min prior to autoradiographic detection of signals by chemiluminescence using a commercially available kit (Lumi-light, Roche Diagnostic, Indianapolis, IN).

2.4 Results

2.4.1 Immunohistochemistry

Visualized by phase contrast microscopy, primary hepatocyte cultured in both monolayer (on collagen) and in Matrigel sandwich configurations (Figure 4A and C, respectively) exhibited morphology indicative of a highly differentiated phenotype, comprised of striking cuboidal cellular architecture with hepatocytes arranged in closely associated networks. Hepatocytes in a Matrigel sandwich culture in particular exhibited enhanced gap junction formation, as assessed with immunohistochemical analyses of connexin 32 protein expression (Figure 4D vs B).
2.4.2 Differentiation marker analysis

Albumin (ALB), a liver-selective protein, is generally used as a marker of the differentiation status of in vitro liver models [3,6,9,11,17]. Although a slight decrease from the liver was suggested, ALB expression was maintained in all in vitro hepatic models within the boundaries defined as unchanged from the liver (less than 4-fold decreased) (Figure 5A). An inverse marker of hepatic differentiation status is α-fetoprotein (AFP), the fetal homologue of albumin. Because synthesis of AFP is turned off shortly after birth, its detection indicates loss of the differentiated liver phenotype and reversion to a more fetal status. Among the seven human hepatocyte donors where AFP was reliably detected, expression of AFP appeared modestly elevated, 4.6 ± 1.2 (SD)-fold from the liver, slightly above the 4-fold cutoff selected for differential expression (Figure 5C). In striking contrast, HepG2 and Huh7 cells exhibited markedly enhanced expression of AFP, with levels increased >600- and 1000-fold, respectively, from levels detected directly in liver tissues.

Figure 4: Detection of connexin 32 using immunohistochemistry in primary human hepatocytes maintained in monolayer and sandwich culture. Hepatocytes were cultured on collagen-coated flasks and were either maintained as a monolayer (A and B) or overlaid with a dilute layer of Matrigel (C and D). After fixation, cells were subjected to immunohistochemical analysis for connexin 32 using an Alexa Fluor secondary antibody conjugated with a FITC molecule. Images were taken as bright field (A and C) or with a FITC-specific filter (B and D). Magnification x 200.
To better estimate the variability of ALB and AFP expression, quantitative RT-PCR (QRT-PCR) was used as a verification of the microarray results. The data derived from both QRT-PCR and microarray analyses indicated that ALB expression was unchanged in the primary cultured hepatocytes relative to liver tissue (Figure 5B). Although the well-documented compression of signal effect is apparent, AFP expression in the different culture systems measured by QRT-PCR closely mimicked the microarray estimation; with the fold enhancement of AFP detected by QRT-PCR even greater than that detected by microarray analysis (59,000 ± 9000- and 130,000 ± 80,000-fold increased in HepG2 and Huh7 cells vs. 600- and 1000-fold increased; Figs. 2C-D).

Figure 5: Albumin (ALB), a differentiation marker, and α-fetoprotein (AFP), a dedifferentiation marker, estimate the differentiation status of the in vitro hepatic models. Data are expressed as fold change (A and C). These markers were verified by QRT-PCR, using the ΔΔCt method for analysis where 18S is the reference gene and the human liver is the reference sample. Data are expressed as fold change, defined by $2^{-\Delta\Delta Ct}$ (B and D). Differential expression is defined as greater than 4-fold change from the human liver (dotted lines).
Among the five hepatocyte donors for which QRT-PCR analysis was performed, *AFP* expression was $4.6 \pm 4.5$-fold higher than the liver, reflecting variability between cultures established from different donors. Thus, both techniques detect *AFP* expression in hepatocytes as nearly unchanged from the liver; while expression in the hepatoma-derived cell lines is dramatically increased relative to the liver. Additionally, verification of the microarray results with a second method of mRNA quantification provides confidence that microarray analysis is an appropriate method for estimation of mRNA expression.

### 2.4.3 Correlation analysis

The pooled human liver replicates exhibited higher correlation with the primary human hepatocytes than with the hepatoma cell lines, although the highest correlation between two samples was apparent for the technical replicates ($p<0.001$; Figure 6G). Correlation between the three technical replicates was quite high; correlation coefficients in all combinations were greater than 0.996 (Figs. 6A-B); all scatter plots are shown using liver replicate B as a representative liver). The six comparisons between the human liver replicates and the hepatoma cell lines revealed very poor correlation in gene expression profiles (HepG2, $0.614 \pm 0.007$; Huh7, $0.566 \pm 0.006$; Figs. 6C-D). For the thirty comparisons between the human liver replicates and the ten human hepatocytes donors, the correlation to human liver among donors ranged from $0.76 \pm 0.01$ in donor HH-G to $0.70 \pm 0.01$ in HH-B (Figs. 6E-F). The variability noted between the liver and the *in vitro* models is likely due to a combination of the absence of certain cell types in the purified hepatocyte preparations that are otherwise present in whole liver tissue, such as Kupffer cells and hepatic stellate cells, and interindividual differences between the hepatocyte donors and the human liver donors.
Figure 6: Correlation of gene expression profiles between human liver and either technical replicates A (A) and C (B); HepG2 cells (C); Huh7 cells (D); two human hepatocyte donors, HH-B (E) and HH-G (F). Panel G summarizes the mean correlation of the individual HepG2, Huh7, and human hepatocyte arrays to the human liver pools and the correlation of technical replicates to a representative array of pooled human liver (liver replicate B). Different letters (a, b, c) indicate significantly different correlation to the human liver pool ($p<0.001$; one-way ANOVA with Tukey’s multiple comparison post-test).
2.4.4 Over-representation analysis

In an effort to examine biological trends among these basal level expression changes in the in vitro models, we used over-representation analysis with the PANTHER Classification System to identify biological processes and molecular pathways fundamental to drug metabolism among the differentially expressed probe sets in all of the models (Table 2). For example, two types of reactions catalyzed by biotransformation enzymes were identified as over-represented; ‘oxidoreductase’ (hepatocytes, $p=3.86 \times 10^{-3}$; HepG2, $p=5.31 \times 10^{-2}$) and ‘transferase’ (HepG2, $p=1.01 \times 10^{-2}$; Huh7, $p=7.03 \times 10^{-4}$). ‘Lipid, fatty acid, and steroid metabolism’ was identified in all three models (HepG2, $p=1.22 \times 10^{-8}$; Huh7, $p=1.24 \times 10^{-7}$; hepatocytes, $p=5.78 \times 10^{-4}$). Additionally, drug metabolism, alternatively referred to as ‘detoxification’ (HepG2, $p=3.44 \times 10^{-2}$, Huh7, $p=1.10 \times 10^{-3}$), is dependent upon signaling pathways, which are carried out by ‘signaling molecules’ (hepatocytes, $p=1.12 \times 10^{-4}$) that often depend upon modifications such as ‘protein phosphorylation’ (HepG2, $p=1.22 \times 10^{-3}$).

2.4.5 Regulation of individual probe sets in the liver-specific GO categories

In the six over-represented GO categories noted above as involved in drug metabolism, the individual probe sets in the primary hepatocyte samples were overwhelmingly unchanged from the liver, while no distinct pattern of regulation was evident among the hepatoma-driven cell lines (Figure 7A-F). Among these six categories, the average number of probe sets in the hepatocytes that were unchanged from the liver was $83.7 \pm 3.7\%$. This observation is also consistent in each of the ten individual hepatocyte donors, as can be seen in two representative ontology categories, ‘oxidoreductase’ and ‘phosphorylation’ (Figure 7G-H). Within each donor, the percentages of probe sets that are scored as unchanged, increased, or decreased emphasize that, while there are slight differences between donors in the regulation of individual probe sets, more probe sets in the hepatocytes are unchanged from the liver.
than are increased or decreased \( (p < 0.001) \). HepG2 and Huh7 cells, on the other hand, exhibited an average of only 42.5 ± 3.1% and 39.2 ± 9.2% unchanged probe sets compared with the liver across the six ontology categories, respectively.

### 2.4.6 Gene-level analysis of liver-specific functions

Expression of individual genes with known biological roles in the over-represented ontology categories supports the hypothesis that in vitro hepatocytes in a sandwich culture are functionally representative of the in vivo liver, in contrast to the

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Table 2: Statistically over-represented gene ontology (GO) categories among the differentially expressed genes in HepG2 cells, Huh7 cells, or human hepatocytes.

<table>
<thead>
<tr>
<th>Panther ID</th>
<th>Panther category</th>
<th>Significant children in this branch</th>
<th>HepG2</th>
<th>Huh7</th>
<th>Hepatocytes</th>
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<td></td>
<td><strong>BIOLOGICAL PROCESS ANNOTATIONS</strong></td>
<td></td>
<td>p-value(^6)</td>
<td></td>
<td></td>
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<tr>
<td>BP00019</td>
<td>Lipid, fatty acid, and steroid metabolism</td>
<td>1.22x10^-8</td>
<td>1.24x10^-7</td>
<td>5.78x10^-4</td>
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<td>BP00295</td>
<td>Steroid metabolism</td>
<td>2.21x10^-3</td>
<td>9.60x10^-6</td>
<td>9.86x10^-6</td>
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<td>BP00020</td>
<td>Fatty acid metabolism</td>
<td>(-)</td>
<td>7.69x10^-4</td>
<td>(-)</td>
<td></td>
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<tr>
<td>BP00063</td>
<td>Protein modification</td>
<td>3.10x10^-3</td>
<td>(-)</td>
<td>(-)</td>
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<tr>
<td>BP00064</td>
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<td>BP00148</td>
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</tr>
<tr>
<td>BP00153</td>
<td>Complement-mediated immunity</td>
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<td>BP00180</td>
<td>Detoxification</td>
<td>3.44x10^-4</td>
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<td>BP00178</td>
<td>Stress response</td>
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<td>BP00203</td>
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<td>BP00282</td>
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<td>BP00035</td>
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<tr>
<td>BP00224</td>
<td>Cell proliferation and differentiation</td>
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<td>2.96x10^-2</td>
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<td></td>
</tr>
<tr>
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<td>MF00016</td>
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<td>MF00107</td>
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<tr>
<td>MF00108</td>
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<td>MF00123</td>
<td>Oxidoreductase</td>
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<td>(-)</td>
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<td>MF00124</td>
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<td>(-)</td>
<td>1.02x10^-3</td>
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<tr>
<td>MF00131</td>
<td>Transferase</td>
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<td>7.03x10^-4</td>
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<tr>
<td>MF00133</td>
<td>Methyltransferase</td>
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<td>1.47x10^-4</td>
<td>(-)</td>
<td></td>
</tr>
</tbody>
</table>

\(^6\) Significance is defined as \( p < 0.05 \).

\(^7\) \(-\); \( p > 0.05 \)
established cell lines. As a case in point, liver-enriched transcription factors, including hepatocyte nuclear factor (HNF) family members, CAAT/enhancer binding protein α (C/EBPα), and members of the nuclear hormone receptor superfamily, are central to maintaining the hepatic phenotype. These transcription factors were tightly regulated in
all ten hepatocyte donors, as expression of all nine genes is maintained at levels less than 4-fold changed from the liver (Figure 8A). Although expression of these transcription factors was also generally regulated in the established cell lines, there were notable exceptions. NR1I2 (pregnane X receptor, or PXR) and NR1I3 (constitutive androstane receptor, or CAR) were >6- and 42-fold decreased in Huh7 cells (Figure 8B). A third nuclear hormone receptor, the retinoid X receptor-α (RXRa), was also decreased in Huh7 cells in comparison to the liver (5.7-fold).

Drug metabolizing enzymes, such as the various CYPs, flavin-containing monooxygenase 5 (FMO5), epoxide hydrolases (EPHXs), glutathione-S-transferase (GSTA1), and glutathione peroxidase (GPX1), catalyze the ‘oxidoreductase’ and ‘transferase’ activities identified by the over-representation analysis and their expression status yields important insight regarding the metabolic functional capacity of in vitro hepatic models. As expected from reports of decreased basal activity of certain CYP enzymes in sandwich culture [4,11], expression of certain enzymes appears to be decreased in culture from the liver; particularly affected are CYP1A2 and CYP2E1 (Figure 8C). However, the remaining enzymes are expressed at levels comparable to or slightly increased from the human liver in the majority of the hepatocyte donors. In contrast, expression of the CYP450 isoforms is generally lost or dramatically decreased in comparison to the liver in both of the hepatoma cell lines that were subjected to analysis (Figure 8D).

Drug transporters, a third functional group of genes whose products perform important roles in ‘detoxification’, have been reported previously to exhibit polarized expression in sandwich culture of hepatocytes. Our results support these findings, as transporters, such as P-glycoprotein (ABCB1), the bile salt export pump (ABCB11), multidrug resistance-associated protein family members (MRPs, or the ABCC family), organic cation and anion transporters (OCTs and OATs, or solute carrier family
Figure 8: Gene-level expression analysis of selected liver-specific categories in human hepatocyte donors and hepatoma-derived cell lines. Distribution of fold change from the liver in the ten hepatocyte donors are shown for genes encoding select transcription factors (A), drug metabolizing enzymes (C), and drug transporters (E). The fold change for the same genes in HepG2 cells and Huh7 cells are reported in B, D and F. Differential expression is defined as greater than four-fold change from the human liver (dotted lines). * indicates that the measured probe set is detected as absent in at least one human hepatocyte donor (PPARA: Absent in two donors; TCF1: two donors; CYP1A2: one donor; ABCB11: one donor; ABCC1: one donor). ** indicates that that probe set is detected as absent in Huh7 cells (NR1I2, NR1I3, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP3A4, ABCB11, SLC22A1, SLC22A7). *** indicates that that probe set is detected as absent in HepG2 (CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2E1, ABCB11, SLC22A1, SLC22A7, SLC02B1).
SLC22A), and organic anion transport proteins (OATP, or solute carrier family SLCO),
were expressed at levels unchanged from the liver in nearly all of the human hepatocyte
donors (Figure 8E). In the established cell lines, however, expression of select
transporters is lost, consistent with the view that these cell models do not maintain
polarized membranes reminiscent of the liver.

2.4.7 Gene-level analysis of a complex biological response

Hepatocytes, but not hepatoma-derived cell lines, retain the ability to respond to
toxic insult by induction of biotransformation enzymes, a critical feature of an
appropriate in vitro model of the liver. Among the ten hepatocyte donors, considerable
interindividual variability was apparent in the basal expression levels of three PB-
inducible target genes; CYP2B6, CYP3A4; and CYP2C9 (Figure 9A-C). Most notably,
basal expression of CYP2B6 ranged in signal intensity from 142 to nearly 2900
fluorescence units; in comparison, the highest signal strength scored after induction by
PB was approximately 4100 units. However, CYP2B6, CYP3A4, and CYP2C9 were
expressed basally at levels unchanged from the human liver in a majority of the donors,
and, overall, PB treatment induced a greater fold change from the liver than treatment
with vehicle (p<0.01; Figure 9D-F). In the established cell lines, only CYP3A4 was
reliably detected at the basal level, albeit more than 45-fold decreased from the human
liver. As for inducibility of these genes in the cell lines, the only suggestion of induction
was observed in Huh7 cells, in which CYP2B6 was expressed at levels comparable to
human liver and CYP3A4 was >45-fold decreased from human liver after PB-treatment
(data not shown). Basal levels of CYP2B6, CYP3A4, and CYP2C9 proteins were clearly
detectable in the human liver pool, but not in the human hepatocyte and established cell
line samples (Figure 9G and data not shown). This apparent discrepancy is likely due to
the nature of the human liver tissues used for Western blotting which, as S9 fractions, are
enriched for microsomal content, while the human hepatocyte and cell line samples
consisted of whole cell extracts. The different protein sources derived from the human
liver tissues vs. in vitro models further explain the absence of RXRα and β-actin
selectively in the human liver tissues. After PB treatment, protein expression of the three target genes was clearly elevated in the human hepatocytes, but not the cell line samples, further supporting the use of primary hepatocytes for studies of drug metabolism.

Figure 9: Gene-level analysis of a complex biological response in human hepatocytes. Data are reported as linear signal intensity values for each untreated and PB-treated array for three target genes: CYP2B6 (A), CYP3A4 (B), and CYP2C9 (C). * indicates that the PB-treated signal intensity in a donor is more than four-fold increased from the untreated signal intensity. Data in D, E, and F are expressed as fold change from the human liver (p<0.01; paired t-test). Differential expression is defined as greater than four-fold change from the human liver (dotted lines). Induction of protein in response to PB treatment is shown for selected donors and hepatoma-derived cells lines (G).
2.4.8 Hierarchical clustering analysis

When subjected to hierarchical clustering analysis, PB treatment caused hepatocytes, but not hepatoma-derived cell lines, to cluster by treatment rather than by sample type. When clustering of the samples was performed using all 39,485 probe sets that remained after removal of those probe sets absent in all 27 arrays, the resulting dendrogram was divided into two major branches: a first branch containing the liver replicates and primary hepatocytes, and a second containing the hepatoma-derived cell lines (Figure 10A). These clustering results indicate that the gene expression patterns occurring in the liver are more similar to hepatocytes than to the heaptoma cell lines, and that, among the human hepatocytes, interindividual variation plays a crucial role in gene expression patterning, as eight of the ten untreated samples clustered most closely with the PB-treated sample from the same donor.

Clustering of the samples was performed a second time, including only probe sets increased more than 4-fold in at least two PB-treated samples. The dendrogram was again divided into the same two main branches described above, emphasizing that the liver replicates are more similar to the hepatocytes than the cell lines (Figure 10B). However, the branch containing the liver pools and hepatocytes was now further divided into two treatment-specific arms; one containing the liver pools, eight of ten untreated samples, and one PB-treated sample, and the second containing nine PB-treated and two untreated hepatocyte samples. Contrary to the hepatocytes, the clustering pattern of the hepatoma cell lines was not altered in this PB-inducible dataset to reflect treatment.

Hierarchical clustering was also used to group the PB-inducible probe sets on the basis of similarity in expression pattern across the 27 samples. One branch of the dendrogram stood out with a distinct expression pattern across the different types of samples (magnified in Figure 10C). This cluster of probe sets was characterized by little change or a slight increase in expression in the untreated hepatocytes, clearly increased expression in the PB-treated hepatocytes, and decreased expression in the hepatoma-
Figure 10: Hierarchical clustering of the human liver replicates, hepatoma-derived cell lines, and human hepatocytes. Hierarchical clustering of the 27 arrays was performed using a dataset consisting of 39,485 probe sets (A), followed by clustering of both the 27 arrays and the PB-inducible probe sets, which were found to be both PB-responsive in at least 2 pairs of samples (untreated and PB-treated) and present in 13 or more of the arrays (B). A cluster of probe sets with a distinct expression pattern across the 27 arrays is magnified in C. All clustering was done using a Euclidean distance measure and an average linkage.
derived cell lines, both treated and untreated. The majority of the probe sets in this cluster are specific for cytochrome P450 family members, specifically, members of the CYP2A, 3A, and 2B families. Three additional non-CYP genes were identified to exhibit this similar overall expression pattern: aminolevulinate, delta-, synthase 1 (ALAS1), leucine-rich repeat containing 54 (LRRC54), and one probe set that is not annotated. Overall, hierarchical clustering confirmed the themes presented thus far in this study; that hepatocytes possess gene expression profiles that are more representative of human liver than the hepatoma cell lines, and that measurement of gene expression character provides novel insight into functional capacity, as hepatocytes, but not hepatoma-derived cell lines, respond robustly to inducer challenge.

2.5 Discussion

To better assess the utility of primary hepatocytes in an ECM sandwich culture configuration as a model for drug metabolism studies, microarray technology was used to gain a global view of the biological processes and molecular functions altered in two commonly used human in vitro hepatic models from the in vivo phenotype. Although it is true that our culture conditions could be described as 3D, in that the hepatocytes are sandwiched between a substratum of collagen and an overlay of Matrigel, the cellular milieu largely consists of a 2D surface provided in the context of a culture plate. Several investigators have devised hollow fiber bioreactors that enable packed cells to share a 3D space, within an environment that may also provide dynamic fluidics forces. In our conceptual framework, packed cells within a bioreactor scheme are more adeptly labeled as 3D cultures. Throughout our manuscript, we have largely referred to our culture scheme as a ‘sandwich’ approach, a term that does accurately describe the culture situation, with 2D being used to reflect that the cells are plated on a flat surface. Our results show that primary hepatocytes cultured using highly defined conditions maintained basal expression of genes involved in liver-specific functions and facilitated a complex biological response to chemical treatment. Hepatoma-derived cell lines were unable to demonstrate either of these features.
Genes examined in this study were chosen based on inclusion in the over-represented ontology categories identified by the PANTHER classification system, an unbiased method of identifying biological trends among large lists of transcriptional changes [14,15,18]. Although many of the over-represented ontology categories described functions integral to drug metabolism, such as ‘oxidoreductase’, ‘transferase’, ‘lipid, fatty acid, and steroid metabolism’, and ‘detoxification’ (Table 2), the prominent pattern of regulation of the individual probe sets within each of these categories was unchanged expression between the hepatocytes and the liver (Figure 7). Interestingly, liver-enriched transcription factors, such as nuclear receptors, were shown to be tightly regulated in all hepatocyte donors (Figure 8A). Conservation of nuclear receptor expression supports the functionality of hepatocytes in sandwich culture to predict drug metabolism, as these receptors have been shown to regulate batteries of genes involved in response to chemical stress [19-21]. Many of the genes regulated by nuclear receptors are drug metabolizing enzymes and drug transporters [22-24], which, under the described culture conditions, were expressed at levels unchanged from the human liver in the majority of the human hepatocyte donors (Figure 8B-C). Again, conserved expression of drug metabolizing enzymes and drug transporters supports the functionality of this culture model for prediction of both drug metabolism and drug disposition.

Throughout this manuscript, differential expression has been defined on the basis of fold change. It is well documented that using fold change alone as a method to identify differentially expressed probe sets does not produce optimal results, as this method has a high probability of identifying false positives [25-27]. However, other methods of detecting differential change (t-statistic, ANOVA, significance analysis of microarrays) require technical replicates in order to assess statistical significance. When we considered our experimental goal, which was to maximize the number of donors in order to address interindividual variability, together with the high level of reproducibility afforded by the Affymetrix arrays, we decided to include as many biological replicates as possible at the expense of technical replicates that would allow significance analysis. In order to limit the number of false positives anticipated to result from using a fold change method to define differential expression, the fold change cutoff was increased above the more
commonly used 2-fold change cutoff. The rationale for this decision was based on results which suggested that small variations in mRNA levels (less than 2-fold) are less accurately reflected by microarray analysis than by more quantitative methods such as QRT-PCR [13]. Thus, a 4-fold cutoff is intended to minimize the number of false positives, and, even though we are likely losing some true positives, we decided to focus only on dramatic gene expression changes in the *in vitro* models from the human liver. Interestingly, analyses performed using a lowered 2-fold cutoff did not alter the conclusion that human hepatocytes maintained in sandwich culture exhibit a gene expression profile more similar to the *in vivo* human liver than hepatoma-derived cell lines with respect to ontology categories involved in drug metabolism. Over-representation analysis using all probe sets changed more than 2-fold in each sample identifies nearly the same ontology categories, although there are a few differences in significance at this cutoff level; ‘lipid, fatty acid, and steroid metabolism’ and ‘signaling molecule’ were no longer identified as significant in Huh7 cells and the hepatocytes, nor was ‘protein phosphorylation’ considered significant in HepG2 cells. Of the probe sets in the six categories of interest, more probe sets remained unchanged from the liver than were increased or decreased in the hepatocytes (*p*<0.001), similar to the results using the 4-fold cutoff. Also, the percentage of probe sets unchanged from liver across these six categories is higher in the hepatocytes than in the hepatoma-derived cell lines at a 2-fold cutoff (65.6±5.1% vs. 40.7±4.1%; *p*<0.001), in agreement with the conclusions drawn at the 4-fold cutoff level.

A second endpoint, complex biological responsiveness to chemical treatment, indicated that large-scale transcriptional analysis can be used to assess an *in vitro* functional response. For example, gene-level analysis of classical PB-inducible markers, *CYP2B6*, *CYP3A4*, and *CYP2C9*, showed that the induction response occurred at both the mRNA and protein levels in the hepatocyte donors but not in the hepatoma-derived cell lines (Figure 9), which are often used as representative models of hepatocyte biology. Hierarchical clustering of the samples also confirmed that the primary hepatocytes retain induction responsiveness to chemical treatment, since treatment-specific clustering was observed only in the hepatocyte samples (Figure 10B), but not in the cell lines. Since
induction of the CYP2B family members by PB treatment has historically been the most difficult P450 response to retain in culture [2,6], these results suggest that, functionally, the primary human hepatocytes can replicate the metabolic capacity of the *in vivo* human liver.

Similar large-scale transcriptional analyses to compare *in vivo* and *in vitro* basal gene expression profiles in rodent models have been conducted, and offer similar results to those presented here. For example, one study established that the global expression profiles between *in vivo* rat liver and primary rat hepatocytes exhibited a correlation coefficient of 0.8023 [28], while another reported a correlation of 0.68 [29]. Both studies are in agreement with our findings, where correlation coefficients ranged from 0.76±0.01 to 0.70±0.01 among the ten human donors (Figure 6G). Hierarchical clustering analysis is a further area of agreement, with Boess et al. [29] finding that rat liver and primary hepatocytes cluster on the same arm of the dendrogram compared to rat liver cell lines that cluster on a separate arm. Our conclusions are further corroborated by reports that primary human hepatocytes in sandwich culture maintain expression of drug metabolizing enzymes similar to that in human liver while HepG2 cells exhibit dramatically decreased levels of these enzymes [30-32].

As anticipated, interindividual variability among the ten donors was noted throughout the study. Hierarchical clustering analysis most greatly illustrated the contribution of interindividual variability among donors, since samples clustered according to donor rather than treatment when all present probe sets were included in the dataset (Figure 10A). Also noted in the clustering analysis was that both untreated and PB-treated samples from donors HH-D and HH-F clustered on the PB-treated arm of the dendrogram (Figure 10B), which was consistent both with the minimal induction response upon chemical treatment and with the higher basal expression of *CYP2B6* and *CYP3A4* in these two donors (Figure 9). These results suggest that, even after almost 5 days in culture, the hepatocytes from these two donors were nearly maximally induced, perhaps because of exposure to environmental factors such as food or drugs [33]. Thus, these hepatocytes were not capable of being further induced by treatment with PB, a trend noted by several other studies [34,35]. A second discrepancy noted was that the
induction response in the PB-treated HH-E sample was minimal, such that this sample clustered more closely with the untreated samples; specifically, with the untreated HH-E sample. Other minor variations included a decrease in albumin expression in a single donors (HH-C, decreased 6.8-fold from liver expression) and undetectable expression of certain transcription factor \((TCF1\) in HH-C and HH-J; \(PPARA\) in HH-E), drug metabolizing enzymes \((CYP1A2\) in HH-J), and drug transporters \((ABCB11\) in HH-E). Further, albumin protein was undetectable in HH-J (Figure 9G). Loss of liver-specific gene and protein expression in hepatocytes from these three donors is likely an indication of loss of differentiation in these samples.

Despite the noted discrepancies, overall, gene expression trends were clearly identified among donors. One such trend was that the regulation of probe ests in each over-represented ontology category was largely unchanged from the liver in all ten donors. Similarly, as indicated above, expression of liver-enriched transcription factors was tightly regulated in the sandwich culture model, such that, when present, none were more than 4-fold changed in any of the donors. This trend also was true for all the drug transporters that were analyzed, with only a single gene \((SLC22A7\) decreased more than 4-fold in a single donor (HH-I). These results suggest that, while interindividual differences will contribute to differences in metabolic capacity \textit{in vitro}, primary hepatocytes cultured in a sandwich model exhibit conserved biological trends from preparation to preparation. These results argue that even with the use of hepatocytes from multiple donors, general trends in response to a chemical or treatment are likely still discernable, despite minor inter-individual differences that inherently will exist.

In conclusion, in this study we conducted a global, unbiased view of the biological processes and molecular functions altered at the transcriptional level in two commonly used \textit{in vitro} models of the human liver. Overall, the results support the utility of primary human hepatocytes cultured in a defined ECM sandwich configuration as a robust model of the \textit{in vivo} hepatic phenotype, as a striking similarity exists in the expression profiles for genes involved in hallmark categories of liver function, such as drug metabolism, between \textit{in vitro} hepatocytes in an ECM sandwich culture model and the liver. Further, this model maintains cellular responses to PB, a unique indicator of a
well-differentiated hepatocyte. Conversely, the results obtained underscore caution when attempting to derive similar biological information from hepatoma-derived cell lines, as the expression levels and profiles of genes involved in liver-specific functions are poorly maintained in these systems.

2.6 References


Chapter 3

Expression profiling of interindividual variability following xenobiotic exposures in primary human hepatocyte cultures

3.1 Abstract

To examine the magnitude of human variability across the entire transcriptome after chemical challenge, we profiled 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.
3.2 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 [1,2]. Although advantages of the human primary hepatocyte model, such as elimination of metabolism extrapolation across 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 [3,4]. 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 [5-7].

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 [8,9], while others have only used hepatocytes from a single donor [10,11]. Variability across donors complicates the determination of which genes are regulated, in that some studies report findings in each individual donor [12], some follow a stringent criteria such that genes of interest must be changed in all donors [3,13,14], some apply a filtering system so that genes of interest are those changed in a particular number of donors [15], while others report average expression across donors [16,17]. Overall, a limited number of donors are generally included (n=2-6), and surprisingly few genes survive the filtering conditions: Richert et al. [15] parsed a 38 gene dataset based on the >12’000 genes on their array; Thum and Borlak [12], 44 of 302 possible genes; Liguori et al. [3], 142 of >22’000; Harris et al. [13], 867 of 31’000; and Radaeva et al. [14], 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 [18,19]. 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 PPARα 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.
3.3 Methods

3.3.1 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 [18]. 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).

3.3.2 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).
3.3.3 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 [20], 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 = $2^{-\Delta\Delta C_t}$.

3.3.4 Competitive PCR

Internal standards were created for CYP1A2 and CYP4A11 by a three step method [21]. Initially, an approximately 500 bp fragment was amplified from human hepatocyte cDNA using the external primers listed in Table 3 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>External FP</td>
<td>TGCGGCAGGGCGACGATTTTC</td>
</tr>
<tr>
<td></td>
<td>External RP</td>
<td>GCAGGGCAGGGTTAGGCAGGTA</td>
</tr>
<tr>
<td></td>
<td>Internal FP</td>
<td>TGATGGCCAGAGCTTGACGACATGAGCAAGGAG</td>
</tr>
<tr>
<td></td>
<td>Internal RP</td>
<td>CTCCCTTGCTCACATGCTCGTCAAGCTCTGGCCATCA</td>
</tr>
<tr>
<td>CYP4A11</td>
<td>External FP</td>
<td>AGGCAAAGTTCGTGTCCAGCTCTA</td>
</tr>
<tr>
<td></td>
<td>External RP</td>
<td>TGGATCACTTGGTCTGTGTCGTA</td>
</tr>
<tr>
<td></td>
<td>Internal FP</td>
<td>TCCTGGCTCCATGGATTGCTGGACACCATCATGAAG</td>
</tr>
<tr>
<td></td>
<td>Internal RP</td>
<td>CTTCATGATGGTGTCACAGCAATCCATGGAGCCAGGA</td>
</tr>
</tbody>
</table>

### 3.3.5 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 [18]. Resulting data was analyzed with GeneChip® Operating Software (GCOS; Affymetrix) to determine the signal intensity of each probe set on each array via the MAS5 algorithm, as well as detection (P, present; A, absent; or M, marginal) and change calls (I, increase; MI, marginal increase; NC, no change; MD, marginal decrease; or D, decrease). The signal log ratio of each probe set on the treated arrays was subsequently calculated 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.

### 3.3.6 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).

### 3.3.7 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 1.9.3 [22] using a Euclidean distance measurement and an average linkage.
3.3.8 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 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 [23]. 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). For the purposes of ontology-based hierarchical clustering, p-values were transformed first by the formula y=1/y in order to label the most significantly over-represented categories with the largest values. To reduce the range of the resulting values, a subsequent transformation was done following either the formula y=log(y) when a category was associated with increased genes or y=-1*log(y) when associated with decreased genes.

3.4 Results

3.4.1 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 11A). The remaining two donors, D and E, had significantly lower correlation with hepatocytes from the other donors, with mean correlation coefficients of $0.887 \pm 0.044$ and $0.881 \pm 0.043$, respectively ($p<0.0001$). The highest correlation between any two donors was $r^2=0.967$ between donors G and H (Figure 11B) and the lowest was $r^2=0.857$ between donors D and E (Figure 11C).

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 12A; $p=0.0793$). 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

Figure 11: 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.
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 12B and 12D; 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

**Figure 12:** 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. 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 \((p<0.05)\).
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 12C and 12E;
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 \( r^2 = 0.919 \)).

3.4.2 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 13A-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 13A), and 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 13B).
The induction of CYP2B6 in these hepatocyte donors is consistent with results generated
via a different algorithm, PLIER, that were reported in a previous study comparing gene
expression in human liver and in primary hepatocytes [18]. Additionally, these induction
responses are in agreement with previous results from a comprehensive study utilizing
hepatocytes from multiple donors (n=62) maintained under conditions comparable to
those described here [24]. 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 13C).

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 13D), and
CYP2B6 was increased in eight donors, ranging from 2.3-fold in donor E to 27-fold in
donor J (Figure 13E). 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 13F). 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.

3.4.3 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 4). 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 [25]. The final chemical in our study, DEHP, consistently regulated only four genes, all of which were also regulated by A1254.

3.4.4 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 14A). 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 PPARα. 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 14A). In our analyses, A1254 was found to inhibit proapoptotic
Table 4: Probe sets either increased or decreased in at least 9 human hepatocyte donors treated with A1254, DEHP, or PB.

<table>
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<th>Gene Title</th>
<th>Gene Symbol</th>
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<td>CYP2B6</td>
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<td>A, D, P</td>
<td>Tskushin</td>
<td>TSKU</td>
<td>Inc</td>
</tr>
<tr>
<td>A, D</td>
<td>Thyroid hormone responsive (SPOT14 homolog, rat)</td>
<td>THRSP</td>
<td>Dec</td>
</tr>
<tr>
<td>A, P</td>
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<td>Inc</td>
</tr>
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</tr>
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<tr>
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<td>Thioredoxin interactin protein</td>
<td>TXNIP</td>
<td>Dec</td>
</tr>
<tr>
<td>P</td>
<td>SEC14-like 4 (S. cerevisiae)</td>
<td>SEC14L4</td>
<td>Inc</td>
</tr>
</tbody>
</table>

1 A, A1254; D, DEHP; P, PB.
2 Inc, Increased; Dec, Decreased.
3 Probe set cannot distinguish between the following UGT1A family members: 1, 3, 4, 5, 6, 7, 8, 9, 10
genes (Figure 14B) and suggested tumor suppressors (Figure 14D) and to increase expression of chaperones (Figure 14C), antioxidants (Figure 14E), and damage response (Figure 14F) genes. Genes within the transport (Figure 14G) and lipid and fatty acid metabolism (Figure 14H) categories were also regulated consistently by A1254.

3.4.5 Hierarchical clustering of reproducible response 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 treatment groups

Figure 14: 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.
clustered according to treatment, despite the evident influence of interindividual variability (Figure 15). 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. Interestingly, genes within this reproducible gene set were often grouped nearest to genes that share similar functions. Specifically, genes were grouped together based on roles in biotransformation/xenobiotic metabolism (Figure 14A; Figure 15, purple line), stress response (Figure 14C, E, and F; Figure 15, blue line), lipid and fatty acid metabolism (Figure 14H; Figure 15, orange line) and tumor suppressor properties (Figure 14D; Figure 15, green line).

3.4.6 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 16), 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).
Figure 15: 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 and horizontal lines indicate chemical-specific clusters: 1, purple line, A1254; 2, blue line, DEHP; and 3, orange line, PB. Vertical lines represent groups of genes with similar function: purple line, biotransformation; blue line, stress response; orange line, lipid and fatty acid metabolism; and green line, tumor suppressor activity. Dataset consisted of 41 genes. Intensity scale represents fold change. * indicates that this probe set cannot distinguish between various UGT1A isoforms. Clustering was done using a Euclidean distance measure and average linkage.
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 over-represented gene ontology categories were compared rather than probe sets or genes (p<0.05; Figure 16B).

Figure 16: 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).

Providing further support for ontology-based analyses, chemical-specific clusters similar to those presented for the reproducible gene set were apparent when ontology categories consistently regulated across donors were used as the basis for clustering (Figure 17). Similar to the gene-level clustering results, A1254-treated samples from the six included donors grouped together, while there was less distinction between DEHP- and PB-treated samples. Not surprisingly, many of the ontology categories found to be
over-represented consistently among A1254-treated donors were closely related to the functional themes identified by the reproducible gene set, such as ‘lipid and fatty acid transport’, ‘fatty acid metabolism’, and ‘chaperone’.

3.5 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 affected 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 13B and 13E; Figure 14). On the other hand DEHP less consistently induced its anticipated target gene, CYP4A11 across individuals (Figure 13C and 13F), 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 [3], 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 [26], two proapoptotic genes were identified as decreased by A1254, TNFSF10 [27] and TXNIP [28]. Similarly, chaperones, particularly Hsp70 family members, also contribute to inhibition of apoptosis [29]. 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 [30], HPGD [31], and ANXA10 [32], and in increased expression of a potential oncogene, AKR1B10 [33].

Possibly related to tumor promotion, PCBs have been implicated in increasing reactive oxygen species (ROS) [34,35]. 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 [36] and UGT1A1 [37]. 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 [35]. Similarly, our study identified two upregulated 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 [38]. 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 [39]. Interestingly, our results identified AIFM2, a closely related homolog to AIF that triggers a functionally similar caspase-independent apoptotic pathway [40], 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 11A). 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 12C and 12E). Donor D was the only donor to not significantly increase the PB target gene, CYP2B6 (Figure 13B). 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 (Figure 15). 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 [4]. 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 [41-43]. 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 [44]. 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 [18,19], 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.

3.6 References


Chapter 4

Transcript profiling reveals conserved and potentially divergent functional processes in human and mouse models after xenobiotic treatment.

4.1 Abstract

Although rodent models are often used to measure potential xenobiotic toxicity, it is unclear how representative these models are for humans. To this end, transcript profiling in primary human hepatocytes from ten donors and in liver tissues from C57BL/6 mice was performed after a 24 hour treatment with two doses of three xenobiotics: Aroclor 1254, an environmental contaminant; phenobarbital, an enzyme-inducing barbiturate drug; and di(2-ethylhexyl) phthalate, a plasticizer. ~12000 orthologous genes on human and mouse genome arrays were identified using the NetAffx Analysis Center (Affymetrix). Using only these genes, <8% of the genes changed in any one human hepatocyte donor were similarly regulated in the comparable mouse model. Of the three chemicals, Aroclor 1254 elicited the greatest number of changes conserved across species. Genes related to oxidative stress response, chaperone activity, and inhibition of apoptosis were increased and genes related to cell cycle control and induction of apoptosis were decreased, consistent with reported generation of reactive oxygen species and promotion of cell growth by Aroclor 1254. In mice, Aroclor 1254 overwhelmingly lowers expression of genes involved both in the TCA cycle, such as citrate synthase and isocitrate dehydrogenase, and in all complexes of the mitochondrial respiratory chain. This reduction in mitochondrial metabolism could ultimately lead to an inhibition of ATP synthesis, thus contributing to the toxic effects of Aroclor 1254. Interestingly, in human hepatocytes from some donors, these genes are predominantly increased. Species-specific regulation of mitochondrial metabolism may mechanistically underlie differential susceptibility to toxic effects of Aroclor 1254 in mice and humans.
4.2 Introduction

It is widely accepted that human and mouse models differ with respect to particular mechanistic responses to xenobiotics. Variation in expression or activity of orthologous enzymes could lead to the generation of species-specific metabolites with varying degrees of toxicity [1,2]. Thus, mechanisms identified in any one species must be critically evaluated for their relevance to man [3]. As this suggests, animal models are not extraordinarily predictive of actual toxicities observed in humans in a clinical setting. In one study, compiled from 12 pharmaceutical companies including 150 compounds, rodent models were predictive of only 43% of actual human toxicities [4]. Events in the liver, an organ central to biotransformation, exhibited one of the poorest correlations to animal studies and one of the highest rates of termination in clinical development. Despite this low concordance, animal models are still integral to the process of safety evaluation, as intact animals provide information that in vitro model systems are ill-suited to provide, such as identification of target organ toxicities and establishment of safety margins [5,6].

Primary human hepatocytes, while subject to the limitations of an in vitro model system, provide a unique view into responses that occur in humans and how those responses may differ from those in animal models. As an in vitro system, hepatocytes have undergone extensive validation in order to establish confidence that drug metabolism studies in this system are relevant to the in vivo condition. Recently, culturing methodologies defined by physiological levels of dexamethasone and a sandwich configuration that embeds hepatocytes between a basal layer of collagen and a commercially available extracellular matrix, Matrigel, have been reported to maintain hepatocytes with improved morphology and increased expression of gap junctional proteins and, thus, cell-cell contacts, in comparison to monolayer cultures [7,8]. Additionally, hepatocytes cultured following this methodology express hepatic marker and biotransformation genes at levels similar to those observed in intact human liver [9]. Although isolated cells may not reflect functional responses identical to those in intact organs, primary hepatocytes have proven useful in measuring certain aspects of in vivo
liver function, such as induction potential of various cytochrome P450s. Actual induction measured \textit{in vivo} has been shown to be reasonably estimated in hepatocytes by parameters such as in vitro clearance and EC50 values [10,11]. Further, \textit{in vitro} primary hepatocytes from rodent species produce metabolites similar to those produced \textit{in vivo} [12], as well as exhibit similar mechanisms leading to hepatotoxicity across a broad range of chemical classes [6]. Additionally, primary hepatocytes improved correlation between \textit{in vitro} cytotoxicity and \textit{in vivo} acute toxicity in comparison to that observed with cell lines derived from hepatomas, since hepatocytes are the only model able to metabolize compounds \textit{in vitro} similarly to that which occurs \textit{in vivo} [13]. Together, these observations support primary hepatocytes as an appropriate model for studying drug metabolism.

As a demonstrated robust model system, primary hepatocytes provide a unique opportunity to compare transcriptional responses in humans and animal models. Through a comparison of orthologous genes in these species, we show that there is a low cross-species concordance in transcriptional responses to xenobiotics, similar to that observed at a clinical level for actual toxicities. Further, a functional analysis approach is used to identify a potential difference in mechanistic response to xenobiotic challenge that could result in differential toxicity in mice and humans.

4.3 Materials and Methods

4.3.1 Cell culture conditions

Primary human hepatocytes from ten human donors were obtained from the Liver Tissue Procurement and Distribution System (LTDPS) at the University of Pittsburgh, through NIH Contract #NO1-DK-9-2310. Hepatocytes were received in collagen-coated T25 flasks, and, upon arrival, the culture media was changed and a matrigel overlay was added as described previously [14]. Selected cultures were treated with either DMSO, 10 \( \mu \)g/ml A1254, 100 \( \mu \)M DEHP, or 500 \( \mu \)M 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. Culturing materials were purchased from Invitrogen (Carlsbad, CA).

4.3.2 Animal experiments

C57BL/6 mice were maintained on a twelve hour light/dark cycle and were given ad libitum access to food and water. Three mice were treated via i.p. injection with each of the following doses: 25 mg/kg A1254, 50 mg/kg A1254, 19.5 mg/kg DEHP, 39 mg/kg DEHP, 37.5 mg/kg PB, and 75 mg/kg PB. After 24 hours, mice were euthanized by CO₂ asphyxiation, and liver tissues were immediately removed and snap-frozen in liquid nitrogen.

4.3.3 Microarray protocol

RNA was isolated from human hepatocytes and frozen liver tissues using 1 ml and 2 ml TRIzol® Reagent (Invitrogen), respectively, according to the manufacturer’s instructions. Contaminating DNA was removed using DNA-free™ DNase Treatment and Removal Reagents (Ambion, Inc., Austin, TX). Final RNA concentrations were determined by UV absorbance at 260 nm using a SmartSpec 3000 Spectrophotometer (BioRad, Hercules, CA). For each mouse treatment, RNA samples were pooled from three individually-treated mice. Paradigm Array Labs, Inc. (Icoria, Research Triangle Park, NC) hybridized the samples either to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) or to Mouse Genome 430A 2.0 Arrays (Affymetrix). Resulting data were analyzed with GeneChip® Operating Software (GCOS; Affymetrix) to determine detection and change calls as described in Goyak et al [14].
4.3.4 Over-representation analysis

The PANTHER classification system version 6.0 [15] was used to determine over-represented gene ontology categories in each human and mouse sample in each treatment group. For each of 241 biological process and 254 molecular function ontology categories, 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 is compared using the binomial test to determine if there are more genes than expected in the differentially regulated list [16].

4.3.5 Functional analysis of cross-species similarities

Databases available through the PANTHER classification system and the Gene Ontology Consortium [15,17], as well as manual literature searches, were used to functionally categorize the genes. Genes were included based on the following definitions from the PANTHER classification system: ‘chaperone’, “a cytoplasmic protein that binds to nascent or unfolded polypeptides and ensures correct folding or transport”; ‘inhibition of apoptosis’, “a regulatory control that inhibits apoptosis”; ‘induction of apoptosis’, “the proteins that are positive regulators in of the process of apoptosis”; ‘cell cycle control’, “a process controlling the initiation and progression of cell cycle events”; and ‘lipid, fatty acid, and steroid metabolism’, “metabolic processes of lipids, including breakdown and biosynthesis of lipids, fatty acids or steroid molecules”. Additionally, functional categories were based on the following definitions from the Gene Ontology Consortium: ‘response to oxidative stress’, “a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of oxidative stress, a state often resulting from exposure to high levels of reactive oxygen species”; and ‘xenobiotic metabolism’, “the chemical reactions and pathways involving a xenobiotic compound, a compound foreign to living organisms”. The remaining functional category, ‘mitochondrial oxidative metabolism’, is based on numerous, overlapping subcategories within ‘generation of precursor metabolites and energy’,
mainly ‘electron transport chain’, ‘oxidative phosphorylation’, and ‘energy derived by oxidation of organic compounds’.

### 4.3.6 Competitive PCR

Internal standards specific for CS, IDH1, and ME1 were used to verify induction of these genes in primary human hepatocytes from select donors. Expression of each gene in the hepatocyte donors was determined by constructing standard curves consisting of PCR reactions containing dilutions of internal standard and constant volumes of hepatocyte cDNA, and the target gene concentration was considered the concentration at which the internal standard and endogenous gene were present in a 1:1 ratio (Table 5).

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4.4 Results

4.4.1 Identification of orthologous genes

In order to compare genes represented on both the human- and mouse-specific arrays, NetAffx Analysis Center (Affymetrix) [18] was used to identify approximately 12,000 orthologous genes, measured by 29,338 probe sets on the human array and 19,277 probe sets on the mouse array. This disparity in the number of probe sets measuring expression of orthologous genes suggests that there may be unequal coverage of the genes on the arrays from each species. Indeed, when a comparison was made of all probe sets or all genes changed on each chemically-treated array, there were significantly fewer transcriptional changes measured in the mouse samples (Figure 18A-B). This trend held true even when the number of over-represented GO categories in samples from each species was compared (Figure 18C), suggesting that unequal gene coverage may result in an underestimation of comparable response across species.

Figure 18: Cross-species comparisons revealed fewer transcriptional changes after chemical challenge on the mouse arrays vs. the human arrays. Each data point indicates either the percentage of probe sets (A) or genes (B) changed or the number of over-represented gene ontology (GO) categories (C) on a single array, in total representing 39 human genome and 6 mouse genome arrays. Only orthologous genes were considered. Significance is defined by $p<0.05$; t-test.
4.4.2 Comparison of transcriptional changes across species

Because interindividual variability in primary hepatocytes from different donors has a large impact on the genes that respond to xenobiotic challenge [14], the xenobiotic-induced changes in each individual hepatocyte donor were compared to changes induced in the mouse model at each of two doses. Among all three chemical treatments, fewer than 8% of the orthologous genes changed in any one human hepatocyte donor were similarly changed in mouse models at either dose (Figure 19). To verify that this low conservation is not an artifact reflecting the choice of orthologous genes, this comparison was repeated using orthologous genes identified by a different source, Keck ARray Manager and Annotator (KARMA) [19]. KARMA listed fewer orthologous genes, only approximately 8400; yet the unequal probe set coverage remained the same (21,167 human probe sets and 14,139 mouse probe sets). Further, although the individual genes identified were different between KARMA and NetAffx, the species comparisons done using KARMA resulted in nearly identical overlap between the genes changed by each

Figure 19: Few of the transcriptional changes induced by Aroclor 1254 (A), DEHP (B), or PB (C) in human hepatocytes were reflected in mice treated with either dose. Each panel shows the range among the ten human hepatocyte donors in percentage of NetAffx orthologous genes changed in each donor that were similarly changed in mice receiving a high or low dose of the same chemical. Significance is defined by p<0.05; paired t-test.
chemical on the human and mouse arrays as that identified using NetAffx (A1254, 4.4% to 8.3% at either dose; DEHP, 1.5% to 5.6%; and PB, 1.1% to 4.9%).

The use of two *in vivo* doses of each chemical did reveal a dose at which comparisons to the *in vitro* model showed a higher degree of similarity. For instance, the lower *in vivo* dose of A1254 had higher similarity to the *in vitro* dose used, and the opposite was true for PB treatment. The sheer number of genes changed commonly between the human hepatocyte donors and mouse models are low; in the highest-similarity comparison, *in vitro* hepatocytes and the low A1254 dose in mouse, the number of genes commonly changed in both models ranged from 71 to 264 genes across donors. In the lowest-similarity comparison, *in vitro*

![Figure 20](image)

**Figure 20:** Comparison of over-represented gene ontology categories and individual response genes increases concordance across species in some but not all cases. Shown are the percentage of changed genes and over-represented ontology categories found in common between each donor and the mouse model treated with a low dose of A1254 (A), PB (B), and DEHP (D).
hepatocytes and the low DEHP dose in mouse, the number of genes ranged from 12 to 54 genes.

As anticipated from previous results, a comparison of over-represented gene ontology categories rather than individual genes increased the concordance in transcriptional response between the mouse and human models when the mechanism of action was similar between species. For example, 6.9-27.1% of ontology categories over-represented in any one donor were also over-represented in the comparable mouse model after A1254 treatment, while 5.1-7.9% of genes were conserved (Figure 20). This trend also held true after PB treatment, which increased concordance in cross-species response in all but a single donor (donor A). Comparison of ontology categories after DEHP treatment, however, eliminated any overlap between mouse and six of the donors, likely due to the highly species specific mechanisms of peroxisome proliferators such as DEHP [20,21].

4.4.3 Functional analysis of cross-species similarities

In order to provide a more comprehensive view of the transcriptional changes occurring in mice and humans, the remainder of our analysis is focused on the highest-similarity comparison, in vitro hepatocytes and the low A1254 dose in mouse. Among the transcriptional changes induced by A1254 in both mouse and humans, four functional categories were consistently identified among genes increased in all ten hepatocyte donors (chaperone, see Appendix 1, Table 7; inhibition of apoptosis, Table 8; oxidative stress response, Table 9; and xenobiotic metabolism, Table 10). Additionally, four functional categories were identified among genes decreased in all ten donors (cell cycle control, see Appendix 1, Table 11; induction of apoptosis, Table 12; lipid, fatty acid, and steroid metabolism, Table 13; and mitochondrial oxidative metabolism, Table 14). These categories are consistent with known effects of A1254, such as production of oxidative stress and tumor promotion.
Within each category, the number of genes changed commonly between the mouse model and the human hepatocytes differed by donor (Figure 21A and 21B), and the individual genes changed were often donor-dependent (Figure 21C and 21D). For instance, within the chaperone category, 13 distinct genes with chaperone activity were increased in both mouse and human hepatocytes. Of these 13 genes, six were increased in at least seven donors (DNAJB1, HSP90AA1, HSPA1A, HSPA1B, HSPA8, HSPH1), one was increased in three to six donors (BAG3), and six were increased in two or fewer donors (DNAJC12, FKBP5, HSP90B1, HSPA5, HSPB1, and HYOU1). The genes responding to A1254 challenge within the oxidative stress response and xenobiotic

**Figure 21**: Genes changed in mice and in all ten human hepatocyte donors revealed eight conserved functional categories. Within each category, the number of genes increased (A) and decreased (B) in each category varies slightly among the ten donors. Panels C and D depict the percentage of genes in each category that are similarly changed in $n$ donors. Abbreviations are as follows: metab, metabolism; Mito, mitochondrial; LFAS, Lipid, fatty acid, and steroid.
metabolism categories exhibited high conservation across human hepatocyte donors, in that a majority of the genes (>65%) were changed in at least 7 donors. On the other hand, mitochondrial oxidative metabolism genes responding to A1254 are extremely donor-dependent, as a majority of these genes (>58%) are changed in only one or two donors.

4.4.4 Regulation of mitochondrial oxidative metabolism

Of the functional categories identified, only mitochondrial oxidative metabolism provides a novel insight into the potential mechanism of A1254. This category was additionally targeted for closer inspection due to the high percentage of genes found to be regulated in only one or two donors; of the 15 distinct genes decreased, none were increased in more than six donors, four were increased in six to three donors (DLAT, PDK1, PDK2, NDUFA5), and 11 were increased in two or fewer donors (MDH1, SDHD, SUCLA2, NDUFA6, NDUFB10, NDUFB3, NDUFB4, NDUFB8, NDUFS1, ATP5C1, ATP5J). After a more thorough examination of all oxidative metabolism genes regulated by A1254, it was determined the initial analysis excluded many oxidative metabolism genes that were indeed changed, albeit divergently, after xenobiotic challenge (see Appendix 1, Table 15, Table 16, Table 17, and Table 18).

Within the tricarboxylic (TCA) acid cycle, genes encoding seven of eight key enzymes were down-regulated by A1254 in the mouse, as well as one enzyme within the pyruvate dehydrogenase (PDH) complex, which supplies the acetyl-CoA substrate to the cycle (Figure 22A). In primary human hepatocytes, however, these same genes were often upregulated, although considerable interindividual variability is exhibited (see Appendix 1; Table 16). Donor A, for instance, exhibited a response similar to that in mice; four of the eight genes were decreased (malate dehydrogenase (MDH), succinyl-CoA ligase (SUCL), succinate dehydrogenase (SDH), and isocitrate dehydrogenase (IDH)) while only citrate synthase (CS) was increased. On the opposite extreme, Donor J had increased expression of MDH, SDH, subunits 1 and 3 of IDH, and oxoglutarate
dehydrogenase (OGDH), while only subunit 2 of IDH was decreased after A1254 challenge.

The remaining donors fell between these two extremes, with a few TCA cycle genes increased and a few decreased by A1254. Particularly divergent between species were CS (decreased in mouse but increased in four donors (A, C, G, I)), IDH subunit 1 (unchanged in mouse but increased in four donors (F, G, H, J)), IDH subunit 3 (decreased in mouse but increased in four donors (B, G, H, J)), and ME1, (decreased in mouse but increased in donors D, E, I and J) (Figure 22B). The A1254-induced expression of these genes in human hepatocytes from select donors was verified by a second method, competitive PCR (Figure 23).

A similar trend was established for genes encoding all complexes of the mitochondrial respiratory chain, such that expression was decreased in the mouse but often increased in human hepatocytes (Figure 24; see Appendix 1, Table 17 and

Figure 22: A1254 primarily decreased genes encoding TCA cycle enzymes in mouse, whereas the overall effect in human hepatocytes was often an increased in expression of these genes. Genes numbered 1 through 8 encode enzymes catalyzing metabolic progression through the TCA cycle, while gene 9 regulates substrate availability. Downward arrows indicate expression was decreased by A1254 while upward arrows indicate increased expression. M, mouse; H, human.
Table 18). Again, donor-specific regulation trends emerged. Some donors, such as Donor B, exhibited regulation similar to that in the mouse: of 57 genes regulated in at least one sample, 19 were decreased by A1254 treatment while none were increased. Hepatocytes from other donors, however, exhibited a very different trend, such as Donor J, in which A1254 treatment up-regulated 22 respiratory chain genes but did not down-regulate a single gene. Among the 57 respiratory chain genes changed in at least one donor, quite a few appeared to be divergently regulated in mice and humans, most notably, genes from complexes I (NDUFA7, NDUFA8, NDUFA9, NDUFB9, NDUFB10, NDUFC1, NDUFS2, NDUFS3, NDUFS6), IV (COX5A, COX6B1, COX7A2, COX8A), and V (ATP5B, ATP5D, ATP5G1, ATP5G2, ATP5G3) (Figure 25).

4.5 Discussion

Among the magnitude of genomics studies that have been published to date, few studies have described cross-species comparisons of xenobiotic response genes, and even fewer are able to compare those responses to a validated human model system. To this end, we compared human and mouse transcriptional responses initiated after challenge with varying doses of three chemical agents, each activating different nuclear receptors associated with different gene batteries. Among any pair of dose comparisons, fewer than
8% of the orthologous genes changed in hepatocytes from any one human donor were similarly altered in the mouse model. As anticipated, comparison of ontology categories overrepresented among transcriptional responses resulted in higher similarity across species when the mechanism of action in both species was similar.

This overall low concordance could be explained by factors that continue to complicate the field of comparative genomics, for one, the identification of orthologous genes, which are those that diverged due to speciation events [22]. For questions of conserved function across species, orthologs are the desired comparator, as gene duplications, which permitted the divergence of paralogs, are also thought to have permitted the divergence of unique functions [23]. However, the ortholog identification process does not lend itself well to high-throughput methods and can be negatively impacted by incomplete sequencing, gene loss, or a myriad of other genome alterations. 

Figure 24: A1254 decreased genes encoding mitochondrial respiratory genes in mouse, whereas these genes were often increased in human hepatocytes. Roman numerals indicate the complex of the respiratory chain, and Arabic numbers indicate the number of genes within each complex that were regulated. Downward arrows indicate expression was decreased by A1254 while upward arrows indicate increased expression. M, mouse; H, human.
Generally, there is a trade-off between sensitivity and specificity, such that sequence-based identification methods often find many orthologous candidates albeit with a high false positive rate while species phylogenetic tree-based methods are quite accurate but may miss many true positives [25,26]. In our study, we employed two different ortholog detection programs, the NetAffx Analysis Center and KARMA, to ensure that results were not biased due to ortholog identification.

Nonetheless, the low concordance across species remained, possibly due to another technical complication: unequal gene coverage across species [27,28]. As observed in Figure 18, significantly fewer probe sets or genes were altered after treatment in the mice than in the humans, which could be a reflection of limited representation of response genes on the mouse array. Another complicating factor that can impact comparative genomics is the constantly evolving state of gene annotation. Although microarray chips were designed using the current version of each genome, biological knowledge increases so quickly that even the most recent versions of microarray chips can contain probes that do not match any transcripts found in the RefSeq database or that match transcripts other than those annotated by the array manufacturer [29,30]. Similarly confounding is that the probe sets are annotated by gene rather than by transcript, which can lead to disagreement in signal among alternatively spliced transcripts [31,32].
Despite these complications, the response genes and ontology categories that were identified agree well with the established mechanistic effects of A1254, suggesting that induction of responses such as xenobiotic metabolism, chaperone activity, and oxidative stress response are conserved in humans and mice. Indeed, PCBs, such as A1254, have an established role in xenobiotic metabolism through activation of both AhR- and CAR-dependent signaling pathways [33-35] and in the production of oxidative stress [36], signaling both the induction of antioxidants and chaperones. Similarly, these results also suggest that processes such as cell cycle control, induction of apoptosis, and lipid, fatty acid, and steroid metabolism are down-regulated by A1254 in both species. Again, these functional categories are in agreement with known mechanisms, as PCBs have been shown to affect cholesterol and lipid content [37], as well as promote tumorigenesis, possibly through inhibition of apoptosis [38] or a decrease in cell cycle control [39,40].

Further, one functional category, mitochondrial oxidative metabolism, was identified as a potential area of divergence in A1254-induced transcriptional response across species. In particular, genes that encode enzymes or subunits with roles in the TCA cycle (Figure 22) or oxidative phosphorylation (Figure 24) were overwhelmingly decreased by A1254 in the mouse model but often increased in certain human hepatocyte donors. Mitochondria, perhaps due to their critical role in supplying cellular energy in the form of ATP, are often targeted by xenobiotics, such as 1,1-dichloroethylene (DCE) [41] and cocaine [42]. Compounds related to A1254, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo-a-pyrene (BaP), that share mechanisms acting, at least in part, through activation of the AhR, have been reported to disrupt rodent intermediary metabolism [27], decrease hepatic ATP levels [43], and decrease activity of mitochondrial enzymes in lung cancer-bearing mice [44]. Our data, together with these reports, could indicate an A1254-induced downregulation of TCA cycle and oxidative phosphorylation genes, ultimately impacting energy production and perhaps leading to cell death. The above studies have all been carried out in rodent species. To the best of our knowledge, in vitro studies in human hepatocytes have not implicated mitochondrial energy loss as a mechanism for A1254-induced toxicity. Additionally supporting a
species-specific regulation is the *in vivo* observation that humans are generally less susceptible to the carcinogenic effects of TCDD than most laboratory animals [45,46].

Although a true species difference in regulation of mitochondrial oxidative metabolism may explain these biological observations, there are additional considerations that may play a role in the observed species differences. Although we are most interested in the *in vivo* human system, two other models (*in vivo* mouse and *in vitro* human hepatocytes) have here been employed to obtain information indirectly about this endpoint (Figure 26) [47]. However, this approach introduces two factors that may influence the comparison between our two measured endpoints: species and *in vivo* vs. *in vitro* differences. Although the described results in primary human hepatocytes showed an interesting parallel to an *in vivo* mouse model and to previously published results, an additional comparison to *in vitro* primary mouse hepatocytes would have provided an ideal control. The results obtained *in vitro* could be different from the *in vivo* situation due to the absence of other cells types *in vitro* that may facilitate *in vivo* signalling. For instance, in rodents, peroxisome proliferators stimulate Kupffer cells to produce cytokines that induce DNA synthesis in hepatocytes, a response that doesn’t occur in primary cultured mouse hepatocytes. Further, finding comparable doses *in vivo* and *in vitro* is notoriously difficult, due to such considerations as the length of time needed for an *in vivo* dose to travel to the target organ or biotransformation that may occur. To overcome these unknowns, our study incorporated two different *in vivo* doses. However, technical limitations in the perfusion procedure in mice restricted our analysis to a comparison of *in vitro* human hepatocytes and *in vivo* mouse liver tissues.

Figure 26: A toxicology parallelogram provides a framework for finding information about endpoints that cannot be measured directly. Circles in bold typeface are endpoints measured in this study, while the dotted circle indicates the indirectly described endpoint.
Despite the unknowns associated with in vitro experiments, our model system has been characterized previously as comparable to the in vivo human liver with respect to characteristic hepatic gene expression and induction responses. Thus, we propose that the observed differences between the two model systems are most likely explained by species differences. However, other studies have concluded that, despite approximately 70-80% correlation in basal gene expression between in vitro and in vivo hepatic models [9,48,49], transcriptional responses to chemical treatment may be diminished or even lost in comparison to in vivo responses [50,51]. Nonetheless, functional categories directly impacted by chemical treatment appear better maintained than those categories related to secondary or tertiary chemical effects [49,51].

In conclusion, this study reports that transcriptional response to chemical treatment appears to be poorly conserved in mouse models in comparison to in vitro primary human hepatocytes when described at a gene-level. As anticipated, the use of ontology categories, designed to be species-independent, increases the comparison across species after A1254 and PB treatment, and also provides a description of which functional categories are conserved across species after xenobiotic challenge. Further, the identification of functional categories that are divergently regulated across species is another useful application of a toxicogenomics approach.

4.6 References


5.1 Abstract

Tsukushin (TSKU), a protein belonging to the small leucine rich proteoglycan family, has been previously described as inducible in response to estrogen and vitamin K, presumably due to activation of ER and SXR, respectively. In this study, we demonstrate that TSKU is a novel inducible target gene in response to phenobarbital (PB) challenge in primary human hepatocytes. Knockdown of TSKU using lentiviral introduction of siRNA in primary human hepatocytes reduced induction of CYP2B6, CYP3A4, and CYP2C9 to 43.5\%+3.1\%, 48.1\%+13.0\%, and 55.2\%+12.4\%, respectively, of induction exhibited in control hepatocytes, as well as detectable protein expression. Reporter assays in the presence and absence of TSKU in COS-1 cells demonstrated that TSKU does not alter activation of the PB-response element in the CYP2B6 promoter. These results suggest that TSKU likely exerts its effects in the extracellular space, as has been demonstrated for this protein in the BMP signaling pathway.

5.2 Introduction

In an expression profiling analysis of primary human hepatocytes, a gene battery was identified as PB-responsive in primary human hepatocytes from ten independent donors. Only two genes in this battery were not genes encoding cytochrome P450 enzymes: delta-aminolevulinate synthase (ALAS1), the rate limiting enzyme in heme biosynthesis [1], and tsukushin (TSKU), a novel PB-responsive gene belonging to the small leucine rich proteoglycan (SLRP) family [2]. Named for the similarity between its expression pattern in chick embryos and the Japanese horsetail plant, tsukushi, TSKU
was originally identified in xenopus and chick as a regulator of germ layer formation and patterning through the coordination of multiple signaling pathways, such as bone morphogenic protein (BMP), Notch, activin, and FGF/MAPK [3-5]. The human ortholog was identified by Charpentier et al [6] as an estrogen responsive gene containing nine leucine-rich repeat regions and a cleavable signaling peptide, making this protein a likely candidate for extracellular secretion. In addition to estrogen responsiveness, TSKU has also been identified as responsive to insulin [7], benzo[a]pyrene [8], and vitamin K2 [9] in whole genome profiling studies. Although identified as inducible by multiple xenobiotics, the only functional role outside of development has been explored by Ichikawa et al [9], who found TSKU to be a primary target gene of the human steroid and xenobiotic receptor (SXR) that enhances collagen accumulation as a part of vitamin K2-regulated bone homeostasis.

A functional role in collagen accumulation is consistent with other SLRP family members that have been shown to interact with collagen to regulate extracellular matrix assembly in tissues as various as bone, skin, and cornea [10,11]. Nonetheless, this is only one of the diverse functions attributed to SLRP family members. Other family members have been shown to modulate activity of growth factors, namely TFGβ family members, and rate of cell proliferation through interaction with tyrosine kinase receptors, specifically with the EGF receptor [12]. Similarly, diverse functional roles have been associated with the defining features of SLRP family members: the leucine rich repeat (LRR). Although involved in processes such as hormone-receptor interactions, cell adhesion, cell trafficking, mammalian development, enzyme inhibition, and apoptosis signaling, the common role that the LRR motif plays is to facilitate protein-protein interactions [11-13].

Combining the information that TSKU plays a role in receptor-mediated signaling pathways through the stabilization of protein-protein interactions and is a target gene of SXR, we hypothesize that TSKU is a target gene of the constitutive androstane receptor (CAR), a closely related member of the nuclear hormone superfamily, which enhances CAR-mediated transcriptional induction through facilitation of protein-protein
interactions. This hypothesis was addressed through knockdown of TSKU in primary human hepatocytes to determine the effect on CYP2B6 induction.

5.3 Materials and Methods

5.3.1 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. 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 hepatocyte maintainence media (HMM), consisting of William’s Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 µM glutamine, 25 mM dexamethasone, 10 mM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, and 5 µg/ml transferrin. Human hepatoma-derived HepG2, Huh7 and COS-1 cells (simian virus-40-transformed green monkey kidney cells) were maintained in Minimum Essential Media (MEM) + Earle’s Salts + L-Glutamax, and 293T/17 transformed human embryonic kidney cells were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) + Glutamax. All cell lines were supplemented with 0.1 mM non-essential amino acids, 0.75 mg/ml sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 1% pencillin/streptomycin, and 10% FBS. Selected cultures were treated with 500 µM PB for 24 h. If not specified otherwise, all culturing materials were purchased from Invitrogen (Carlsbad, CA).

5.3.2 Quantitative mRNA analysis

RNA was isolated and quantified as described in Olsavsky et al. [2]. 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.
Microarray analyses were done by Paradigm Array Labs, Inc. (Icoria, Research Triangle Park, NC) using Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA). 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, CYP3A4, CYP2C9, and CYP1A2 was calculated using the ∆∆Ct method using 18S, expression in the vehicle-treated sample from the appropriate donor as the reference standard. ∆∆Ct was transformed into fold-change using the formula, fold change = 2^{−∆∆Ct}. TSKU expression was quantified using a competitive PCR method, via an internal standard created according to the method described in Anderson et al. [14] with the primers listed in Table 6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>TSKU</td>
<td>External FP</td>
<td>GCGGGGGCGGGCTACACGAC</td>
</tr>
<tr>
<td></td>
<td>External RP</td>
<td>CGAAGGCACCGGACCAATGACAG</td>
</tr>
<tr>
<td></td>
<td>Internal FP</td>
<td>GAGCGACGTGACCCGGACCAACCTCATTACCAGCG</td>
</tr>
<tr>
<td></td>
<td>Internal RP</td>
<td>GCGGTGAAATGAGTTGTCAGACGCAGTCGCTC</td>
</tr>
</tbody>
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### 5.3.3 Plasmids

For knockdown experiments, small interfering RNA (siRNA) target sequences within the TSKU transcript were identified using the web-based service siRNA Wizard (InvivoGen, San Diego, CA; [http://www.sirnawizard.com](http://www.sirnawizard.com)). For both a TSKU target (nucleotides 48-66) and a scrambled sequence, complementary oligonucleotides encoding a short hairpin RNA (sense target sequence, loop sequence, antisense target sequence, terminator sequence) were obtained from Integrated DNA Technologies (Carlsbad, CA). The TSKU-specific oligonucleotide sequences were as follows: oligonucleotide 1, 5’-ACCGGATCCACCGGACCATGCTTCCTCCGGGTCAGACCCGGGAAGCA
TGGCCGGGTTTTTGTGAATTC
GGT-3'; oligonucleotide 2, 5'-ACCGAATTC
AAAAA
AACCCGGCCCATGCTTCCCCCGGTCTGACAGGAAGCCGGGAAGCATGGCCGGG
TTGGATCCGGT-3' (restriction sites underlined; loop sequence italicized).
Complementary oligonucleotides were annealed, digested with BamHI and EcoRI,
electrophoresed through a 2% agarose gel, purified using QIAquick Gel Extraction Kit
(Qiagen Inc., Valencia, CA) and ligated into the pSIH1-H1-copGFP shRNA lentivector
(Systems Biosciences, Mountain View, CA). For reporter assays, TSKU was amplified
from primary human hepatocyte cDNA using the following primer sequences: forward,
5'-GCCTACATGCCCGTGCCGCTGTCCTGCTGCTG-3'; reverse, 5'-GATCTAGATCACAGATGTCGTCCTGCTGCTG-3'
(restriction sites underlined). The resulting amplicon was
digested with KpnI and XbaI and cloned by ligation into p3XFLAG-CMV-10 (Sigma-
Aldrich, St. Louis, MO). Other plasmids used in this study include pTracer-CMV2,
pTracer-CMV2-CAR, pcDNA3.1+, pcDNA3.1+-RXRα, and pGL3-2B6-XREM-PBREM
(described in [15]). Plasmids were purified using the Qiagen Plasmid Maxi Kit (Qiagen
Inc.).

5.3.4 TSKU knockdown in primary human hepatocytes

Lentivirus production and subsequent target cell infection was performed
according to the manufacturer’s recommendations, with the exception that Lipofectamine
2000 (Invitrogen) was used for transfection of 293T/17 packaging cells with pPACKH1
packaging plasmids (Systems Biosciences) and pSIH1-H1-copGFP lentiviral vectors. Six
hours after transfection, the medium was changed to HMM for collection of pseudoviral
particles. After 48 hrs, the pseudoviral supernatant was harvested from the packaging
cells, filtered and added directly to primary human hepatocytes. At either 24 hr- (donor
L) or 48 hr-post infection (donors K and M), the pseudoviral supernatant was replaced
with fresh HMM. It should be noted that the 48 hour incubation period resulted in a
higher infection rate than the 24 hour period (~40% infection vs. ~10% infection,
respectively), as measured by GFP expression. Selected cultures were treated with 500
µM PB six days post-infection, and cells were harvested after a 24-hour treatment period.
5.3.5 Western blotting

Protein was isolated from the organic phase of the TRIzol® Reagent remaining after RNA isolation according to the protocol provided by the manufacturer. Protein concentrations were determined according to the Bradford method, using a commercially available kit (BioRad Protein Assay) and bovine serum albumin as the standard. A total of 40 µg of protein per sample was separated by electrophoresis on a precast Ready Gel (10% Tris-HCl, BioRad) and transferred to Immun-Blot PVDF membrane (BioRad). The PVDF membrane was subjected to a blocking procedure by incubation in 5% non-fat dry milk in TBS-Tween 20 (0.1%) for 1 h. Primary antibodies were diluted in blocking buffer as follows: CYP2B6, 1:700 (monoclonal antibody clone 49-10-20); CYP3A4, 1:700 (monoclonal antibody 275-1-2); and, RXRα, 1:1000 (polyclonal antibody D-20 Santa Cruz Biotechnology, Santa Cruz, CA). Incubation in primary antibody was carried out overnight at 4°C. Antibodies for CYP2B6 and CYP3A4 were obtained through Dr. Harry C. Gelboin at the NCI/NIH. The PVDF membranes were then washed 4 times for 5 min each, followed by incubation in the appropriate secondary antibody, either goat anti-rabbit IgG-HRP (Santa Cruz, #sc-2004; 1:3000 for detection of RXRα,) or goat anti-mouse IgG-HRP (Santa Cruz, #sc-2005; 1:3000 for detection CYP2B6 and CYP3A4). Subsequently, the membranes were washed 4 times for 5 min prior to autoradiographic detection of signals by chemiluminescence using a commercially available kit (Lumi-light, Roche Diagnostic, Indianapolis, IN).

5.3.6 CYP2B6 reporter assays

Transfection of COS-1 cells was performed in a 48-well format, at a cell density of approximately 50,000 cells per well. DNA transfection mixtures were prepared using Fugene6 transfection reagent (Roche, Indianapolis, IN) in a 3:1 (reagent:DNA) ratio. Assays consisted of 25 ng of CMV2 or CMV2-CAR expression plasmids, 25 ng of pcDNA3.1+ or pcDNA3.1+-RXRα expression plasmids, 100 ng 2B6-XREM luciferase reporter, and 10 ng pRL-CMV (Promega, Madison, WI). Transfections were done
approximately 2 hours after plating. After 24 hrs, cells were washed with PBS and luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega) and quantified with a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase assay and stop and glow reagents were diluted with 1x Tris-buffered saline, pH 8.0, to a 0.5x concentration. Other than these exceptions, the assays were done according to the instructions provided by the manufacturer. Dilution of luciferase reagent had no effect on normalized luciferase values.

5.3.7 Statistics

All statistical analyses were performed using GraphPad Prism v4.00 for Windows (GraphPad Software, San Diego, CA).

5.4 Results

5.4.1 Identification of TSKU as a PB-responsive gene

TSKU was significantly increased from 1.3- to 7.5-fold in primary human hepatocytes (n=10 donors) after PB treatment, measured by an increase in signal intensity units of 541 to 12,900 units to 4490 to 20,500 units (Figure 27A). In addition to PB, both A1254 and DEHP treatment also increased TSKU expression in hepatocytes from nine or more donors. In hepatocytes from six of nine donors, this induction was verified with a second method of mRNA quantification, competitive PCR using a homologous internal standard (Figure 27B). Results in the remaining three donors show a trend of induction in donor C, although not significant, while TSKU was decreased in the remaining two donors (F and I). Additionally, basal expression of TSKU varies more than 10-fold across the donors, and expression in the hepatoma-derived cell lines, HepG2 and Huh7, is at the low end of this range. Further, TSKU was not increased in either of these cell lines.
TSKU expression was increased after A1254, DEHP, or PB challenge. Expression is representative of ten human hepatocyte donors in comparison to vehicle-treated hepatocytes as measured by microarray analysis using Affymetrix Human Genome U133 Plus 2.0 arrays (A). TSKU induction after PB treatment in human hepatocytes was verified with a second method of mRNA quantification, competitive PCR using a homologous internal standard (B). Significance is defined as *, p<0.05; **, p<0.01; ***, p<0.001; and ****, p<0.0001.
5.4.2 TSKU knockdown in primary human hepatocytes

To test the hypothesis that TSKU enhances CAR-mediated transcriptional induction, endogenous TSKU in primary human hepatocytes from three independent donors was knocked down using lentiviral particles expressing siRNA specific for TSKU. Lentiviral infection resulted in expression of siRNA constructs in approximately 10% to 40% of the hepatocytes, as measured by GFP expression (Figure 28A-D). Expression of

![Image of Figure 28 showing GFP expression in primary human hepatocytes from a representative donor (HH-M) expressing GFP indicating lentiviral infection with siRNA-expressing plasmids. Hepatocytes infected with siRNA specific for TSKU are shown in panels A and B, while hepatocytes infected with a scrambled siRNA sequence are shown in C and D. TSKU expression in hepatocytes is summarized from results in three independent donors (E), and TSKU expression in hepatocytes infected with TSKU-specific or scrambled siRNA is relative to that in control cells. Lower case letters denote differences in TSKU expression (one-way ANOVA; p<0.05).]
TSKU-specific siRNA resulted in reduction of TSKU expression to levels 46.4±4.7% lower than expression in control hepatocytes, while the scrambled siRNA constructs did not significantly affect TSKU expression (Figure 28). When TSKU expression was reduced, the robustness of the cytochrome P450 induction response was diminished, such that the induction of classic PB-response genes, CYP2B6 [16-19], CYP3A4 [16,18,20], and CYP2C9 [21,22], was reduced to 43.5±3.1%, 48.1±13.0%, and 55.2±12.4%, respectively, of induction exhibited in control hepatocytes (Figure 29A-F). Reduced CYP2B6 induction at the transcript level also led to a diminished induction response at the protein level (Figure 30). In contrast to PB-inducible genes, induction of CYP1A2, mediated through the arylhydrocarbon receptor (AhR) [23,24], was not altered after TSKU knockdown (Figure 29G-H).

5.4.3 CYP2B6 reporter assays

Because knockdown experiments suggested that TSKU contributes to the robustness of the cytochrome P450 induction response to PB, the ability of TSKU to directly affect transactivation of the PB-responsive enhancer module (PBREM) in the promoter region of CYP2B6 was investigated in COS-1 cells. Although COS-1 cells were chosen for ease of transfection, these cells do not express physiological levels of CAR or RXRα. Thus, to mimic physiological conditions, these proteins were overexpressed in conjunction with TSKU. However, exogenous expression of CAR in cell lines results in constitutive activity of this receptor, even in the absence of ligand. To ensure that any effect of TSKU on transactivation would not be masked by maximal activation of the CYP2B6-PBREM reporter construct by CAR alone, CAR was titrated into the experiments to show differential activation of the reporter. As reported previously [15], the presence of exogenous RXRα significantly increased luciferase activity driven by activation of the CYP2B6-PBREM (Figure 31A vs. 31B). However, when both CAR and RXRα were co-expressed, the presence of TSKU did not significantly affect activation of the CYP2B6-PBREM (Figure 31). These results argue
Figure 29: TSKU knockdown in primary human hepatocytes reduced induction of the PB-response genes, CYP2B6, CYP3A4, and CYP2C9. Magnitude of fold change is shown in panels A, C, E, and G, while induction in cells infected with either scrambled or TSKU-specific siRNA oligos is show relative to control cells in panels B, D, F, and H. Significance is denoted by *, p<0.05 or **, p<0.01 (one-way ANOVA). Lowercase letters indicate significant differences in expression.
against a direct effect of TSKU in stabilization of proteins at the PBREM, instead suggesting that TSKU exerts its effects in the extracellular space.

5.5 Discussion

This study presents the novel finding that TSKU is a PB-response gene with an indirect role in the CAR-mediated signaling pathway leading to induction of biotransformation enzymes. An expression profiling study of PB-responsive genes in primary human hepatocytes identified TSKU as a novel gene consistently induced in hepatocytes from ten human donors (Figure 27). Although it has been identified as responsive to a variety of xenobiotics, the only functional role attributed to TSKU outside of embryonic development is the enhancement of collagen accumulation in SXR-regulated bone homeostasis. The current study demonstrates that TSKU enhances CAR-mediated transcriptional induction of the phase I biotransformation enzymes, CYP2B6, CYP3A4, and CYP2C9. When TSKU-specific siRNA was introduced into primary human hepatocytes through lentiviral particles, the mRNA and protein induction of these PB-responsive genes was diminished (Figure 29A-F and Figure 30). Additionally, this effect was specific for the PB-response, as induction of CYP1A2, an AhR response gene, was not altered after TSKU knockdown (Figure 29G-H).

Sequence analysis of the human TSKU protein revealed nine leucine-rich repeats (LRRs) [6], which function to stabilize protein-protein interactions in a diverse array of biological processes. Based on these observations, we hypothesized that TSKU could stabilize interactions between proteins present at the promoter regions of PB-responsive
genes, such as CAR, RXRa, and coactivator proteins, with an overall effect of enhancing transcriptional activation at these promoters. To investigate this hypothesis, reporter assays were conducted in COS-1 cells to determine if the presence of TSKU altered the activation of the PBREM in the CYP2B6 promoter region. However, no difference in luciferase activity driven by the CYP2B6-PBREM was observed after TSKU overexpression (Figure 31).

Since a direct intracellular role in stabilizing the enhanceosome is unlikely based on the results of the reporter assay, it is possible that TSKU exerts its effects in the extracellular space, consistent with the presence of a signalling peptide typical of secreted proteins within the N-terminal region [6]. The chick ortholog of TSKU contains a similar signalling peptide, and this protein has been demonstrated to bind the TFGβ-like ligand BMP4 in the extracellular space, ultimately preventing binding to its membrane-bound receptor and subsequent activation of BMP signalling [5]. In addition to the BMP pathway, *Xenopus* TSKU integrates multiple signalling pathways to coordinate embryonic germ layer formation; while binding and potentiating Xnr2 signalling and

![Figure 31: TSKU does not directly alter activation the PB-response element in the CYP2B6 promoter. COS-1 cells were transiently transfected with increasing concentrations of CAR in the presence and absence of TSKU in the absence (A) or presence of exogenous RXRa (B). Significance was determined by t-tests (p<0.05). ***, p<0.0001.](image-url)
subsequent Smad2 activity, TSKU also binds extracellular FGF8b, interfering with the ability of this ligand to bind to and activate signalling through the FGF receptor [4].

Further exploration of the role of TSKU in the PB response could provide valuable insight into the molecular mechanism of CAR activation. In the intact liver, CAR is sequestered in a cytosolic complex consisting of heat shock protein 90 (HSP90) and the tetratricopeptide repeat protein cytoplasmic CAR retention protein (CCRP) [25,26], and PB treatment initiates a signaling cascade resulting in nuclear translocation of CAR [27,28]. Intriguingly, although CAR/ RXRα heterodimers are bound to the PBREM fragments isolated only from PB-treated nuclear extracts [17,19], neither coactivator binding nor fluorescence resonance energy transfer (FRET) studies have established direct binding between PB and CAR [29,30]. The discovery that okadaic acid, a protein phosphatase inhibitor, prevented CAR translocation [27,31,32] led to the identification of the PP2A phosphatase as a factor bound to CAR after PB treatment [26] and of Ser-202 in mCAR as an essential phosphorylation site in the translocation process [33]. The upstream factor that presumably interacts with PB to initiate this signalling cascade has remained elusive, however.

Recently, a membrane-associated subunit of protein phosphatase 1 (R16A) was identified as a possible candidate for this upstream factor [34]. Expression of R16A stimulates nuclear translocation of CAR in the absence of ligand, and, in the presence of ligand, dimerization of R16A is enhanced and correlated with CAR translocation. Further, R16A inhibits PP1b phosphatase activity, which normally blocks CAR translocation. If TSKU interacts with a membrane-bound factor to stabilize and enhance PB-mediated signaling, R16A seems a likely target for this interaction. Further study is required to determine if R16A provides a link between TSKU and CYP2B6 induction in the CAR-mediated signaling pathway.

In summary, the current study has identified TSKU as a novel PB-inducible gene that plays an indirect role in the CYP450 induction response. As TSKU did not directly enhance transcriptional activation of the PBREM that drives CYP2B6 induction, we propose that TSKU likely exerts its effects in the extracellular space, consistent both with
its identification as a secreted protein and with previous findings regarding its role in
signalling during embryonic development. Further studies are necessary to determine the
protein with which TSKU interacts to enhance CAR-mediated signalling.

5.6 References

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Chapter 6

Significance of toxicogenomic analyses in hepatic model systems

6.1 Hepatic models to infer human health risk

A common goal in toxicity research is the inference of human health risks. Without the incorporation of validated human models into risk analysis, research only creates safer conditions for rodents. However, the in vitro nature of primary human hepatocytes introduces confounding factors. Toward this goal, the first investigation in this study addressed the relevance of primary human hepatocytes as a model for processes that occur in the human liver. As demonstrated in chapter 2, primary human hepatocytes, maintained under conditions consisting of physiologically relevant levels of glucocorticoids and in a sandwich culture configuration, exhibit expression levels of liver-enriched transcription factors, transporters, and drug-metabolizing enzymes comparable to that seen in liver tissues (Figure 5). After xenobiotic challenge, the induction response of cytochrome P450 enzymes, both transcriptionally and translationally, was exhibited in primary human hepatocytes, demonstrating that this model is a better predictor of the in vivo situation than cell lines established from hepatomas, such as HepG2 and Huh7 cells, in which this induction response is absent (Figure 6; [1-3]).

6.2 Adaptation of hepatocytes to in vitro conditions

Despite the marked improvement in liver-specific gene expression and induction in hepatocytes vs. established cell lines, there are clearly changes in primary hepatocytes in comparison to liver tissues, as correlation ranged from 0.70 ± 0.01 to 0.76 ± 0.01 between liver tissues and the individual donors. As noted previously, this difference in gene expression is likely due in part to the presence of cells in liver tissues that are absent
in the enriched hepatocyte preparations, such as Kuppfer cells, as well as to interindividual differences due to inherent donor-specific differences between hepatocytes and human liver tissues. However, these differences likely also reflect, at least in part, functional changes that occur in vitro after the trauma of the isolation process and the adaptation to in vitro conditions. Along these lines, other studies have attributed changes in rodent primary hepatocytes vs. rodent liver tissues to fundamental differences in physiology between these models [4,5]. Specifically, these studies found that expression of particular categories were vastly diminished or lost in vitro, including 'cell proliferation', 'immune response', and 'blood coagulation', among others. An important point to keep in mind, however, is that the hepatocytes used in these studies were likely of sub-optimal quality due to the lack of sandwich culture conditions; an observation supported by the reported lack of differentiation status and biotransformation enzyme expression.

Our study offers a similar in vivo-in vitro comparison, yet with the extra confidence afforded by a thoroughly described, differentiated model system. Further, the use of hepatocytes from ten independent donors provides additional insight into this question, as functional categories commonly changed in vitro among the different donors are more likely to represent change related to the in vitro adaptation process rather than to inter-sample differences. In comparison to the human liver tissues, 160 and 534 distinct genes were more than 2-fold increased and decreased, respectively, in all ten human hepatocyte donors. Gene ontology analyses using the PANTHER Classification System found that the 'cell structure and motility', 'induction of apoptosis', and 'cell cycle control' ontology categories were represented more often than anticipated among the genes consistently increased in the human hepatocytes, while such categories as 'oncogenesis', 'receptor protein serine/threonine kinase signaling pathway', and 'nuclear hormone receptor' were overrepresented among genes decreased in the hepatocytes.

Although the significance of these categories cannot be determined without additional experimentation, cell structure and motility upregulation could reflect the structural adaptation to the in vitro microenvironment, perhaps providing clues to the
mechanisms behind the phenotypic descriptions of *in vitro* adaptation [6-8]. Apoptosis and cell cycle control genes could reflect cell stress [9,10].

### 6.3 Interindividual variability in primary human hepatocytes

One major complication with the primary human hepatocytes is the vast amount of interindividual variability in metabolism across the human population. The impact of this variability is exemplified through clinical observations; for example, comparable doses of codeine often do not produce the same magnitude of relief among individuals [11] and some individuals experience a high occurrence of hemolysis when treated with anti-malarial drugs [12]. This variation has been explained by documented differences in cytochrome P450 (CYP450) expression and activity, as these enzymes play a major role in the detoxification response. Variation among individuals has been noted in total microsomal protein content per gram of liver [13], mRNA and protein expression of various CYP450 enzymes [14-17], and in overall kinetics for the elimination of phase I- and II-specific substrates [18]. Further, genetic polymorphisms, like those in *CYP2D6*, can result in different metabolic phenotypes; such as, in this example, poor, extensive, and ultra-rapid metabolizers. These phenotypes explain the observation mentioned above in the variable codeine response. A poor metabolizer will not be able to efficiently metabolize codeine to its activated metabolite and therefore will not receive adequate pain relief, while an ultra-rapid metabolizer will have an exaggerated and potentially life-threatening response, as the active metabolite will be formed at higher-than-anticipated rates [11,12,19].

Further, drug metabolism requires the coordinated interaction of many types of molecules, not just enzymes. Drug uptake and efflux can be altered by levels of drug transporters, variation among transcription factor activity can ultimately affect the levels of inducible genes such as the drug metabolizing enzymes, and the amount of enzyme induction and inhibition in response to substrates can vary among individuals [11,12,15,19]. In addition to genetic explanations for variability, environmental factors such as age, gender, nutritional status, concomitant illness, renal and liver functional
status, and recreational drug use can all contribute to inter-individual differences in metabolism [20,21].

These variables are often controlled for by using rodent models, which eliminate variation among subjects with respect to genetic differences, age, gender, diet, immune status, and exposure to foreign inducers or inhibitors, such as nicotine from cigarette smoke or alcohol [22,23]. By removing the intra-group variation caused by these factors, toxicogenomics studies have clearly demonstrated chemical-specific expression “fingerprints” in mice and rats [24-26]. In humans, however, metabolic variability has led to a disagreement regarding the ability of primary human hepatocytes to mount a robust, consistent response to xenobiotic treatment regardless of donor source. Some studies support this hypothesis [27] while others suggest that transcriptional response varies so considerably by donor that identifying biologically consistent trends may be improbable without prior mechanistic knowledge [28,29].

To this end, our study uses a biologically relevant data set to demonstrate that the nature of the chemical may play a key role in its ability to engender a robust response. Similar to the study by Liguori et al [27], we found that a consistent transcriptional response was exhibited after treatment with A1254, which resulted in a clear clustering of hepatocytes based on chemical treatment when subjected to hierarchical clustering analysis (Table 4; Figure 12). Nonetheless, hepatocytes treated with PB regulated only 4 genes in nine or more donors (Table 4). Despite the small number of consistent response genes, the identification of classic PB response genes, such as \textit{CYP2B6} and \textit{CYP3A4}, lends confidence that results are accurate (Figure 10).

Like PB, DEHP did not produce a robust, consistent gene signature across the donors (Table 4). As the effects of DEHP are thought to be carried out primarily by its active metabolite, mono(2-ethylhexyl) phthalate (MEHP) [30-32], this inconsistent response could indicate that DEHP is not metabolized to levels sufficient for induction of response genes. Alternatively, these results could be a reflection of the weak activation of human PPARα by phthalate metabolites in comparison to the mouse ortholog [33].
6.4 Reproducibility of microarray data

In our study, donor source appears to be a substantial factor in the consistency of transcriptional response, such that only approximately 10% of all changed genes were similarly changed in half of the donors (Figure 13A). In general terms, however, response genes identified through genomics studies often seem to be widely variable, a point that may play a role in the variations seen in our study [34,35]. For instance, in a comparison of genomics studies examining CAR response genes, only half of the identified transcripts had similar expression changes across the studies [36-38]. These differences were attributed to variation in dosing regimens and microarray platforms [39]. Even when similar platforms are used, different laboratories do not always identify the same differentially expressed genes, as was the case for two profiling studies of "stemness" markers in embryonic and adult stem cells, which commonly identified only six genes between the two studies [40-42].

Recently, it has been suggested that the low reproducibility in the above studies reflects the method used to identify differentially expressed genes; namely, reliance on statistical methods alone rather than incorporation of fold change values [43,44]. In combination with a non-stringent p-value to increase specificity, rank ordering genes of interest based on fold change significantly increased reproducibility across test sites and microarray platforms in comparison to methods using only a t-statistic to calculate a p-value [43]. Further, even when the individual genes found to be differentially expressed were not consistent across sets of either platform/laboratory pairs [34] or highly variable samples [45], a comparison of the gene ontology categories enriched among those differentially expressed genes increased reproducibility.

6.5 Gene ontology-based analyses

One method used to identify enriched gene ontology categories among differentially expressed genes is through over-representation analysis. This approach takes advantage of the existing framework created by the Gene Ontology (GO) project, a
A collaborative effort that has resulted in the development of a controlled vocabulary to describe genes and their products across all eukaryotes. This vocabulary fits genes from different species into species-independent ontology categories, consisting of ‘biological process’ terms, which describe a biological objective accomplished through ordered assemblies of genes or gene products such as ‘apoptosis’ or ‘cell cycle control’, and ‘molecular function’ terms, which describe the biochemical activity of a gene product, for instance, ‘oxidoreductase’ or ‘Hsp 90 family chaperone’ [46]. In addition to the ability to quickly search for gene products with similar function across multiple species, a controlled vocabulary is desirable in that it provides more consistent search results than a database with a free text search mode [46,47]. Further, it has been suggested that the use of these higher level descriptors may result in a more reproducible “fingerprint” across individuals than by using individual genes as descriptors [48].

Throughout the current study, the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System (ABI, Foster City, CA) was used for identification of functional ontology categories of interest. The overall goal of this classification system is to annotate genes with respect to biological function, much in the same way that other databases have mapped functions to particular domains within a protein [47]. The current version of the PANTHER classification system has annotated over 75% of the gene products listed in the RefSeq database into over 5000 protein families and 30,000 subfamilies. After a list of differentially expressed genes has been determined from a microarray experiment, PANTHER fits these experimental genes into biological process and molecular function ontology categories. Similarly, genes from a reference list are also fit into these categories, and the binomial test is performed to determine which ontology categories have more hits than expected in the experimental list, based on results in the reference list [49]. It can be assumed then that these overrepresented categories are also altered at a functional level, representing the biological processes affected by toxicant exposure. This approach has been used successfully both to identify modes of action of a toxicant in an unbiased manner and to provide new insights into the molecular mechanisms induced by toxicants [48,50-52].
Analyses performed using the PANTHER Classification System resulted in a more consistent, biologically relevant dataset than analyses of individual response genes, especially for PB and DEHP treatment. Treatment with these xenobiotics resulted in fewer than 10 consistently altered response genes across the donors (Table 4), yet PANTHER analyses identified a core set of categories consistently changed, despite the source of the donor (Figure 14). For example, ‘fatty acid metabolism’ was enriched among the response to DEHP treatment, which is consistent with the well-documented activation of fatty acid-metabolizing enzymes, such as long chain acyl-CoA dehydrogenase, peroxisomal keto-acyl-CoA thiolase, and long chain acyl-CoA synthetase, by DEHP and other peroxisome proliferators [53,54]. Interestingly, these response genes have been described in rodents; our results suggest that at least some of the human donors retain a similar induction in response to DEHP.

Further, the results of this study suggest that ontology-based analyses may be a useful strategy for identifying biologically-relevant effects of new chemical entities, such as those developed as potential pharameuticals, in the midst of the considerable noise associated with interindividual varability in response genes. An argument could be made that the interindividual variability in transcriptional response may be too great to overcome without existing mechanistic knowledge. However, our results were able to show consistent change in alteration of functional categories by a single xenobiotic, despite highly donor-dependent individual response genes.

6.6 Benefits of interindividual variability

Although ontology-based analyses are useful for minimizing its impact, the donor-specific variability associated with in vitro hepatocytes may provide useful information for risk analysis. Risk analysis, as defined by the National Academy of Science (NAS), is a complex series of assessments for predicting health risks in human populations caused by chemical exposure, resulting in the determination of “safe” exposure levels “without appreciable health risk” [20,55]. Generally, appropriate safe levels in humans are determined by dividing a dose identified in animal species by a 100-
fold uncertainty factor, which incorporates a 10-fold factor to allow for both interspecies variability and for human variability (Lehman and Fitzhugh, 1954, Assoc. Food Drug Off. U.S.Q. Bull. 18, 33-35). Each of these factors of 10 can be further subdivided into components to describe toxicokinetic and toxicodynamic variability, and these default values can be replaced with chemical-specific adjustment factors whenever experimental data are available [56-58].

Nonetheless, experimental data regarding human variability is often a limiting factor in risk analysis, and data derived in primary human hepatocytes could be critical for determining variability across either the population as a whole or in subsets of the population [20,21,59]. \textit{In vivo} parameters such as clearance rates (C\textsubscript{max}) and area under the plasma concentration vs. time curve (AUC) have demonstrated that the default values used in risk analysis often do not cover human variability, especially for xenobiotics metabolized by polymorphic enzymes, like CYP2D6 or CYP2C19, or for certain subpopulations, like neonates or the elderly [55,60]. Although \textit{in vitro} observations are not directly applicable for the \textit{in vivo} situation, certain parameters determined \textit{in vitro} are predictive of \textit{in vivo} effects, especially when used in combination with physiologically-based pharmacokinetic (PBPK) modelling [61-63]. For instance, CYP450 inhibition data determined \textit{in vitro} can be useful to determine the likelihood and magnitude of \textit{in vivo} drug interactions [64,65], and \textit{in vitro} parameters such as half-life and intrinsic clearance can be used in PBPK modelling to estimate bioavailability and \textit{in vivo} half life [66].

6.7 Donor-specific factors

Although hepatocytes from only ten donors were used in this study, it is tempting to speculate what factors may contribute to the noted variability. Particularly interesting among the donor-specific differences is that two of the donors in our study, donors D and E, were repeatedly marked as outliers (Figure 8C, Figure 9C and 9E, and Figure 10B). Age of the donor is a possible source contributing to this outlier status, as donors D and E are the second and third oldest donors (ages 61 and 63, respectively) included in our study, and age has been shown to be inversely associated with hepatocyte yield and
viability [67,68]. Yet, this trend did not always hold true within our study, as donor G, the oldest donor, exhibited the largest induction of CYP2B6 in response to PB treatment.

A consideration of the age of hepatocyte donors brings up another interesting point: should there be an age limit below which hepatocytes should not be used? In our study, the youngest donor in our study at 0.75 years, was not detected as an outlier in any of the analyses; yet donor F, the second youngest donor (3 years) was noted to have the lowest inducibility of A1254 and PB target genes (Figure 10). Although the donor age and hepatocyte viability are negatively correlated [67,68], dynamic changes in CYP450 gene expression occur during fetal and childhood development [69,70]. For example, infants less than a year old have exhibit approximately 50% and 80% of CYP2C19 and CYP2E1 levels observed in adults, and express a different CYP3A isoform than adults (CYP3A7 in infants vs. CYP3A4 in adults) [71]. Developmental differences in biotransformation enzyme profiles could impact results obtained in hepatocytes from donors of varying ages.

Another interesting factor unique to donors D and E was the presence of 50% steatosis; livers from all other donors had fewer than 10% steatosis levels. Livers are in high demand for organ transplantation, with more than 12,500 US patients on the waiting list and approximately 6600 transplants performed in 2006 [72]. Nonetheless, many potential donor livers are rejected for transplant, with the leading cause for rejection being the presence of high-grade macrosteatosis [73]. Macrosteatosis is characterized by abnormal storage of triacylglycerol in macrovacuoles within hepatocytes, impairing hepatic blood flow and increasing the likelihood of ischemia-reperfusion injury [74,75]. Although debatable, the presence of high-grade macrosteatosis is often inversely associated with patient survival after transplant [76-79], which likely leads to a high percentage of hepatocytes from these rejected livers being used in in vitro research. Fewer studies have been done to determine the impact of macrosteatosis in vitro, although these hepatocytes have be found to be metabolically competent, despite decreased CYP activities [73,80,81].
6.8 Future directions

Important directions for future research that extend from these investigations include a functional assessment of the striking interspecies in mitochondrial oxidative metabolism. Although this study focused purely on gene expression, endpoints such as oxygen consumption, ATP concentrations, or activity of the individual complexes in the electron transport chain could clarify whether gene expression changes in the TCA cycle and the electron transport chain truly result in a species-specific susceptibility to toxicity. Our results, described in the fourth chapter of this thesis, suggest that A1254 lowers expression of TCA cycle and electron transport genes in the mouse. We hypothesize that these lower expression of these energy production genes decreases flux through these pathways, ultimately resulting in decreased ATP production and cell death \[82,83\]. One way to address this hypothesis would be through a direct measurement of ATP; however, changes in ATP concentrations may not be detected if flux through the glycolytic pathway is increased to compensate \[84,85\]. A more specific approach would be to use antibodies to assess the functionality of the individual complexes of the electron transport chain through a recently described immunocapture technique \[86,87\]. If a functional affect is determined to occur in mice, a similar assay can be done using hepatocytes to determine if a similar decrease in oxidative phosphorylation occurs after A1254 challenge in humans.

Another significant impact originating from this research is the identification of a novel gene with a role in the CAR-mediated CYP induction response. Intriguingly, although TSKU did not directly enhance transcriptional activation of the CYP2B6 promoter, TSKU knockdown in primary human hepatocytes clearly demonstrates that TSKU is a part of the PB signaling pathway. Based on sequence analysis, TSKU is likely a secreted protein, so it is likely that TSKU enhances signaling in the extracellular space. In this way, further experimentation to identify the extracellular receptor with which TSKU interacts could help elucidate the signalling pathway leading to CAR activation, as direct interaction between PB and CAR has not been demonstrated to date.
6.9 Conclusion

The results described represent important steps in the validation of a primary human hepatocyte culturing method. While human hepatocytes are generally viewed as a standard model system for xenobiotic metabolism research, numerous sources of variation are encountered with this model, including lack of a standardized culturing method, variation in quality of human tissue, and interindividual variability among donors. Obtaining consistent data from human model systems is an ultimate goal for risk assessment and for establishing mechanistic determinants of toxic response. In these respects, my research was focused on addressing the impact of the variation among human hepatocyte donors. These results support the use of a highly defined model system as one that maintains differentiation characteristics, expression of liver-specific genes at levels comparable to those in vivo, and gene induction capabilities across donors, regardless of the source/quality of the donor. Further, this research provides a global measure of variation among donors as well as a comparison of transcriptional responses to chemical treatment in humans and in mice. Importantly, direct applications of this primary hepatocyte culture model include pharmaceutical and academic research in xenobiotic toxicology, together with applications for risk assessment and regulatory toxicology.

6.10 References


### Appendix A

**Supplementary Data for Chapter 4**

Table 7: Orthologous ‘chaperone’ genes increased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2-associated athanogene 3</td>
<td>BAG3</td>
<td>CDHJ</td>
<td>4</td>
</tr>
<tr>
<td>DnaJ (Hsp40) homolog, subfamily B, member 1</td>
<td>DNAJB1</td>
<td>ADEGHIJ</td>
<td>7</td>
</tr>
<tr>
<td>DnaJ (Hsp40) homolog, subfamily C, member 12</td>
<td>DNAJC12</td>
<td>D</td>
<td>1</td>
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<td>FK506 binding protein 5</td>
<td>FKBP5</td>
<td>DF</td>
<td>2</td>
</tr>
<tr>
<td>Heat shock protein 90kDa (cytosolic) class A member 1</td>
<td>HSP90AA1</td>
<td>ABDEFGHIJ</td>
<td>9</td>
</tr>
<tr>
<td>Heat shock protein 90kDa beta (Grp94), member 1</td>
<td>HSP90B1</td>
<td>BD</td>
<td>2</td>
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<tr>
<td>Heat shock 70 kDa protein 1A</td>
<td>HSPA1A</td>
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<td>9</td>
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<td>CD</td>
<td>2</td>
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<tr>
<td>Heat shock 70 kDa protein 8</td>
<td>HSPA8</td>
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<td>9</td>
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<td>Hypoxia up-regulated 1</td>
<td>HYOU1</td>
<td>C</td>
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Table 8: Orthologous ‘inhibition of apoptosis’ genes increased by A1254 in both mouse and human hepatocytes.

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<th>Donors</th>
<th>Number of donors</th>
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<td>Bcl2-associated athanogene 3</td>
<td>BAG3</td>
<td>CDHJ</td>
<td>4</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein 1A</td>
<td>HSPA1A</td>
<td>ABCDFGHJ</td>
<td>8</td>
</tr>
<tr>
<td>Heat shock 105 kDa/110 kDa protein 1</td>
<td>HSPH1</td>
<td>ABDEGHJI</td>
<td>8</td>
</tr>
<tr>
<td>Metallothionein 2A</td>
<td>MT2A</td>
<td>ACF</td>
<td>3</td>
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<td>Ornithine decarboxylase 1</td>
<td>ODC1</td>
<td>AEGHJ</td>
<td>5</td>
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<tr>
<td>Suppressor of cytokine signaling 3</td>
<td>SOCS3</td>
<td>AC</td>
<td>2</td>
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<tr>
<td>Signal transducer and activator of transcription 3 (acute phase response factor)</td>
<td>STAT3</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>Thioredoxin domain containing 5</td>
<td>TXNDC5</td>
<td>ACE</td>
<td>3</td>
</tr>
</tbody>
</table>
### Table 9: Orthologous ‘oxidative stress’ genes increased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate-cysteine ligase, modifier subunit</td>
<td>GCLM</td>
<td>ABCDGHJJ</td>
<td>8</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein 1A</td>
<td>HSPA1A</td>
<td>ABCDFGHJ</td>
<td>8</td>
</tr>
<tr>
<td>Metallothionein 1E (functional)</td>
<td>MT1E</td>
<td>ACF</td>
<td>3</td>
</tr>
<tr>
<td>Metallothionein 2A</td>
<td>MT2A</td>
<td>ACF</td>
<td>3</td>
</tr>
<tr>
<td>Sulfiredoxin</td>
<td>SRXN1</td>
<td>ABCDEGHJJ</td>
<td>9</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>TFRC</td>
<td>ABCDEGHJJ</td>
<td>9</td>
</tr>
<tr>
<td>Thioredoxin domain containing 5</td>
<td>TXNDC5</td>
<td>ACE</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 10: Orthologous ‘xenobiotic metabolism’ genes increased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehyde dehydrogenase 1 family, member L1</td>
<td>ALDH1L1</td>
<td>CF</td>
<td>2</td>
</tr>
<tr>
<td>Cytochrome P450, family 1, subfamily A, polypeptide 1</td>
<td>CYP1A1</td>
<td>ABCDEGHJJ</td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome P450, family 1, subfamily A, polypeptide 2</td>
<td>CYP1A2</td>
<td>ABCDFGHJ</td>
<td>10</td>
</tr>
<tr>
<td>Cytochrome P450, family 2, subfamily B, polypeptide 6</td>
<td>CYP2B6</td>
<td>ABCDFGHJ</td>
<td>10</td>
</tr>
<tr>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 18</td>
<td>CYP2C18</td>
<td>DGIJ</td>
<td>4</td>
</tr>
<tr>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 19</td>
<td>CYP2C19</td>
<td>ABCDFGHJ</td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 9</td>
<td>CYP2C9</td>
<td>ABCDFGHJ</td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 7</td>
<td>CYP3A7</td>
<td>ABCDFGHJ</td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome P450, family 51, subfamily A, polypeptide 1</td>
<td>CYP51A1</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxysteroid (17-beta) dehydrogenase 2</td>
<td>HSD17B2</td>
<td>ACEFGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Nuclear receptor subfamily 0, group B, member 2</td>
<td>NR0B2</td>
<td>ABCDEHJJ</td>
<td>7</td>
</tr>
<tr>
<td>Nuclear receptor subfamily 1, group 1, member 3</td>
<td>NR1I3</td>
<td>CFHIJ</td>
<td>5</td>
</tr>
<tr>
<td>P450 (cytochrome) oxidoreductase</td>
<td>POR</td>
<td>ABCEGHJJ</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 11: Orthologous ‘cell cycle control’ genes decreased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyprostaglandin dehydrogenase 15-(NAD)</td>
<td>HPGD</td>
<td>ABCDEGHJI</td>
<td>9</td>
</tr>
<tr>
<td>Thioredoxin interacting protein</td>
<td>TXNIP</td>
<td>ABCDEGHJI</td>
<td>9</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)</td>
<td>EGFR</td>
<td>CDFGHJI</td>
<td>7</td>
</tr>
<tr>
<td>Forkhead box O3A</td>
<td>FOXO3A</td>
<td>ACFGHJI</td>
<td>7</td>
</tr>
<tr>
<td>Nuclear factor I/B</td>
<td>NFIB</td>
<td>ACFGHJI</td>
<td>7</td>
</tr>
<tr>
<td>Forkhead box P1</td>
<td>FOXP1</td>
<td>ACFDFI</td>
<td>5</td>
</tr>
<tr>
<td>G0/G1 switch 2</td>
<td>G0S2</td>
<td>FGHJ</td>
<td>4</td>
</tr>
<tr>
<td>Dual specificity phosphatase 1</td>
<td>DUSP1</td>
<td>HJ</td>
<td>3</td>
</tr>
<tr>
<td>Growth arrest-specific 2</td>
<td>GAS2</td>
<td>ABC</td>
<td>3</td>
</tr>
<tr>
<td>Transfucer of ERBB2, 2</td>
<td>TOB2</td>
<td>ABH</td>
<td>3</td>
</tr>
<tr>
<td>Tumor protein p53 inducible nuclear protein 1</td>
<td>TP53INP1</td>
<td>ACF</td>
<td>3</td>
</tr>
<tr>
<td>Endothelial cell growth factor 1</td>
<td>ECGF1</td>
<td>DF</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 12: Orthologous ‘induction of apoptosis’ genes decreased by A1254 in mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioredoxin interacting protein</td>
<td>TXNIP</td>
<td>ABCDEGHJI</td>
<td>9</td>
</tr>
<tr>
<td>Forkhead box O3A</td>
<td>FOXO3A</td>
<td>ACFGHJI</td>
<td>7</td>
</tr>
<tr>
<td>PHD finger protein 17</td>
<td>PHF17</td>
<td>ACFIJ</td>
<td>5</td>
</tr>
<tr>
<td>Tumor protein p53 inducible nuclear protein 1</td>
<td>TP53INP1</td>
<td>ACF</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 13: Orthologous ‘lipid, fatty acid, and steroid metabolism’ genes decreased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid hormone responsive</td>
<td>THRSP</td>
<td>ABDEFGHIJ</td>
<td>9</td>
</tr>
<tr>
<td>Lipin 2</td>
<td>LPIN2</td>
<td>ACDGIJ</td>
<td>6</td>
</tr>
<tr>
<td><strong>Lipid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipin 1</td>
<td>LPIN1</td>
<td>ACDFGHIJ</td>
<td>8</td>
</tr>
<tr>
<td>Lipase, hepatic</td>
<td>LIPC</td>
<td>SGI</td>
<td>3</td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)</td>
<td>GPD2</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td><strong>Fatty acid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyl-CoA synthetase short-chain family member 2</td>
<td>ACSS2</td>
<td>ADFGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Cytochrome P450, family 4, subfamily A, polypeptide 11</td>
<td>CYP4A11</td>
<td>ABEGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>FASN</td>
<td>ADFGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Stearoyl-CoA desaturase (delta-9-desaturase)</td>
<td>SCD</td>
<td>ABDGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Acyl-CoA synthetase long-chain family member 4</td>
<td>ACSL4</td>
<td>ADEPHI</td>
<td>6</td>
</tr>
<tr>
<td>Acyl-CoA synthetase long-chain family member 1</td>
<td>ACSL1</td>
<td>AFHJ</td>
<td>5</td>
</tr>
<tr>
<td>Enoyl coenzyme A, hydratase/domain containing 2</td>
<td>ECHDC2</td>
<td>BFGHI</td>
<td>5</td>
</tr>
<tr>
<td>Enoyl coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase</td>
<td>EHHADH</td>
<td>ABDGH</td>
<td>5</td>
</tr>
<tr>
<td>ELOVL family member 6, elongation of long chain fatty acids</td>
<td>ELOVL6</td>
<td>ADFGH</td>
<td>5</td>
</tr>
<tr>
<td>Cytochrome b5 reductase 3</td>
<td>CYB5R3</td>
<td>BDGI</td>
<td>4</td>
</tr>
<tr>
<td>Cytochrome P450, family 8, subfamily B, polypeptide 1</td>
<td>CYP8B1</td>
<td>BFHJ</td>
<td>4</td>
</tr>
<tr>
<td>Acyl-CoA thioesterase 12</td>
<td>ACOT12</td>
<td>ACI</td>
<td>3</td>
</tr>
<tr>
<td>ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)</td>
<td>ELOVL5</td>
<td>ACI</td>
<td>3</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa</td>
<td>NDUFAB1</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td><strong>Steroid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl-coenzyme A synthase 2</td>
<td>HMGCS2</td>
<td>ABEGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)</td>
<td>HSD17B6</td>
<td>ABEGHJ</td>
<td>7</td>
</tr>
<tr>
<td>Phosphatidic acid phosphatase type 2B</td>
<td>PPAP2B</td>
<td>ADGHJ</td>
<td>6</td>
</tr>
<tr>
<td>Retinol dehydrogenase 16 (all-trans)</td>
<td>RDH16</td>
<td>ABEGHJ</td>
<td>6</td>
</tr>
<tr>
<td>Sterol-C-5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like</td>
<td>SC5DL</td>
<td>ACGHI</td>
<td>5</td>
</tr>
<tr>
<td>Cytochrome P450, family 4, subfamily V, polypeptide 2</td>
<td>CYP4V2</td>
<td>ACGL</td>
<td>4</td>
</tr>
<tr>
<td>Insulin induced gene 2</td>
<td>INSIG2</td>
<td>ACG</td>
<td>3</td>
</tr>
<tr>
<td>UDP glucuronosyltransferase 2 family, polypeptide B15</td>
<td>UGT2B15</td>
<td>ABC</td>
<td>3</td>
</tr>
<tr>
<td>Sulfotransferase family, cytosolic, 1A, phenol-prefering, member 1</td>
<td>SULT1A1</td>
<td>BI</td>
<td>2</td>
</tr>
<tr>
<td>Sulfotransferase family, cytosolic, 1B, member 1</td>
<td>SULT1B1</td>
<td>C</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 14: Orthologous ‘mitochondrial oxidative metabolism’ genes decreased by A1254 in both mouse and human hepatocytes.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Donors</th>
<th>Number of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCA cycle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 1</td>
<td>PDK1</td>
<td>DFGHIJ</td>
<td>6</td>
</tr>
<tr>
<td>Dihydrolipoamide S-acetyltransferase (E2 component of the pyruvate dehydrogenase complex)</td>
<td>DLAT</td>
<td>AGHIJ</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 2</td>
<td>PDK2</td>
<td>SBG</td>
<td>3</td>
</tr>
<tr>
<td>Malate dehydrogenase 1, NAD (soluble)</td>
<td>MDH1</td>
<td>AC</td>
<td>2</td>
</tr>
<tr>
<td>Succinate-CoA ligase, ADP-forming, beta subunit</td>
<td>SUCLA2</td>
<td>AC</td>
<td>2</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit D, integral membrane protein</td>
<td>SDHD</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5, 13kDa</td>
<td>NDUFA5</td>
<td>ACF</td>
<td>3</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3, 12kDa</td>
<td>NDUFB3</td>
<td>AC</td>
<td>2</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6, 14kDa</td>
<td>NDUFA6</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 10, 22kDa</td>
<td>NDUFB10</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4, 15kDa</td>
<td>NDUFB4</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8, 19kDa</td>
<td>NDUFB8</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa</td>
<td>NDUFS1</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit D, integral membrane protein</td>
<td>SDHD</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex V (ATP synthase)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1</td>
<td>ATP5C1</td>
<td>F</td>
<td>1</td>
</tr>
<tr>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6</td>
<td>ATP5J</td>
<td>A</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 15: Orthologous ‘mitochondrial oxidative metabolism’ genes regulated by A1254 specifically in mouse.

<table>
<thead>
<tr>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCA cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aconitase 2</td>
<td>Aco2</td>
<td>Dec</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>Cs</td>
<td>Dec</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 3 (NAD+) beta</td>
<td>Idh3b</td>
<td>Dec</td>
</tr>
<tr>
<td>Malic enzyme, supernatant</td>
<td>Mod1</td>
<td>Dec</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)</td>
<td>Sdhb</td>
<td>Dec</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) I alpha subcomplex 12</td>
<td>Ndufa12</td>
<td>Dec</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) I beta subcomplex 5</td>
<td>Ndufb5</td>
<td>Dec</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) I beta subcomplex 6</td>
<td>Ndufb6</td>
<td>Dec</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) I beta subcomplex unknown 2</td>
<td>Ndufc2</td>
<td>Dec</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)</td>
<td>Sdhb</td>
<td>Dec</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome C-1</td>
<td>Cyc1</td>
<td>Dec</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Shuttling protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome C, somatic</td>
<td>Cycs</td>
<td>Dec</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase, subunit Va</td>
<td>Cox5a</td>
<td>Dec</td>
</tr>
<tr>
<td>Cytochrome c oxidase, subunit VI a, polypeptide 2</td>
<td>Cox6a2</td>
<td>Inc</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex V</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2</td>
<td>Atp5j2</td>
<td>Dec</td>
</tr>
</tbody>
</table>
Table 16: Orthologous ‘TCA cycle’ genes regulated in A1254 specifically by human hepatocytes.

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Donors Increased</th>
<th>Donors Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase</td>
<td>CS</td>
<td>ACGI</td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 3 (NAD+) alpha</td>
<td>IDH3A</td>
<td>BGHJ</td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 3 (NAD+) beta</td>
<td>IDH3B</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 3 (NAD+) gamma</td>
<td>IDH3G</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 1 (NAD+) soluble</td>
<td>IDH1</td>
<td>FGHJ</td>
<td></td>
</tr>
<tr>
<td>Malic enzyme 1, NADP(+)-dependent, cytosolic</td>
<td>ME1</td>
<td>DEIJ</td>
<td></td>
</tr>
<tr>
<td>Malic enzyme 3, NADP(+)-dependent, mitochondrial</td>
<td>ME3</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 2</td>
<td>PDK2</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 3</td>
<td>PDK3</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>Succinate-CoA ligase, ADP-forming, beta subunit</td>
<td>SUCLA2</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SUCLG1</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Fumarate hydratase</td>
<td>FH</td>
<td>D</td>
<td>I</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 2 (NADP+), mitochondrial</td>
<td>IDH2</td>
<td>C</td>
<td>ABDGHIJ</td>
</tr>
<tr>
<td>Malate dehydrogenase 2, NAD (Mitochondrial)</td>
<td>MDH2</td>
<td>CJ</td>
<td>B</td>
</tr>
<tr>
<td>Malic enzyme 2, NAD(+)-dependent, mitochondrial</td>
<td>ME2</td>
<td>BDGO</td>
<td>C</td>
</tr>
<tr>
<td>Oxoglutarate (alpha-ketoglutarate)dehydogenase (lipoamide)</td>
<td>OGDH</td>
<td>CJ</td>
<td>G</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isozyme 4</td>
<td>PDK4</td>
<td>BF</td>
<td>HIJ</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa</td>
<td>SDHC</td>
<td>CJ</td>
<td>BGI</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit D, integral membrane protein</td>
<td>SDHD+</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)</td>
<td>SDHA</td>
<td></td>
<td>F</td>
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<tr>
<td>Succinate-CoA ligase, GSP-forming, beta subunit</td>
<td>SUCLG2</td>
<td>ACGH</td>
<td></td>
</tr>
</tbody>
</table>

* Gene is also decreased in expression in A1254-treated mice
Table 17: Orthologous ‘Oxidative phosphorylation-Complex I’ genes regulated by A1254 specifically in human hepatocytes.

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Donors Increased</th>
<th>Donors Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 12</td>
<td>NDUFA12</td>
<td>GI</td>
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</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 8</td>
<td>NDUFA8</td>
<td>IJ</td>
<td></td>
</tr>
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<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9</td>
<td>NDUFA9</td>
<td>J</td>
<td></td>
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<td>NDUFA10</td>
<td>J</td>
<td></td>
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<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9</td>
<td>NDUFB9</td>
<td>HJ</td>
<td></td>
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<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NADH-coenzyme Q reductase)</td>
<td>NDUFS2</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NADH-coenzyme Q reductase)</td>
<td>NDUFS3</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 5 (NADH-coenzyme Q reductase)</td>
<td>NDUFS5</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 6 (NADH-coenzyme Q reductase)</td>
<td>NDUFS6</td>
<td>HJ</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 3</td>
<td>NDUFV3</td>
<td>CHJ</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 3</td>
<td>NDUFA3</td>
<td>H</td>
<td>B</td>
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<tr>
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<td>NDUFA5'</td>
<td>D</td>
<td>ACF</td>
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<td>C</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7</td>
<td>NDUFA7</td>
<td>GH</td>
<td>B</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4</td>
<td>NDUFB4'</td>
<td>J</td>
<td>B</td>
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<td>B</td>
</tr>
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<td>NADH dehydrogenase (ubiquinone) 1, subcomplex unknown</td>
<td>NDUFC1</td>
<td>HJ</td>
<td>B</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 7 (NADH-coenzyme Q reductase)</td>
<td>NDUFS7</td>
<td>H</td>
<td>B</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 8 (NADH-coenzyme Q reductase)</td>
<td>NDUFS8</td>
<td>CHJ</td>
<td>BF</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 1</td>
<td>NDUFV1</td>
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<td>B</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2</td>
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<td>BCI</td>
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<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4</td>
<td>NDUFA4</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) 1 beta subcomplex 2</td>
<td>NDUFB2</td>
<td>ABC</td>
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<td>D</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 4 (NADH-coenzyme Q reductase)</td>
<td>NDUFS4</td>
<td>ACF</td>
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</table>
Table 18: Orthologous ‘oxidative phosphorylation-complex II-V’ genes regulated by A1254 specifically in human hepatocytes.

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Symbol</th>
<th>Increase</th>
<th>Decrease</th>
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<tbody>
<tr>
<td><strong>Oxidative phosphorylation, Complex II</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Succinate dehydrogenase complex, subunit D, integral membrane protein</td>
<td>SDHD</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa</td>
<td>SDHC</td>
<td>CJ</td>
<td>BGI</td>
</tr>
<tr>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)</td>
<td>SDHA</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Complex III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome C-1</td>
<td>CYC1</td>
<td>AIJ</td>
<td></td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation, Shuttling protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome C, somatic</td>
<td>CYCS</td>
<td>BDGIJ</td>
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<tr>
<td><strong>Oxidative phosphorylation, Complex IV</strong></td>
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<td></td>
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<td>Cytochrome c oxidase, subunit Va</td>
<td>COX5A</td>
<td>AHJ</td>
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<tr>
<td>Cytochrome c oxidase subunit 8A (ubiquitous)</td>
<td>COX8A</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>COX15 homolog, cytochrome c oxidase assembly protein (yeast)</td>
<td>COX15</td>
<td>CFGI</td>
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<td>Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)</td>
<td>COX7A2</td>
<td>J</td>
<td>B</td>
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<td>COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)</td>
<td>COX17</td>
<td>DHJ</td>
<td>C</td>
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<tr>
<td>Cytochrome c oxidase subunit IV isoform 1</td>
<td>COX4H1</td>
<td>B</td>
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<tr>
<td>Cytochrome c oxidase subunit Via polypeptide 1</td>
<td>COX6A1</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)</td>
<td>COX6B1</td>
<td>B</td>
<td></td>
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<tr>
<td>Cytochrome c oxidase subunit Vlc</td>
<td>COX6C</td>
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<tr>
<td>Cytochrome c oxidase subunit VIIc</td>
<td>COX7C</td>
<td>C</td>
<td></td>
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<td>COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)</td>
<td>COX18</td>
<td>A</td>
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<tr>
<td><strong>Oxidative phosphorylation, Complex V</strong></td>
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<td>ATP synthase, H+transporting, mitochondrial F1 complex, beta polypeptide</td>
<td>ATP5B</td>
<td>CG</td>
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<td>ATP5G1</td>
<td>ACDG</td>
<td>HJ</td>
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<tr>
<td>ATP synthase H+transporting mitochondrial F0 complex subunit C3 (subunit 9)</td>
<td>ATP5G3</td>
<td>GJ</td>
<td></td>
</tr>
<tr>
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<td>ATP5H</td>
<td>J</td>
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<td>ATP5J2</td>
<td>J</td>
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<td>ATP5C1</td>
<td>I</td>
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<td>ATP synthase, H+transporting mitochondrial F1 complex delta subunit</td>
<td>ATP5D</td>
<td>ACG</td>
<td>B</td>
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<td>ATP synthase H+transporting mitochondrial F0 complex subunit B1</td>
<td>ATP5F1</td>
<td>GJ</td>
<td>C</td>
</tr>
<tr>
<td>ATP synthase, H+transporting mitochondrial F0 complex subunit C2 (subunit 9)</td>
<td>ATP5G2</td>
<td>GH</td>
<td>B</td>
</tr>
<tr>
<td>ATP synthase, H+transporting mitochondrial F0 complex subunit s (factor B)</td>
<td>ATP5S</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>ATP synthase, H+transporting mitochondrial F0 complex subunit E</td>
<td>ATP5I</td>
<td>C</td>
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</tr>
<tr>
<td>ATP synthase, H+transporting, mitochondrial F1, O subunit (oligomycin sensitivity conferring protein)</td>
<td>ATP5O</td>
<td>C</td>
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</table>
Appendix B

Gene Expression Profiling of Extracellular Matrix as an Effector of Human Hepatocyte Phenotype in Primary Cell Culture

B.1 Abstract

Previously, we demonstrated that primary cultures of rat hepatocytes evidence higher levels of differentiated function when cultured in the presence of a dilute overlay of extracellular matrix (ECM; Matrigel). In this investigation, we used DNA microarrays, quantitative RT-PCR, immunoblotting, and cell morphology analyses to evaluate the biological responses imparted by Matrigel overlays on primary cultures of human hepatocytes from five independent donors. Although inter-individual variability in responses was evident, our results demonstrated that Matrigel additions typically improved hepatocyte morphology and differentiation character. Results from RNA profiling experiments indicated that Matrigel additions enhanced hepatocyte RNA expression levels associated with a battery of differentiated features, to levels comparable to those seen in vivo, for genes such as the cytochrome P450s, solute carrier family members, sulfotransferases, certain nuclear transcription factors, and other liver specific markers, such as albumin, transferrin and response to the inducer, phenobarbital. In contrast, Matrigel additions were generally associated with reduced RNA expression levels for several cytokeratins, integrins and a number of stress-related pathways. Decreases in integrin protein expression were similarly detected, although enhanced levels of the gap-junction-associated protein, connexin 32, were detected in Matrigel -treated cultures. These data support the concept that by mediating a reduction in cellular stress and stress-signaling pathways, together with and enhancement of gap junctional cell-cell communication, ECM functions mechanistically to facilitate the differentiation character of primary human hepatocytes in culture.
B.2 Introduction

In biological tissues, the importance of microenvironment on cell function is well established. The interactions between cells and the extracellular matrix (ECM) together with cell-cell interactions can have profound effects on cell morphology, function, proliferation, differentiation and responses to stimuli [1-9]. ECM is comprised of a complex mixture of biomaterials, including collagens, proteins, proteoglycans and glycosoaminoglycans, and supports the growth, attachment and migration of cells in their tissue microenvironments [10].

Several studies have demonstrated that ECM additions to cultures of hepatocellular carcinoma (HCC) cell lines and primary hepatocytes largely enhance cell functional criteria [3,4,6-9]. The importance of an ECM component in maintenance of cell polarity and morphology has been reported in rat hepatocytes [1,2,5-7], as has a modulatory role of ECM in the regulation of rat hepatocyte responses to signaling ligands [5]. Rat primary hepatocytes cultured in the presence of an ECM appear to display an actin filament organization similar to that of intact liver [8], and rat hepatocytes cultured in collagen or Matrigel sandwich configurations demonstrate improved morphology and enhanced levels of albumin gene expression [1,2,6,7]. Similarly, improved levels of albumin, transferrin and transthyretin - markers of differentiated phenotype - were associated with ECM and dexamethasone treated hepatocytes, together with the concomitant suppression of de-differentiation markers, such as alpha-fetoprotein (AFP) and glutathione transferase π (GSTπ) [9]. It has been reported that cell-ECM contacts in culture enhance signaling and transcription factor binding within the albumin gene promoter [11]. Whereas primary rat hepatocytes typically lose the expression of the cytochrome P450 genes gradually in culture, the presence of Matrigel was noted to re-establish and maintain P450 expression levels [3,12]. Presence of Matrigel also greatly facilitates the gene induction response to phenobarbital (PB), a feature that manifests only in highly differentiated hepatocytes [9,13-16].

Human hepatocytes are more difficult to obtain than those of rodents and are often more difficult to attach in 2-dimensional cultures; however, human hepatocytes
offer a more accurate reflection of species-specific responses to stimuli, such as pharmaceutical exposures, and provide important insight regarding population variability in chemical response [17-19]. Due to the scarcity of liver donors for primary human hepatocyte cultures, resulting in small sample sizes, it is essential that optimal and consistent culture conditions be defined. Gene expression profiles and response to drug challenge have been investigated in both attached and suspension-cultured human hepatocytes. Although both cell types were viable and offer at least limited responses to challenge, the gene expression profiles of the two types of culture can be quite different, and illustrate the importance of determining optimal culture conditions for human predictive modeling [19]. Hamilton et al [20] examined responses of human hepatocytes to various conditions of culture including ECM additions and cellular plating densities. They reported that although presence of ECM resulted in phenotypic differences in the cells, maintenance of the drug induction response was more dependent on plating density than ECM. In contrast, others have evaluated the effects of Matrigel overlay on human hepatocyte culture and concluded that the presence of ECM markedly facilitated xenobiotic responsiveness [21].

Despite these observations, the underlying mechanisms of ECM effects on cellular function are largely unknown. Experimental evidence suggests that cues received from the ECM are important in maintaining hepatocyte cell morphology, function and differentiation status [1-9]. The integrins play a prominent role in mediating cell-ECM interactions and modulate the signal transduction of extracellular cues. Integrins are transmembrane proteins composed of non-covalently linked alpha and beta subunits. Each alpha and beta subunit contains an extracellular ligand binding domain, a transmembrane domain and a short cytoplasmic tail. Although integrins possess no intrinsic enzymatic activity, they function to transduce extracellular signals through direct contact with the cellular cytoskeleton, facilitating the redistribution of intracellular proteins and activation of associated signaling cascades [22-24]. Aggregations of integrins cause accumulation and activation of signaling molecules [25], activating Rac, Rho, Cdc42 [26-28] and the MAPK signaling module [29,30]. Overexpression of the beta-1 and beta-3 integrin subunits enhance Rho and Rac activity, respectively, and these
activation events are often accompanied by morphological alterations as well as and changes in stress fiber formation [31].

We conducted this study in order to better assess the biological impact of ECM on primary human hepatocytes in culture, and to examine the underlying mechanisms associated with these effects. We hypothesized that ECM additions enhance the differentiation patterning of hepatocytes in culture and their resulting responsiveness to environmental cues through the modulation of cellular integrin signaling networks and associated formation of focal adhesions.

B.3 Methods

B.3.1 Cell culture

Enriched primary human hepatocyte cultures plated on collagen were obtained through the Liver Tissue Procurement and Distribution System, Pittsburgh, which was funded by NIH Contract #NO1-DK-9-2310. Cells were photographed upon arrival and the cells were placed in fresh William’s E media containing: 1% penicillin/streptomycin, 1% HEPES, 20 µM glutamine, 25 nM dexamethasone, 10 nM insulin, 1% linoleic acid/BSA, 5 ng/mL selenious acid and 5 µg/mL transferrin. Selected cultures were overlayed with 225 µg/mL BD Matrigel™ Basement Membrane Matrix (BD Biosciences; San Jose, CA), as described previously [9]. All other culturing materials were purchased from Invitrogen (Carlsbad, CA). Maintenance media was changed once per two days. Selected cultures were treated with 500 µM PB, on day 4 of culture.

B.3.2 RNA isolation and purification

One mL of Trizol reagent (Invitrogen) was pipetted onto hepatocytes following media aspiration. The cell monolayer was scraped after a 2 min incubation at room temperature (RT). Trizol/cell mix was pipetted ten times and then transferred to a
microcentrifuge tube and vortexed for 10 sec. Lysates were incubated at RT for five min. Chloroform, 200 µL, was added and tubes were shaken for 15 seconds followed by a 3 min incubation at RT. Samples were centrifuged for 15 min at 12,000 x g at 4 ºC. The aqueous phase was transferred to a clean tube and organic phase was stored at -80 ºC for DNA and protein isolation. Isopropyl alcohol, 500 µL, was added to each sample and incubated at RT for 10 min followed by centrifugation at 4ºC for 10 min at 12,000 x g. The RNA pellet was washed with 1 mL of 75% ethanol, vortexed, and centrifuged for 5 min at 4 ºC at 7500 x g. Pellets were air dried and then resuspended in nuclease-free water. Samples were incubated at 60ºC for 10 min. RNA integrity was confirmed by ethidium bromide visualization following formaldehyde gel electrophoresis.

RNA was further purified using DNA-free™ (Ambion). Briefly, 3 µL of DNA-free DNase and 10 µL of DNase-free DNase buffer were added to each sample. Reactions were incubated at 37ºC for 20 min. Inactivator, 20 µL, was added and samples were incubated at RT for 2 min followed by centrifugation. Supernatants were transferred to fresh tubes. Ammonium acetate (7.5M) and 100% ethanol were added at 0.1 volumes and 2.5 volumes respectively. Samples were mixed and incubated at -20 ºC for 90 min followed by centrifugation at 12,500 rpm for 10 min at 4 ºC. The resulting pellet was washed in 75% ethanol and then air dried. Pellets were dissolved in nuclease-free water and incubated at 60ºC for 10 min. Concentrations were determined by spectrophotometry using a SmartSpec 3000 spectrophotometer (BioRad).

**B.3.3 cDNA preparation**

cDNA was prepared using the High Capacity cDNA Archive Kit (cat# 4322171, Applied Biosystems). Briefly, ten µL of 10X Reverse Transcription Buffer, 4 µL 25X dNTPs, 10 µL of 10X random primers, 5 µL of Multiscribe Reverse Transcriptase (50U/µL) and 21 µL of nuclease free water were combined in a 0.5 mL microcentrifuge tube. RNA (2 µg) and water were added for a total reaction volume of 100 µL. Reverse transcription was carried out at 25ºC for 10 min followed by incubation at 37ºC for 2 h.
B.3.4 RT-PCR

Reaction components were prepared for duplicate 25 µL reactions in 96-well plates: 2X TaqMan Universal PCR Master Mix (ABI, P/N 4304437), 25 µL, 20X Assays-on-Demand Gene Expression Assay Mix, 2.5 µL, and cDNA diluted in RNase-free water, 22.5 µL. Samples were mixed and then split into individual wells. Using the Applied Biosystems 7300 Real-Time PCR System, plate documents were configured with the appropriate assay and sample information. Thermocycling conditions were as follows: UNG activation, 2 min 50ºC, hold 10 min 95ºC, and for each of 40 cycles, 15 sec 95ºC and 1 min 60ºC. Sequence Detection System software (ABI) was used to collect and organize the fluorescence data for analysis.

B.3.5 RT-PCR data analysis

Data were analyzed using the ∆∆CT method [32]. Briefly, CT values for genes of interest were normalized to 18S by generating a ∆CT for each gene (ex. ∆CTalb\textsubscript{mg} = \text{average}CT\textsubscript{alb\textsubscript{mg}} - \text{average}CT\textsubscript{18S\textsubscript{mg}}). The ∆∆CT was calculated by normalizing the ∆CT values for treatments to control (ΔΔCT\textsubscript{alb} = ΔCT\textsubscript{alb\textsubscript{mg}} - ΔCT\textsubscript{alb\textsubscript{c}}). Results were then expressed as fold change over control samples by raising $2^{-\Delta\Delta CT}$.

B.3.6 Microarray analysis

Five µg of purified RNA was processed for microarray analysis by Paradigm Array Labs (Icoria). After all samples were subjected to and passed quality control measures, RNA was hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays and results were analyzed by GeneChip Operating Software (Affymetrix). Raw cell intensity values were computed by the Cell Analysis algorithm and present, absent and marginal calls were generated. All individual Matrigel samples were then baselined back to their corresponding controls generating change calls and change call p-values. Outputs were
transferred to Microsoft Access and filtered for presence in both Matrigel and control samples. Surviving probe sets were used for further study.

**B.3.7 Cluster analysis**

Microarray results were analyzed by ArrayAssist 4.0 (Stratagene) with hierarchical clustering. Raw cell intensities were clustered based on expression in donor samples. Results were then filtered for two-fold changes across all donors and clustered again by sample.

**B.3.8 GoStat analysis**

Lists of changed probe sets were batch analyzed by GOstat [33] for overrepresented categories of genes. The entire list of probe sets from the Affymetrix Human Genome U133 Plus 2.0 array was used as a reference list to determine overrepresentation. The maximum p-value cut-off was set at 0.05 and correction for multiple testing was performed using the Benjamini false discovery rate.

**B.3.9 Protein isolation**

Isopropanol, 1.5 mls per 1mL Trizol used, was added to the leftover organic phase after RNA and DNA isolation by Trizol. Samples were incubated at room temperature for 10 min. Protein was sedimented by centrifugation at 12,000 x g at 4°C for 10 min. After removal of the supernatant, protein pellet was washed three times in 2 mLs of 0.3 M guanidine hydrochloride in 95% ethanol. The pellets were centrifuged at 7500 x g for 5 min at 4°C following each wash. After the final wash, protein pellets were vortexed in 2 mLs of 100% ethanol per 1 mL Trizol used followed by incubation at RT for 20 min and centrifugation at 7500 x g for 5 min at 4°C. After removing the supernatant, the pellets were air dried for 15 min and resuspended in 50 µL of 2X Buffer (8M urea, 2M
thiourea, 0.05M Tris, pH 6.8, 75mM dithiothreitol, 3% sodium dodecyl sulfate, 0.05% Bromphenol blue). After the protein samples were completely redissolved, 50 µL of water was added and protein concentrations were quantified by a modified Bradford assay (BioRad, cat#500-0006).

B.3.10 Western immunoblotting

Twenty µg of total protein were heated at 95°C prior to loading onto a precast 10% Tris-HCl SDS-PAGE gel (BioRad). Proteins were separated by denaturing SDS-PAGE (100 volts for 1.5 hours in 0.03M Tris, 0.2M glycine, 0.025% SDS) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes, (120 volts for 1 h in 0.03M Tris, 0.2M glycine, 20% methanol). Membranes were blocked in 5% non-fat dry milk and TBS-T (0.1% Tween) for 1 h prior to the addition of primary antibody diluted in blocking buffer. Membranes were incubated with primary antibody overnight at 4°C with rocking and then washed 3 X 5 min in TBS-T. Membranes were then incubated with the appropriate secondary horseradish peroxidase conjugated antibody diluted 1:5000 in blocking buffer for 1 h at RT with rocking. After 3 X 5 min washes in TBS-T, protein-antibody complexes were visualized by chemiluminescence (Lumilight, Roche) and exposed to X-ray film. Primary antibodies and dilutions were as follows: α-Keratin 8 (MS-997, Neomarkers, Fremont, CA) 1:200; α-Keratin 18 (MS-142, Neomarkers) 1:200; α-phosphoKeratin 8 (MS-1241, Neomarkers) 1:200; α-phosphoKeratin 18 (MS-1242, Neomarkers) 1:200; α-integrin α5 (AF1864, R&DSystems, Minneapolis, MN) 1:250; α-connexin 32 (13-8200, Zymed Laboratories Inc, San Francisco, CA) 1:500.
B.4 Results

B.4.1 Matrigel overlay elicits phenotypic change in individual hepatocyte samples.

To assess the morphological impact of ECM addition, photomicrographs were obtained from control (Figure 32A, 32C and 32E) and Matrigel treated (32B, 32D and 32F) samples each day over the course of culturing. Photomicrographic results from Donors A, B and C illustrate that the addition of a Matrigel overlay improved hepatocyte morphology, as the cells exhibited more defined cuboidal shape and improved definition of cell borders. In contrast, cells without a Matrigel overlay exhibited a flattened appearance, poorly defined borders and elaborated spinous processes resembling a fibroblastic character. These cells also failed to form the highly organized networks of cells cultured in the presence of an ECM.

Figure 33 displays photomicrographs taken from Donor D and Donor E hepatocytes cultured in the presence (B and D) and absence of Matrigel (A and C). Matrigel additions to these cultures appeared to result in little distinguishable morphological change. However, even from their initial culture, these samples exhibited numerous aggregations of necrotic and apoptotic hepatocytes, both the in presence and absence of ECM. These cells demonstrated disorganized cellular architecture, flattened morphology and poorly defined cell borders. Therefore, the compromised initial quality of hepatocytes obtained from these two donors was not rescued by Matrigel additions.

Overall, our observations indicated that morphological changes in primary human hepatocytes demonstrating low quality morphology were not phenotypically responsive to ECM, whereas higher quality cultures exhibited further defined improvement in morphology when cultured in the presence of a Matrigel overlay. After reviewing the donor descriptions, no obvious correlation between donor characteristics with culture quality or response to Matrigel could be established (Table 19).
Figure 32: Matrigel enhances cellular morphology of primary human hepatocyte cultures for Donor A, Donor B and Donor C. Primary human hepatocytes from Donor A (A and B), Donor D (C and D) and Donor C (E and F) were cultured in the presence (B, D, F) and absence (A, C, E) of a Matrigel overlay. Photomicrographs were taken under 20X magnification using phase contrast imaging. Arrows indicate compromised morphology in the absence of a Matrigel overlay.
B.4.2 Matrigel enhances the differentiation status of primary human hepatocytes.

Previous reports have established a supportive role for ECM/Matrigel in hepatocyte culture [3,4,6-9]. Studies in rat showed that addition of Matrigel to primary hepatocyte cultures increased the expression of differentiation markers [9]. To examine the effects of ECM on primary cultures of human hepatocytes, cells were grown in the presence or absence of a Matrigel overlay. Cultures were monitored over the course of several days and total RNA was collected. Real time quantitative PCR was performed on RT reactions from purified RNA. Results were compared to untreated controls using the

Figure 33: Matrigel overlay exhibits minimal impact on cellular morphology for primary human hepatocyte cultures obtained from Donor D or Donor E. Primary human hepatocytes from Donor D (A and B) and Donor E (C and D) were cultured in the presence (B and D) and absence (A and C) of a matrigel overlay. Photomicrographs were taken under 20X magnification using phase contrast imaging.
\[ \Delta \Delta C_T \] method [32]. The addition of ECM improved the expression of three markers of differentiation: albumin, transferrin, and transthyretin in all four hepatocyte samples tested (Figure 34A). In addition to enhanced expression of differentiation markers, culturing the hepatocytes in the presence of Matrigel appropriately repressed expression of the de-differentiation markers, GST\(\pi\) and AFP (Figure 34B).

<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Age</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Cause of Death</th>
<th>Smoker/Alcohol Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH-A</td>
<td>0.75</td>
<td>M</td>
<td>C</td>
<td>Anoxia</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-B</td>
<td>61</td>
<td>M</td>
<td>C</td>
<td>Gunshot wound</td>
<td>No/Yes (21 drinks/week)</td>
</tr>
<tr>
<td>HH-C</td>
<td>3</td>
<td>F</td>
<td>C</td>
<td>Anoxia</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-D</td>
<td>29</td>
<td>F</td>
<td>C</td>
<td>N/A</td>
<td>No/No</td>
</tr>
<tr>
<td>HH-E</td>
<td>46</td>
<td>F</td>
<td>C</td>
<td>Head injury</td>
<td>Yes (13 years)/Yes (3 drinks/week)</td>
</tr>
</tbody>
</table>

1 Human hepatocytes were obtained from Dr. Steven Strom at the University of Pittsburgh, following the regulations of the Liver Tissue Procurement and Distribution System (LPDS)
2 C; Caucasian
3 N/A; not available

The induction of drug metabolizing enzymes in response to an external stimulus, in particular PB, represents a complex biological response indicative of a highly differentiated hepatic phenotype. When primary human hepatocytes cultured in the presence or absence of Matrigel (MG and control, respectively) were treated with PB for 24 h prior to isolation of total RNA, induction of the PB-responsive genes, CYP2B6 and CYP3A4, were enhanced in cells cultured in the presence of Matrigel (PB+MG). HepG2 cells, a human hepatocellular carcinoma cell line, treated in the same fashion exhibited no induction response and no improvement in response in the presence of Matrigel (Figure 34C). Total RNA was collected from a section of whole human liver and the expression profile of differentiation and de-differentiation markers was determined through quantitative RT-PCR. A comparison of human liver, human hepatocytes cultured with Matrigel and hepatocellular carcinoma cells was performed. The results demonstrated that primary human hepatocytes cultured in the presence of a Matrigel overlay most closely resemble the expression profile of the human liver while HepG2 cells, although
Figure 34: Effects of Matrigel addition on differentiation status of primary human hepatocyte cultures. Total RNA was isolated from primary human hepatocytes cultured for five days in the presence or absence of a Matrigel overlay. (A) Transcript levels for the hepatocyte differentiation markers, albumin, transferrin and transthyretin, were assessed -/+ Matrigel additions using quantitative RT-PCR and the ΔΔCt method. (B) Transcript levels for the hepatocyte de-differentiation markers, alpha fetoprotein and GSTP1 were similarly ascertained, -/+ Matrigel additions. (C) Primary human hepatocytes were cultured in the absence (control) or presence of Matrigel (MG). Cultures were treated on day 4 with phenobarbital (PB and PB+MG) or DMSO (control) for 24 hours prior to RNA isolation. Relative fold changes in transcript levels for the PB-inducible marker genes, CYP2B6 and CYP3A4, are indicated. (D) Total RNA was isolated from HepG2 cells (indicated by arrows), a section of human liver #154 as well as three different donor samples of primary human hepatocytes that were cultured with a Matrigel overlay. Relative expression analyses for a panel of differentiation and de-differentiation markers were determined by quantitative RT-PCR analysis and the results are graphically depicted.
expressing certain markers, differ from the expression levels of the liver by at least 10-fold and as much as 200-fold (Figure 34D). This result was apparent in both Donors D and E, despite the lack of morphological impact of Matrigel on these cultures (Figure 33). In studies to be reported elsewhere, further comparisons to additional human liver tissues, from 6 different donors, were also conducted, with similar conclusions derived as that for the representative HL#154 liver presented here. Therefore, the cumulative evidence indicated that a Matrigel overlay was a positive regulator of differentiation status of primary human hepatocytes, facilitating the up-regulation of differentiation makers, down-regulation of de-differentiation markers and enhancement of the response to PB.

**B.4.3 Interindividual variability contributes to expression profile of donors.**

Sample sets, with and without Matrigel, from all donors were subjected to hierarchical clustering. In Figure 35A, the raw intensity values of all present probe sets in all samples are shown in a heat map. Donors A, C, D and E clustered together regardless of treatment, indicating the dominance of interindividual variability. When the probe set list was filtered for dramatic changes (2-fold or more, either increased or decreased) all of the samples tended to cluster by treatment. Matrigel-treated cultures from Donors A, B and C shared similar expression patterns and Matrigel cultures from Donors D and E clustered together (Figure 35B). These clustering patterns reflected the similarity in morphological assessments of the individual cultures (Figure 32 and Figure 33). In contrast, the clustering of cultures lacking ECM displayed no clear pattern. These results highlight the dominance of inter-individual variability in the absence of ECM treatment; however, the ability of Matrigel to effect similar patterns of gene expression irrespective of donor is also well apparent.
Figure 35: Hierarchical clustering analyses of inter-individual differences and treatment regimens of primary human hepatocyte samples. Total RNA was isolated from cultures of primary human hepatocytes from five different donors, +/- Matrigel overlay. RNA transcript profiles were analyzed by microarray hybridizations, as described in Methods. Results were hierarchically clustered (Array Assist, Stratagene) before (A) and after (B) filtering for two-fold changes in gene expression level between Matrigel treated samples and untreated controls.
B.4.4 Addition of Matrigel to primary human hepatocyte cultures elicits changes in gene expression.

To characterize the changes in gene expression influenced by ECM additions to the primary hepatocyte cultures, individual RNA samples were analyzed by microarray profiling. Gene expression profiles from five donors cultured in the presence or the absence of a Matrigel overlay were examined using the GeneChip Operating Software (GCOS; Affymetrix). Initially, regression scatterplots were generated by plotting raw intensity values of -/+ ECM data and correlation coefficients were generated (Figure 36A-36E). The R-squared values obtained -/+ Matrigel were 0.9480, 0.9607, 0.9753, 0.9816, and 0.9926 for Donor A, Donor B, Donor C, Donor D, and Donor E, respectively. Although the control and Matrigel samples were highly correlated, measurable gene expression changes were apparent in each donor set. Donor A exhibited 5193 changed probe sets; Donor B, 4492 changed probe sets; Donor C, 2769 changed probe sets; Donor D, 2740 altered probe sets; with Donor E exhibiting the fewest numbers of changed probe sets, with 1024 (Figure 36F). In summary for these analyses, although overall levels of gene expression changes consequent to Matrigel overlay additions were subtle, as evidenced by the high R-squared values, a minimum of 1000 probe sets were indeed altered as a result of that respective culture condition.

B.4.5 Matrigel treatment contributes to changed gene expression profiles of primary human hepatocytes.

Table 20 and Table 22 list the probe sets and corresponding genes altered in response to ECM addition among all five hepatocyte donor samples. Table 20 illustrates that several cytochrome P450s: 2C8, 2C9 and 2C19, and epoxide hydrolase 1 (EPHX1) were each increased in all five donor samples treated with Matrigel. Since donors A, B and C are closely related in expression profiles and demonstrated similar morphological
Figure 36: Biological impact of Matrigel additions in primary human hepatocyte cultures. Microarray analyses were performed on total RNA collected from primary human hepatocytes cultured in the presence or absence of a Matrigel overlay for either 4 or 5 days. Raw signal intensities for every probe set were plotted for control and Matrigel samples for each donor (A-E). A correlation coefficient was generated for each scatterplot. Total numbers of changed genes (change call p≤0.00267) were reported for each donor (F).
response to Matrigel, the common genes that were upregulated by presence of an ECM in these samples were examined. A total of 193 probe sets, corresponding to 153 genes, were increased by addition of Matrigel to hepatocyte cultures in these three donors. Selected genes are displayed in Table 21. Eleven of the cytochrome P450 gene products were represented by at least one probe set, along with probe sets corresponding to other liver specific genes like CEBPalpha, FMO5, the nuclear receptors NR1D2 and NR1I3, the solute carrier family proteins and the sulfotransferases.

**Table 21:** Universal gene expression increases due to addition of Matrigel in five human hepatocyte cultures.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>Description</th>
<th>Gene Symbol</th>
<th>Donor A</th>
<th>Donor B</th>
<th>Donor C</th>
<th>Donor D</th>
<th>Donor E</th>
</tr>
</thead>
<tbody>
<tr>
<td>202017_at</td>
<td>Epoxide hydrolase 1, microsomal</td>
<td>EPHX1</td>
<td>2.297</td>
<td>2.639</td>
<td>3.732</td>
<td>1.149</td>
<td>1.414</td>
</tr>
<tr>
<td>208147_s_at</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 8</td>
<td>CYP2C8</td>
<td>3.249</td>
<td>3.249</td>
<td>2.828</td>
<td>2.000</td>
<td>2.144</td>
</tr>
<tr>
<td>214421_x_at</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 9</td>
<td>CYP2C9</td>
<td>1.625</td>
<td>2.144</td>
<td>1.866</td>
<td>1.414</td>
<td>1.231</td>
</tr>
<tr>
<td>216025_x_at</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 19 // Cytochrome P450, family 2, subfamily C, polypeptide 9</td>
<td>CYP2C19 // CYP2C9</td>
<td>1.625</td>
<td>2.144</td>
<td>1.866</td>
<td>1.414</td>
<td>1.231</td>
</tr>
<tr>
<td>220017_x_at</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 9</td>
<td>CYP2C9</td>
<td>1.625</td>
<td>2.000</td>
<td>1.866</td>
<td>1.414</td>
<td>1.231</td>
</tr>
<tr>
<td>226147_s_at</td>
<td>Polymeric immunoglobulin receptor</td>
<td>PIGR</td>
<td>1.866</td>
<td>3.482</td>
<td>1.231</td>
<td>1.516</td>
<td>1.320</td>
</tr>
</tbody>
</table>

Microarray results were filtered for probe sets up-regulated among all five donor Matrigel samples. Amount of fold change compared to control for each probe set is reported.

The presence of an ECM also resulted in the decrease of four probe sets among all individuals (Table 22), corresponding to beta 5-tubulin (OK/SW-cl.56), hypothetical protein H41 (H41), pleckstrin homology-like domain family A member 1 (PHLDA1), and likely ortholog of rat vacuole membrane protein 1 (VMP1). Among Donors A, B and C, those most responsive to ECM addition, 272 probe sets corresponding to 209 genes exhibited decreased expression in the presence of Matrigel, including actinin, tropomyosin, BMP2, Cdc42, Ras and Ras-related proteins, chemokines, keratins,
Table 21: Selected increased probe sets in Matrigel responsive human hepatocyte cultures.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>Gene symbol</th>
<th>Description</th>
<th>Donor A</th>
<th>Donor B</th>
<th>Donor C</th>
</tr>
</thead>
<tbody>
<tr>
<td>206262_at</td>
<td>ADH1B</td>
<td>Alcohol dehydrogenase 1B (class I), beta polypeptide</td>
<td>5.657</td>
<td>1.625</td>
<td>3.031</td>
</tr>
<tr>
<td>204039_at</td>
<td>CEBPA</td>
<td>CCAAT/enhancer binding protein (C/EBP), alpha</td>
<td>2.297</td>
<td>1.866</td>
<td>1.866</td>
</tr>
<tr>
<td>209366_x_at*</td>
<td>CYP2A6</td>
<td>Cytochrome P450, family 2, subfamily A, polypeptide 6</td>
<td>4.925</td>
<td>6.063</td>
<td>14.929</td>
</tr>
<tr>
<td>203979_at</td>
<td>CYP2A7</td>
<td>Cytochrome P450, family 2, subfamily A, polypeptide 7</td>
<td>2.639</td>
<td>6.063</td>
<td>12.996</td>
</tr>
<tr>
<td>206754_s_at*</td>
<td>CYP2B6</td>
<td>Cytochrome P450, family 2, subfamily B, polypeptide 6</td>
<td>1.414</td>
<td>9.849</td>
<td>8.574</td>
</tr>
<tr>
<td>216661_x_at*</td>
<td>CYP2C19</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 19</td>
<td>1.741</td>
<td>2.144</td>
<td>2.000</td>
</tr>
<tr>
<td>208147_s_at</td>
<td>CYP2C8</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 8</td>
<td>3.249</td>
<td>3.249</td>
<td>2.828</td>
</tr>
<tr>
<td>220017_x_at*</td>
<td>CYP2C9</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 9</td>
<td>1.625</td>
<td>2.000</td>
<td>1.866</td>
</tr>
<tr>
<td>205999_x_at*</td>
<td>CYP3A4</td>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 4</td>
<td>4.000</td>
<td>2.000</td>
<td>3.482</td>
</tr>
<tr>
<td>211442_x_at</td>
<td>CYP3A43</td>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 43</td>
<td>1.414</td>
<td>2.144</td>
<td>2.639</td>
</tr>
<tr>
<td>211843_x_at*</td>
<td>CYP3A7</td>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 7</td>
<td>2.828</td>
<td>1.866</td>
<td>3.031</td>
</tr>
<tr>
<td>202017_at</td>
<td>EPHX1</td>
<td>Epoxide hydrolase 1, microsomal</td>
<td>2.297</td>
<td>2.639</td>
<td>3.732</td>
</tr>
<tr>
<td>205776_at*</td>
<td>FMO5</td>
<td>Flavin containing monooxygenase 5</td>
<td>1.741</td>
<td>1.866</td>
<td>1.625</td>
</tr>
<tr>
<td>225768_at</td>
<td>NR1D2</td>
<td>Nuclear receptor subfamily 1, group D, member 2</td>
<td>1.741</td>
<td>2.000</td>
<td>1.741</td>
</tr>
<tr>
<td>207007_at</td>
<td>NR1H3</td>
<td>Nuclear receptor subfamily 1, group I, member 3</td>
<td>3.249</td>
<td>1.866</td>
<td>1.625</td>
</tr>
<tr>
<td>207097_s_at</td>
<td>SLC17A2</td>
<td>Solute carrier family 17, member 2</td>
<td>1.866</td>
<td>2.144</td>
<td>1.414</td>
</tr>
<tr>
<td>201920_at</td>
<td>SLC20A1</td>
<td>Solute carrier family 20, member 1</td>
<td>1.320</td>
<td>2.639</td>
<td>1.625</td>
</tr>
<tr>
<td>205972_at</td>
<td>SLC38A3</td>
<td>Solute carrier family 38, member 3</td>
<td>3.249</td>
<td>2.144</td>
<td>1.866</td>
</tr>
<tr>
<td>225516_at</td>
<td>SLC7A2</td>
<td>Solute carrier family 7, member 2</td>
<td>1.866</td>
<td>1.741</td>
<td>1.414</td>
</tr>
<tr>
<td>222071_s_at</td>
<td>SLCO4C1</td>
<td>Solute carrier organic anion transporter family member 4C1</td>
<td>1.866</td>
<td>1.625</td>
<td>3.031</td>
</tr>
<tr>
<td>203615_x_at</td>
<td>SULT1A1</td>
<td>Sulphotransferase family, cytosolic 1A, member 1</td>
<td>1.414</td>
<td>1.320</td>
<td>1.149</td>
</tr>
<tr>
<td>206292_s_at</td>
<td>SULT2A1</td>
<td>Sulphotransferase family, cytosolic 2A, member 1</td>
<td>1.625</td>
<td>1.516</td>
<td>1.625</td>
</tr>
</tbody>
</table>

Microarray results were filtered for probe sets up-regulated among Matigel samples for Donor A, Donor B, and Donor C (B). Amount of fold change compared to control for each probe set is reported. An asterisk (*) denotes those genes which had multiple probe sets increased; a single representative probe set with fold changes with respect to control is reported.
integrins, JAK1, TGFbeta receptor I and II, as well as others. A list of selected genes demonstrating reduced expression levels in the presence of Matrigel is provided in Table 23.

Table 22: Universal gene expression decreases due to Matrigel addition in five human hepatocyte primary cell cultures.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Description</th>
<th>Gene Symbol</th>
<th>Donor A</th>
<th>Donor B</th>
<th>Donor C</th>
<th>Donor D</th>
<th>Donor E</th>
</tr>
</thead>
<tbody>
<tr>
<td>209026_x_at</td>
<td>Beta 5-tubulin</td>
<td>OK/SW-cl56</td>
<td>0.812</td>
<td>0.871</td>
<td>0.660</td>
<td>0.812</td>
<td>0.812</td>
</tr>
<tr>
<td>213548_s_at</td>
<td>Hypothetical protein H41</td>
<td>H41</td>
<td>0.758</td>
<td>0.379</td>
<td>0.660</td>
<td>0.758</td>
<td>0.616</td>
</tr>
<tr>
<td>217996_at</td>
<td>Pleckstrin homology-like</td>
<td>PHLDA1</td>
<td>0.707</td>
<td>0.354</td>
<td>0.500</td>
<td>0.707</td>
<td>0.812</td>
</tr>
<tr>
<td>224917_at</td>
<td>Likely ortholog of rat vacuole membrane protein 1</td>
<td>VMP1</td>
<td>0.268</td>
<td>0.707</td>
<td>0.707</td>
<td>0.758</td>
<td>0.758</td>
</tr>
</tbody>
</table>

When considering changes occurring in all five individuals, there were certain categories of genes for which expression was either increased or decreased in every donor in response to Matrigel. To investigate common themes of gene expression changes, all of the probe sets exhibiting either increased or decreased change in all five donors were analyzed by GOstat [33]. Oxidoreductase-associated genes were the major overrepresented group of genes exhibiting enhanced expression levels (Table 24). Genes in the xenobiotic metabolism ontology category also exhibited increased expression. Overrepresented genes demonstrating reduced expression level in the presence of ECM included actin cytoskeleton, cytoskeleton organization and biogenesis, actin binding, cytoskeletal protein binding and cellular morphogenesis (Table 25). Overall then, ECM up-regulated gene categories within several liver-specific functions, while cytoskeletal and stress related categories comprised the down-regulated list.
Imaging analyses indicated the presence of necrotic and apoptotic cellular content in two of the five donor samples, Donors D and E (Figure 33). Strong associations with expression markers for apoptosis and necrosis were not evident for these donors in our GOstat analyses; however, the control vs. Matrigel-treated GOstat comparisons

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Description</th>
<th>Gene Symbol</th>
<th>Donor A</th>
<th>Donor B</th>
<th>Donor C</th>
</tr>
</thead>
<tbody>
<tr>
<td>208636_at*</td>
<td>Actinin, alpha 1</td>
<td>ACTN1</td>
<td>0.660</td>
<td>0.758</td>
<td>0.574</td>
</tr>
<tr>
<td>205289_at*</td>
<td>Bone morphogenetic protein 2</td>
<td>BMP2</td>
<td>0.500</td>
<td>0.406</td>
<td>0.574</td>
</tr>
<tr>
<td>211367_s_at*</td>
<td>Caspase 1, apoptosis-related cysteine protease</td>
<td>CASP1</td>
<td>0.707</td>
<td>0.660</td>
<td>0.707</td>
</tr>
<tr>
<td>203065_s_at*</td>
<td>Caveolin 1, caveolae protein, 22kDa</td>
<td>CAV1</td>
<td>0.435</td>
<td>0.406</td>
<td>0.574</td>
</tr>
<tr>
<td>203324_s_at</td>
<td>Caveolin 2</td>
<td>CAV2</td>
<td>0.660</td>
<td>0.616</td>
<td>0.812</td>
</tr>
<tr>
<td>208727_s_at</td>
<td>Cell division cycle 42</td>
<td>CDC42</td>
<td>0.660</td>
<td>0.758</td>
<td>0.574</td>
</tr>
<tr>
<td>823_at</td>
<td>Chemokine (C-X3-C motif) ligand 1</td>
<td>CX3CL1</td>
<td>0.707</td>
<td>0.758</td>
<td>0.536</td>
</tr>
<tr>
<td>204470_at</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>CXCL1</td>
<td>0.406</td>
<td>0.660</td>
<td>0.500</td>
</tr>
<tr>
<td>206336_at</td>
<td>Chemokine (C-X-C motif) ligand 6</td>
<td>CXCL6</td>
<td>0.406</td>
<td>0.574</td>
<td>0.435</td>
</tr>
<tr>
<td>200859_x_at</td>
<td>Filamin A, alpha</td>
<td>FLNA</td>
<td>0.308</td>
<td>0.707</td>
<td>0.616</td>
</tr>
<tr>
<td>210338_s_at</td>
<td>Heat shock 70kDa protein 8</td>
<td>HSPA8</td>
<td>0.871</td>
<td>0.812</td>
<td>0.812</td>
</tr>
<tr>
<td>201841_s_at</td>
<td>Heat shock 27kDa protein 1</td>
<td>HSPB1</td>
<td>0.536</td>
<td>0.707</td>
<td>0.536</td>
</tr>
<tr>
<td>221667_s_at</td>
<td>Heat shock 22kDa protein 8</td>
<td>HSPB8</td>
<td>0.354</td>
<td>0.574</td>
<td>0.536</td>
</tr>
<tr>
<td>202727_s_at*</td>
<td>Interferon gamma receptor 1</td>
<td>IFNGR1</td>
<td>0.536</td>
<td>0.660</td>
<td>0.707</td>
</tr>
<tr>
<td>206295_at</td>
<td>Interleukin 17</td>
<td>IL18</td>
<td>0.287</td>
<td>0.287</td>
<td>0.379</td>
</tr>
<tr>
<td>202859_x_at*</td>
<td>Interleukin 8</td>
<td>IL8</td>
<td>0.218</td>
<td>0.616</td>
<td>0.536</td>
</tr>
<tr>
<td>202351_at</td>
<td>Integrin, alpha V</td>
<td>ITGAV</td>
<td>0.500</td>
<td>0.758</td>
<td>0.616</td>
</tr>
<tr>
<td>1553530_a_at*</td>
<td>Integrin, beta 1</td>
<td>ITGB1</td>
<td>0.574</td>
<td>0.467</td>
<td>0.707</td>
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<td>226535_at</td>
<td>Integrin, beta 6</td>
<td>ITGB6</td>
<td>0.354</td>
<td>0.467</td>
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<td>1552611_a_at*</td>
<td>Janus kinase 1</td>
<td>JAK1</td>
<td>0.616</td>
<td>0.574</td>
<td>0.707</td>
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<tr>
<td>201596_x_at</td>
<td>Keratin 18</td>
<td>KRT18</td>
<td>0.758</td>
<td>0.812</td>
<td>0.536</td>
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<td>201650_at</td>
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<td>0.406</td>
<td>0.330</td>
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<td>KRT7</td>
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<td>0.616</td>
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<td>209008_x_at</td>
<td>Keratin 8</td>
<td>KRT8</td>
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<td>0.812</td>
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<tr>
<td>212119_at</td>
<td>Ras homolog gene family, member Q</td>
<td>RHOQ</td>
<td>0.812</td>
<td>0.812</td>
<td>0.758</td>
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<tr>
<td>224793_s_at</td>
<td>Transforming growth factor, beta receptor 1</td>
<td>TGFBR1</td>
<td>0.660</td>
<td>0.707</td>
<td>0.707</td>
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<td>208944_at</td>
<td>Transforming growth factor, beta receptor II (70/80kDa)</td>
<td>TGFBR2</td>
<td>1.00</td>
<td>0.707</td>
<td>0.707</td>
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<td>218856_at</td>
<td>Tumor necrosis factor receptor superfamily, member 21</td>
<td>TNFRSF21</td>
<td>0.379</td>
<td>0.467</td>
<td>0.536</td>
</tr>
<tr>
<td>210987_x_at*</td>
<td>Tropomyosin 1 (alpha)</td>
<td>TPM1</td>
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<td>0.660</td>
<td>0.500</td>
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<td>209118_s_at</td>
<td>Tubulin, alpha 3</td>
<td>TUBA3</td>
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<td>0.812</td>
<td>0.616</td>
</tr>
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<td>200931_s_at</td>
<td>Vinculin</td>
<td>VCL</td>
<td>0.574</td>
<td>0.660</td>
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</table>

Microarray results were filtered for probe sets down-regulated among Matrigel samples for Donor A, Donor B, and Donor C. Amount of fold change compared to control for each probe set is reported. An asterisk (*) denotes those genes which had multiple probe sets decreased; a single representative probe set with fold changes with respect to control is reported.
performed referenced the initially compromised cellular state, one where these expression markers were already elevated. Although Matrigel supplementation was not able to rescue these cultures from their apoptotic or necrotic phenotype, the addition of an ECM did enhance the expression of other cellular markers thereby promoting a more closer reflection to that of intact human liver (Figure 34D).

**B.4.6 Impact of Matrigel on protein expression.**

To further characterize mechanistic effects of Matrigel as a determinant of phenotypic and genotypic changes in primary human hepatocytes, we performed targeted protein level investigations in the culture system (Figure 37). In Donor C on Day 5 (114 h), Matrigel additions appeared to have little effect on the levels of protein expression of keratin 8 or 18, and little effect on the phosphorylation status of these proteins.
Table 25: Selected GO terms from decreased gene list among all human hepatocyte samples.

<table>
<thead>
<tr>
<th>Best GOs</th>
<th>GO Description</th>
<th>Count</th>
<th>Total</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0003924</td>
<td>GTPase activity</td>
<td>48</td>
<td>137</td>
<td>6.11E-07</td>
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<tr>
<td>GO:0005525</td>
<td>GTP binding</td>
<td>85</td>
<td>300</td>
<td>2.23E-06</td>
</tr>
<tr>
<td>GO:0015629</td>
<td>Actin cytoskeleton</td>
<td>55</td>
<td>183</td>
<td>5.39E-05</td>
</tr>
<tr>
<td>GO:0007010</td>
<td>Cytoskeleton organization and biogenesis</td>
<td>83</td>
<td>322</td>
<td>0.000294</td>
</tr>
<tr>
<td>GO:0003779</td>
<td>Actin binding</td>
<td>56</td>
<td>214</td>
<td>0.00467</td>
</tr>
<tr>
<td>GO:0008092</td>
<td>Cytoskeletal protein binding</td>
<td>72</td>
<td>296</td>
<td>0.00771</td>
</tr>
<tr>
<td>GO:000902</td>
<td>Cellular morphogenesis</td>
<td>58</td>
<td>228</td>
<td>0.00804</td>
</tr>
</tbody>
</table>

All decreased probe sets for all five donors were combined and batch analyzed by GOstat. Results show the confidence level associated with the GO term overrepresentation.

Figure 37: Protein expression profiling of primary human hepatocytes and HepG2 cells. Total cellular protein was isolated from primary human hepatocyte cultures and cultures of HepG2 cells, and equal quantities of protein from the respective samples were applied to 10% denaturing gels, separated by SDS-PAGE and then transferred to PVDF membranes and assessed by immunoblotting analyses, as described in Methods.
However, integrin α5 levels were dramatically decreased as a consequence of Matrigel addition; in fact, this protein was undetectable in hepatocytes cultured with Matrigel. Hepatocytes cultured for 7 days (162 h) -/+ Matrigel were also examined for protein expression levels. Integrin α5 was detectable in the Matrigel-treated sample but exhibited an apparent decrease in its respective expression level. Day 5 expression of connexin 32 in control samples was modest, but displayed a slight increase in expression in the presence of Matrigel. This increase was more clearly evident in the Day 7 samples where Matrigel additions produced a robust increase in the amount of connexin 32 protein. These results reflect trends observed in the microarray data, for example, decreased integrin levels and increased connexin levels were apparent in the mRNA profiling data as well.

Protein expression for Donor E also reflected the trends observed in both the microarray data and in the phenotypic assessments, as this individual exhibited only minimal response to ECM. No apparent changes in keratin 8 or 18 phosphorylation status, or in levels of the respective proteins, were noted. The level of integrin α5 appeared mildly decreased in response to Matrigel at Day 2 (42 h), but at no other time point. Connexin 32 expression levels were low at all time points, although detectable at Day 6 (138 h) and Day 8 (186 h), and were increased over controls.

Protein expression profiles were evaluated in two other individuals, Donor B and Donor D, but were more varied (data not shown). In both of these individuals, little change was apparent, -/+ Matrigel, in keratin 8 or 18 protein levels, although the trend was toward decreased expression levels in the presence of ECM. Integrin alpha 5 appeared reduced in the Matrigel-treated samples, whereas, connexin protein levels were largely increased in the presence of Matrigel.

Selected Day 5 protein assessments were also performed in HepG2 human hepatoma cells. Although keratin 8 and 18 protein expression was maintained in these cells, no phosphorylation of keratins 8 and 18 was detected. Similarly, there was a complete lack of connexin 32 protein expression in these cells.
Therefore, protein expression data for primary human hepatocytes cultured in the presence of a Matrigel overlay showed the same general trends as microarray data. Decreases in integrin expression and increases in connexin expression were noted in several samples. Conversely, HepG2 cells demonstrated a lack of connexin expression.

**B.5 Discussion**

Addition of an ECM/Matrigel overlay to cultured primary rat hepatocytes has been reported to enhance cellular differentiation status, xenobiotic responsiveness and hepatocyte morphology [7,9,13,15,16]. However, evidence supporting a facilitating role of ECM in primary human hepatocyte culture is mixed, with certain studies indicating a positive role for ECM in human hepatocyte cultures, [21], while others maintain that plating density and not ECM is the primary effector of hepatocyte differentiation status and phenotypic character [20]. In this study, we used gene expression profiling, protein analysis and morphological characterization to evaluate the biological impact of ECM additions to culture of primary human hepatocytes and demonstrate that ECM indeed enhances the differentiation status of the human hepatocyte and further, facilitates the gene responsiveness of these cells to chemical inducer challenge. Our results establish that primary human hepatocytes, when cultured with Matrigel and in a highly defined culture medium, allow cellular expression character that closely resembles of human liver tissues in vivo, and therefore serve as robust model systems for analysis of liver functional biology, toxicological assessment and biotransformation function.

With respect to cellular morphology, we show that Matrigel additions often facilitate formation of more pronounced cuboidal cell architecture, together with establishment of more highly organized networks of cells possessing clearly defined cell borders and bile canalicular features (Figure 32). These phenotypic changes are likely the result of changes in cytoskeletal structure and the ability of the cells to form focal adhesions with the extracellular matrix. Interindividual differences in responsiveness to ECM were noted, particularly in donors that yielded cells of compromised quality and viability. In these latter cases, it appeared that ECM additions were not sufficient to
‘rescue’ the otherwise poor quality of starting cellular material. Results of hierarchical clustering of gene expression highlight the variability among donors. However the shift in clustering from individual to treatment supports the conclusion that ECM additions positively impact gene expression character in hepatocytes cultured from donors yielding cells of relatively higher quality (Figure 35). Reflections of poorer cell quality include the presence of rafts of apoptotic and/or necrotic cells in both control and Matrigel-treated samples. Even hepatocyte samples evidencing poorer cellular morphology demonstrated increases in differentiation marker gene expression, in the presence of Matrigel, and the overall expression profiles of these Matrigel-treated cultures were reasonably well reflective of the intact human liver samples (Figures 34A and 34D).

Although the overall expression profiles between control and Matrigel samples within a donor were highly correlated, many specific genes demonstrated altered expression character in response to Matrigel (Figure 36). In particular, the cytochrome P450 genes represented a major group whose expression profile was up-regulated by ECM addition in hepatocytes. Epoxide hydrolase 1, another drug metabolizing enzyme, was up-regulated by the presence of ECM in hepatocyte cultures, as were two nuclear receptors, NR1D2 and NR1I3 [34]. NR1I3, the constitutive androstane receptor, plays an important role mediating PB induction responsiveness [9,35]. Many other genes were increased in the presence of Matrigel, most of them liver specific, including: CEBPalpha, FMO5, solute carrier family members, sulfotransferases and transferrin receptor 2 (Table 21). The enhanced expression of these gene batteries in the presence of Matrigel establishes the utility of this model system for toxicology and biotransformation investigations, demonstrates that the cellular microenvironment in an important determinant for cultured human hepatocytes and that ECM addition enables cellular responses that more accurately reflect those of an intact human liver.

ECM also contributed to the decreased expression of several genes, including certain keratins, integrins, signaling molecules and chaperones. Consistent with the idea that ECM additions impact the cellular microenvironment, we hypothesized that overrepresented groups demonstrating altered gene expression in the presence of Matrigel would include genes contributing to the cellular cytoskeleton. Indeed, several gene
references in this category exhibited decreased expression levels consequent to Matrigel treatment (Table 25). We suggest that Matrigel-induced reductions in cytoskeletal-related gene expression likely manifests from the enhanced set of interactions enabled through focal adhesion contacts with cell membrane proteins that are now stimulated by the ECM microenvironment provided. In support of this contention, morphogenesis-related cell surface markers and membrane-associated transcripts were downregulated in response to Matrigel, as were a cadre of stress-related genes. These latter results are consistent with those obtained in primary rat hepatocyte studies, that demonstrated the ability of an ECM microenvironment to effectively ‘quiet’ the stress response [9,36-38]. Our microarray data indicate that several stress-related molecules, such as the interleukins, chemokines and a number of stress-related protein kinases exhibit reduced transcript expression in primary human hepatocytes cultured in the presence of Matrigel (Table 23), results supporting the concept that the more in vivo-like microenvironment enables a less perturbed cellular state.

Overall, protein expression profiles were reflective of the patterns seen within the microarray data sets. For example, connexin expression levels were increased in the presence of Matrigel while integrin α5 protein levels were consistently reduced in Matrigel-treated samples (Figure 37). The mRNA expression levels of several integrin pathway associated genes were decreased in three of five human hepatocyte samples cultured in the presence of Matrigel. Integrin signaling genes effected included integrins, caveolin, various forms of Ras, Rho, Cdc42, Rac, filamen, actinin, actin, vinculin and Arp2/3 (data not shown). The number of affected components of the integrin signaling pathway indicates a selective down-regulation of this module in response to ECM. We propose that this pathway is specifically affected by the presence of Matrigel since the integrins constitute the main interacting module with the extracellular matrix. The down-regulation effects of ECM on integrin levels were coupled to reductions in expression levels of their associated downstream signaling cascades. Since integrin signaling pathways are directly linked with intracellular stress signaling, via activation of molecules such as Rho, Rac and Cdc42, [22-24,29-31,39,40], the down-regulation of
integrin signaling is likely the major force behind the suppression of cellular stress cascades evident in the Matrigel-cultured hepatocytes.

Levels of connexin mRNA as well as protein were increased in hepatocytes cultured in the presence of Matrigel. Connexins are gap junction proteins that are responsible for cell-cell communication and play important roles in cell growth and differentiation [41,42]. Previous reports demonstrated that only hepatocytes cultured in the presence of Matrigel treatment demonstrated connexin 32 expression [7]. In addition, an inverse relationship has been established between cell proliferation and gap junction communication [43]. Hepatocytes are normally non-proliferative \textit{in vivo}, and remain so under our primary culture conditions [44,45]. We suggest that hepatocytes cultured in the presence of an ECM likely exhibit increased capacity for cell-cell communication, an enhanced state of differentiation and improved responsiveness to complex external stimuli, such as that manifested in the PB induction response.

The results of this investigation demonstrate a positive role for Matrigel in primary cultures of human hepatocytes, in particular in donor samples of higher initial cell quality. In the hepatocyte samples, Matrigel induced both subtle and dramatic changes in mRNA expression in hepatocyte cultures, changes of clear biological impact. ECM additions tended to improve hepatocyte morphology as reflected by enhanced cuboidal shape, more highly defined cell borders and formation of highly organized cellular networks observed in the presence of matrix. Matrigel additions also resulted in improved hepatocyte differentiation status, as evidenced by enhanced expression of differentiation markers, decreased expression of de-differentiation markers and an improved capacity of the cellular response to PB. The mRNA expression of several cytochrome P450s, epoxide hydrolase 1, FMO5, SLCs, CAR and PB responsiveness were enhanced in hepatocytes cultured in the presence of ECM, supporting the utility of this culture model for both toxicology and drug metabolism research.

Despite some differences in gene expression character, primary human hepatocytes cultured in defined media conditions and in the presence of Matrigel exhibited strikingly similar profiles to those of the intact human liver. In contrast, human hepatocellular carcinoma cells, often used in liver-based \textit{in vitro} model studies, exhibited
substantially disparate expression profiles when compared intact human liver (results to be published elsewhere). Mechanistically, we propose that Matrigel facilitates these biological alterations in cultured primary hepatocytes through modification of cell surface marker protein expression, changes that prime the cell for improved \textit{in vivo}-like behaviors. Gap junction mRNA and protein expression are augmented in Matrigel-treated samples, likely resulting in improved ability of the cultured cells to engage in appropriate cell-cell communication, facilitated as well by the formation of complex cellular networks. Although expression levels of several cell surface markers were down-regulated in the presence of matrix, including the integrins and keratins, as were several stress-related gene pathways, these profiles were more highly reflective of those seen in liver tissues themselves. We postulate that provision of a more native configuration of ECM to the cultured cells results in a microenvironment where the cells no longer need to overcompensate for the lack of proper matrix interactions, and thereby exhibit reduced stress responses and reduced compensatory need to synthesize cell surface components.

B.6 References


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EDUCATIONAL BACKGROUND

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  Award: First place, Graduate Student Abstract Competition, Molecular Biology Specialty Section, Society of Toxicology, March 2008

Society of Toxicology, Charlotte, NC, Poster March 2007
  Title: “Gene expression profiling and differentiation assessment in primary hepatocyte cultures, established hepatoma cell lines, and liver tissues”

AWARDS

Mr. and Mrs. Bosworth E. Grier Scholarship, College of Agricultural Sciences, Pennsylvania State University, September 2007

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