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Assessing the metabolic competence of sandwich-cultured mouse primary hepatocytes.

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The metabolic competence of primary mouse hepatocytes

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Abbreviations: Cyp450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethylsulfoxide; RMA, Robust Multi-array Average; SAM, significance analysis of microarrays; PCA, principal component analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia for Genes and Genomes.

Abstract

Primary human and rat hepatocyte cultures are well established *in vitro* systems used in toxicological studies. However, while transgenic mouse models provide an opportunity for studying mechanisms of toxicity, mouse primary hepatocyte cultures are less well described. The potential usefulness of a mouse hepatocyte-based *in vitro* model was assessed in this study by investigating time-dependent competence for xenobiotic metabolism and gene expression profiles. Primary mouse hepatocytes, isolated using 2-step collagenase perfusion, were cultured in a collagen sandwich configuration. Gene expression profiles and the activities of various Cyp450 enzymes were determined after 0 h, 42 h and 90 h in culture. Principal component analysis of gene expression profiles shows that replicates per time point are quite similar. Gene expression levels of most phase I biotransformation enzymes decrease to ca 69 % and 57 % of the original levels at 42 h and 90 h, respectively, while enzyme activities for most of the studied Cyp450s decrease to 59 % and 34 %. The decrease for phase II gene expression is only to 96 % and 92 % of the original levels at 42 h and 90 h, respectively. Pathway analysis reveals initial effects at the level of proteins, external signaling pathways and energy production. Later effects are observed for transcription, translation, membranes and cell cycle related gene sets. These results indicate that the sandwich-cultured primary mouse hepatocyte system is robust and appears to maintain its metabolic competence better as compared to rat hepatocytes.

Introduction

Toxicological studies designed to assess safety and possible toxicity of compounds in human populations predominantly rely on the use of animal systems. As animal experiments are expensive, time-consuming and connected with ethical drawbacks, numerous attempts have been made to find a reliable way of predicting *in vivo* toxicity in humans by means of *in vitro* models in recent years.

Traditionally, since the liver plays a major role in the metabolism of many compounds and also represents an important target organ in systemic toxicity, hepatic models are frequently used as an *in vitro* alternative in pharmacological, toxicological and metabolic studies (Blaauboer et al., 1994, Davila et al., 1998). Hepatic cell lines, primary hepatocyte cultures and precision-cut liver slices from various species are well established *in vitro* systems for these studies (Blaauboer et al., 1994, Davila et al., 1998, Schaeffner et al., 2005). As precision-cut liver slices have a very short live span and immortalized cell lines have lost many liver specific functions, primary cultures of hepatocytes are preferentially used for *in vitro* studies on liver toxicity (Schaeffner et al., 2005, Richert et al., 2002, Berry et al., 1997).

Primary mammalian hepatocytes largely retain their liver-specific functions when freshly derived from the animal. Long-term cultures of functional hepatocytes however are difficult to establish. To increase the longevity and maintain differentiated functions of hepatocytes in primary cultures, cells are cultured in a sandwich configuration of collagen-collagen and in serum-free culture medium (Tuschl and Mueller, 2006). Hepatocytes cultured in a sandwich configuration reorganize to form an architecture similar to that found in the liver

and are able to form functional bile canalicular networks and gap junctions. In such a sandwich configuration, hepatocytes can be cultured for a longer time period compared to cultures on single layers of collagen (LeCluyse et al., 2000a, LeCluyse et al., 2000b, Boess et al., 2003, Kienhuis et al., 2007).

The use of primary human hepatocytes is preferential in order to predict *in vivo* toxicity in humans but is hampered by the limited availability of donor material and the large variability between the donors (Schaeffner et al., 2005). Therefore, primary hepatocytes isolated from other mammals are used as an alternative to human tissue. However, the rapid decline in liver specific functions, in particular cytochrome P450 (Cyp450) enzyme activity, in rat liver *in vitro* systems limits the use of these in studies for testing chemicals for which metabolism depends on the Cyp450 enzyme system (Boess et al., 2003, Hoen et al., 2000). Human hepatocytes also tend to show a decrease in most Cyp450 enzymes, but some levels of Cyp450 gene expressions can be restored after a few days (Morel et al., 1990, LeCluyse, 2001). A mouse *in vitro* hepatocyte system might be an alternative to the rat and human systems, especially if the metabolic competence is preserved. In addition, since the complete sequence of the mouse genome is known (Waterston et al., 2002) and transgenic mouse models are widely available, primary mouse hepatocytes are usable for the purpose of performing mechanistic investigations of liver toxicity. However, information about stability of liver specific functions in mice is scarce.

It was the aim of the current study to investigate the robustness of a mouse hepatocyte-based *in vitro* model, especially with respect to the biotransformation functions (evaluated by assessing gene expression and enzyme activities) in order to get more insight in its potential usefulness in toxicology.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer, insulin and Trizol were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone (50-23-7), collagenase type IV, dimethylsulfoxide (DMSO; 67-68-5) and Trypan blue (72-57-1) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA).

The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). The 5x MegaScript T7 Kit was obtained from Ambion (Austin, TX). The GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit and Reagents, the Hybridization, Wash and Stain Kit and the Mouse Genome 430 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA). The CYP450 AL4 cocktail was delivered by Advanced In Vitro Cell Technologies (Valencia, Spain).

Animals

Permission for animal studies was obtained from the Animal Ethical Committee. Adult male C57/B6 mice (Charles River), weighing 20-25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. This mouse strain was chosen because it is frequently used in toxicological and pharmacological investigations, and it is a common background for transgenic mouse strains. The animals were housed in macrolon cages with sawdust

bedding at 22°C and 50-60% humidity. The light cycle was 12 h light/12 h dark. Feed and tap water were available *ad libitum*.

Isolation of hepatocytes.

Hepatocytes were isolated from adult male C57/B6 mice by a two-step collagenase perfusion method according to Seglen and Casciano (Seglen, 1976, Casciano, 2000), with modifications. The liver was perfused after cannulation of the abdominal inferior vena cava. The hepatic portal vein was cut through and the thoracic inferior vena cava was occluded with forceps. The organ was washed with Hanks' calcium- and magnesium-free buffer for 3 min. After the liver had been freed of blood the calcium-free buffer was replaced by a collagenase buffer (0.5 mg/ml) for 7-10 min. A perfusion rate of 7 ml/min and a temperature around 39°C was maintained for both perfusates during the entire procedure. After the perfusion had been terminated, the liver was rapidly excised from the body cavity and transferred to a sterile Petri dish. The gall bladder and remnants of the diaphragm were removed and cells were released by disrupting the liver capsule mechanically and by shaking the cells into attachment medium. The cells were separated from undigested tissue with a sterile 50-µm mesh nylon filter. After washing by low-speed centrifugation at 50 g for 3 min at 4°C several times, cell viability and yield were determined by trypan blue exclusion.

Cell culture.

Cells from three independent biological replicates with viability >85% were used and cultured on collagen gel precoated 6-well plates at a density of 6.5×10^5 cells/ml (Koebe et

al., 1994). Cells were allowed to attach for 2-4 h at 37°C in a humidified chamber with 95%/5% air/CO_2 in DMEM supplemented with 10% FCS, insulin (0.5 U/ml), glucagon (7 ng/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 µm/ml streptomycin). After attachment, medium containing unattached cells and debris was removed by washing and the cultures were overlaid with a second collagen layer to form a collagen-collagen sandwich culture. Primary cultures of mouse hepatocytes were cultured at 37°C in a humidified chamber with 95%/5% air/CO_2 in serum-free culture medium supplemented with insulin 0.5 U/ml), glucagon (7 ng/ml), hydrocortisone (7.5 µg/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 µm/ml streptomycin). Cells from three independent biological experiments (each from a different animal) were harvested at 0, 42 and 90 h after isolation for gene expression analysis.

RNA isolation.

Total RNA was isolated from cultured mouse hepatocytes using Trizol reagent with the RNeasy kit according to the manufacturer's protocol. Total cellular levels were measured on a spectrophotometer and the quality of each RNA preparation was determined with a bio-analyzer (Agilent Technologies, The Netherlands). Extracted RNA was stored at −80°C.

Target preparation and microarray hybridization.

cRNA targets were prepared according to the Affymetrix protocol. The targets were hybridized according to the manufacturer's recommended procedures on high-density oligonucleotide genechips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays). The

genechips were washed and stained using a fluidics station by Affymetrix and scanned in an Affymetrix GeneArray scanner.

A total of nine targets (3 time points from 3 animals) were prepared, and each preparation was analyzed using one GeneChip array. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values all were within acceptable limits. Hybridization controls BioB, BioC, BioD, and CreX, were called present on all chips and yielded the expected increases in intensities.

Data analysis.

Nine datasets were obtained from this experiment. The raw data were imported into ArrayTrack (Tong et al., 2003) and converted and normalized by Robust Multi-array Average (RMA, integrated into ArrayTrack) (Irizarry et al., 2003). Spots of poor quality and control spots were omitted. Subsequently, the remaining probe sets (25,084) were logarithm (base 2) transformed. Processed and normalized gene expression values were evaluated for identifying differentially expressed probe sets with the multiclass significance analysis of microarrays (SAM, integrated into ArrayTrack; p-value < 0.05) (Shi et al., 2006). Multiclass SAM resulted in 6560 remaining differentially expressed probe sets and these were used for principal component analysis (PCA).

Pathway analysis.

Gene expressions at 42 vs 0 h, 90 vs 0 h and 90 vs 42 h were uploaded in T-profiler (http://www.t-profiler.org; Boorsma et al., 2005) to identify transcriptional regulation of biochemical pathways and biological processes which included genes sets from Gene

Ontology (GO) terms, curated gene sets, Motifs and Kyoto Encyclopedia for Genes and Genomes (KEGG) pathways. T-profiler uses the T test to score the difference between the mean expression level of predefined groups of genes and that of all other genes without any statistical pre-selection of modulated genes (Boorsma et al., 2005). Significance was determined by generating an E value, a Bonferroni corrected P value. Pathways and processes were determined significant if E values were below 0.05, and were thereupon used for hierarchical clustering and gene grouping in GenePattern (http://www.broad.mit.edu/cancer/software/genepattern/).

In order to increase specificity of the transcriptomics changes, the 6560 differentially expressed probe sets, determined by Multiclass SAM, were uploaded into MetaCore (GeneGo, San Diego, CA) for identifying the involvement of differentially expressed genes in specific cellular pathways by overrepresentation analyses compared to the total amount of genes involved in the particular pathways.

Analysis of CYP450s enzyme activities

Cyp450 activity assays at 0 h, 42 h and 90 h were conducted by the direct incubation of three independent sandwich-cultured mouse hepatocyte preparations with a cocktail of Cyp450 substrates (Lahoz et al., 2008). Substrates mixture stock was prepared in DMSO, and conveniently diluted in incubation media to obtain the optimal assay concentrations (Table 1). The final concentration of DMSO during incubation was 0.5% (v/v). After 2h of incubation enzymatic reactions were stopped by aspirating the incubation medium. Samples were subsequently centrifuged at 2500 rpm for 5 min. The supernatants were transferred to clean tubes and frozen at -80ºC until analysis. Sample analysis and cocktail preparation was

carried out in the Unidad Mixta Hospital La Fe-Advancell, Valencia, Spain. Samples were subjected to hydrolysis before analysis by incubation with β-glucuronidase and arylsulfatase for 2h at 37ºC. One volume of ice-cold acetonitrile was added and centrifuged samples were transferred to clean vials for further analysis. Metabolites formed and released into the culture medium were quantified by high performance liquid chromatography tandem mass spectrometry (Lahoz et al., 2007). Enzymatic activities were expressed as pmol of metabolite formed/incubation time/number of cells. For the statistical analysis the T-Test was used with a significance level of 5%.

Results

Principal component analysis of all genes

A PCA on the 6560 differentially expressed probe sets, identified with the multiclass SAM analysis (p-value < 0.05), was used to visualize inter-individual and time-dependent differences in gene expression profiles of primary mouse hepatocytes cultured in sandwich configuration (Figure 1). PCA shows a clear discrimination between the three time groups and only limited differences between replicates of the 42 h and 90 h groups are observed, while more variance in gene expression is seen between the 0 h replicates.

Pathway analysis

T-profiler was used for identifying gene groups that were differentially expressed. The gene groups significantly altered in hepatocytes from at least two experiments and modified in the same direction in liver cells from all experiments were selected for each time period. In total, 384 gene sets were identified as being modulated. Hierarchical clustering was used to generate more comprehensible groups of these genes (Figure 2, and see supplemental data). Most gene sets were either only downregulated or only upregulated. A small amount of these gene sets appeared to be upregulated during the first 42 h, and downregulated in the period from 42 to 90 h.

An additional analysis of affected cellular pathways was conducted using MetaCore, which showed similar results compared to the results from T-profiler (data not shown). Most prominent pathways significantly altered in MetaCore were involved in several catabolic,

metabolic and biosynthetic processes, immune responses and cellular, nuclear and macromolecule related processes.

Biotransformation enzymes

Gene expression and activity of phase I and phase II drug-metabolizing enzymes were quantified to assess the metabolic capacity of the primary mouse hepatocyte system (see Table 2 and Table 3). For the majority of the Cyp450 genes, the gene expression level decreased during culturing. For a small group of Cyp450s, however, the expression levels remained relatively constant while for an even smaller group, gene expression levels increased in all time periods.

For the phase II genes, large variations were seen in their expression levels between the different time points, even within groups of phase II genes belonging to the same family. Most phase II enzyme groups were down-regulated or hardly changed over time. Only genes involved in glutathione metabolism and N-acetyltransferases were generally up-regulated.

Cyp450 enzyme activities of 8 selected enzymes were measured in each sample. These activities are presented as Log2 mean ± SD values in Figure 3. Enzyme activities for Cyp2a4 and Cyp2a1 were not detected. For the majority of the studied Cyp450 enzymes, a significant decrease in enzyme activity was measured in both time periods. Only one group of enzymes (Cyp2B9-10/13/19/23) showed an increase in activity and expression levels. After 90 h in culture, all Cyp450 enzymes remained active in the primary hepatocytes.

These Cyp450 enzyme activities and their gene expression are presented in Figure 3. In all cases gene expression and enzyme activity changed in the same directions, and mostly in the same order.

Discussion

As human and rat sandwich-cultured hepatocytes have their disadvantages because of the limited availability of donor material and/or a rapid decline in Cyp450 enzyme activities, primary mouse hepatocytes may be an option to overcome this shortcoming (Schaeffner et al., 2005, Hoen et al., 2000). Moreover, the growing availability of transgenic models can be a reason to use mouse primary hepatocytes. As information on the stability of liver specific functions in mouse hepatocytes is scarce, the present study investigated the stability of a mouse hepatocyte-based *in vitro* model, especially with respect to the biotransformation functions (gene expression and enzyme activities), in order to get more insight in its potential usefulness in toxicology.

PCA was used to elucidate changes in gene expression profiles between donor animals and between cultivation times. This revealed that profiles are very different between the three time points. These time-dependent changes can be attributed to the adaptation of the primary hepatocytes to their *in vitro* environment. Noteworthy, the hepatocytes at time point 0 h show larger inter-animal differences compared to those at later time points. This observation might be caused by initial stress induced by the enzymatic separation and isolation of hepatocytes (Pillar and Seitz, 1999). This inter-animal variability is less clear in the later time points, indicating that the stress responses are fading.

Pathway analysis, used to identify biochemical pathways and biological processes that were differentially expressed, showed again that differences exist between the two cultivation periods. Some gene sets were affected in both periods, whereas others in only the first 42h or only in the 48h thereafter. During only the first 42 h in culture, gene sets in

mitochondrial processes, hormonal processes and chemotaxis were downregulated, whereas those in protein related processes are upregulated during only this period. Pathways mostly affected after the first 42h are downregulation of membrane related processes, and upregulation of transcription and replication and cell cycle related processes. Gene sets that are continuously downregulated are on humoral and cell mediated immunity, CYP450 related responses and liver-specific genes. A continuous upregulation is observed in nuclear and nucleic acid related metabolism, cellular organization, and in parts of cell cycle related processes. The overall picture based on these pathway analyses, is that liver-specific functions are continuously decreasing and that gross cellular organizations are continuously upregulated. Initial effects are more at the level of protein synthesis, external signaling pathways and energy production, while later on transcription, translation, membranes and cell cycle related gene sets are affected.

Microscopic observations showed that in this 42 h period, cells were changing their periphery, cellular components and structures, and forming aggregates (not shown). These morphological changes at microscopic level confirm previously described studies on human hepatocytes and on the histology of rat and mouse hepatocytes. The morphological changes include attachment to the collagen layer, the remodeling of sandwich-cultured hepatocytes to a polygonal shape, the appearance of a clear, less granular, cytoplasm and the aggregation of the cells including the formation of gap junctions for intercellular communication (Tuschl and Mueller, 2006, Klaunig et al., 1981a, Klaunig et al., 1981b, Lu et al., 2007). Membrane and mitochondrial changes have also been demonstrated before in freshly isolated hepatocytes and hepatocytes cultured for 3 days (Berry and Simpson, 1962, Ikeda et al., 1992). Unfortunately, all these morphological changes were poorly represented

by changes at the level of pathways as analyzed by T-profiler or MetaCore. These morphological changes were perhaps too specific and the annotation of genes with respect to that is too limited. In human and rat hepatocytes cultured in a sandwich configuration, others have found genes and pathways involved in cell structure to be highly expressed after 24 h in culture (Waring et al., 2003, Schuetz et al., 1988, Ben-Ze'ev et al., 1988; Liu et al., 1991).

To investigate the metabolic competence of sandwich-cultured mouse hepatocytes in more detail, gene expression profiles from phase I and phase II biotransformation enzymes were evaluated during the cultivation period. In general the expression values of all phase I biotransformation enzymes are decreasing over the whole cultivation period. During the first 42 h these phase I enzyme gene expression values are decreasing by 31% (ca 69 % remaining) from the original values at 0 h. After 90 h in culture the hepatocytes exhibit clearly lower levels (ca 57%) of Cyp450s compared to the original values at 0 h.

It was described before that many cultures of mammalian hepatocytes exhibit low levels of Cyp450 but still possess the ability to respond to Cyp450 inducers (Cervenkova et al., 2001). The expression of Cyp1a1 and Cyp1a2 during cultivation has been studied in detail by Northern blotting (Tamaki et al., 2005). In primary mouse hepatocytes cultured in dishes without collagen, Cyp1a1 expression increased during cultivation. Cyp1a2 was constitutively expressed in these cells, but insulin suppressed this expression slightly. Our results show a steady level in Cyp1a1 gene expression and a down-regulation in Cyp1a2 gene expression. In human hepatocytes, most Cyp450s also showed a decrease in expression levels with the exception of a few Cyp450s which had elevated levels of expression (Richert et al., 2006). Cyp1A2 appeared to be more stable compared to the

mouse model during cultivation, while Cyp2E1 and Cyp3A4, which is the homologue for Cyp3a11 in mice (Nelson et al., 2004), were shown to be decreasing in time, comparable to our results (George et al., 1997). Most Cyp450s in conventional sandwich-cultured rat hepatocytes appeared to be decreasing in a time-dependent manner (Kienhuis et al., 2007). Even within the short time period of 24 h after isolation, the expression levels of many Cyp450s decreased tremendously in rat hepatocytes (Boess et al., 2003). While in our study gene expression levels showed a slower decrease over time indicating that mouse hepatocytes remain more stable with respect to Cyp450 gene expression as compared to rat hepatocytes but not to human hepatocytes.

As several rat studies suggest that phase II enzyme activities are better preserved in culture than phase I enzyme activities, we decided to only measure enzyme activities of most important phase I enzymes involved in xenobiotic biotransformation to strengthen our gene expression results (Rogiers and Vercruysse, 1993, Kern et al., 1997). These results show a general decrease in Cyp450 enzyme activity, with only one exception. Activity levels were decreasing the first 42 h to 59% from the original activities. After 90 h, activity levels were decreased to 34 % in comparison to 0 h. Compared to our results, Cyp450 enzyme activities in rat hepatocytes are decreasing very rapidly. Already after 6 h, less than 50% of the initial Cyp450 enzyme activity was left in rat hepatocytes (Lopez-Garcia, 1998; Lopez-Garcia and Sanz-Gonzalez, 2000). These rat hepatocytes, however, were cultured on a collagen monolayer, which may affect the stability of the cells.

Phase II biotransformation enzymes demonstrate a less clear pattern. Enzymes were either up- or down-regulated or remain unchanged. Even within a group of enzymes, differences in gene expression can be seen. In general, the expression levels are decreasing to 96 % and

92 % as compared to levels at 0 h after 42 h and 90 h, respectively. In rat hepatocytes, however, phase II enzymes in general showed a decrease in gene expression level after 72 h compared to gene expression in liver (Kienhuis et al., 2007). For rat hepatocytes, it is known that the phase II enzymes are better preserved in culture than phase I enzymes (Rogiers and Vercruysse, 1993, Kern et al., 1997). Our data confirm these findings, thus showing that mouse hepatocytes are also stable in expression of genes encoding phase II enzymes.

An overall comparison of the gene expression levels from phase I and phase II enzymes from rat and mouse hepatocytes demonstrated that mouse hepatocytes exhibit a more stable gene expression pattern than primary rat hepatocytes.

Conclusion

In summary, our findings show that the sandwich-cultured primary mouse hepatocyte *in vitro* model has little or no inter-animal variations, demonstrating its reproducibility. The system is still metabolic competent even after 90 h culturing, despite that phase I enzyme gene expression and activity are generally decreasing. In rat hepatocytes, this decrease in phase I and II gene expression appears to be faster. Human hepatocytes, on the other hand, also show a decrease in these phase I enzymes, but are able to restore the gene expression levels of some Cyp450s. Taken together, our results indicate that the primary mouse hepatocyte system is relatively stable and might maintain its metabolic competence longer than rat hepatocytes, and roughly similar to that of human hepatocytes. Based on our gene

expression data, we recommend applying a 42 h recovery period before using the mouse hepatocytes.

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Footnotes

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Legends for figures

Figure 1 Principal component analysis using the data of all differentially expressed genes from primary mouse hepatocytes at 0, 42 and 90 h cultivation.

Figure 2 Hierarchical clustering of gene sets significantly modified in primary mouse hepatocytes in the time periods from 0 to 42 h, from 42 to 90 h and from 0 to 90 h. Clustering is based on the t-values, as provided by T-profiler. Yellow and blue mean up- and downregulation, respectively.

Figure 3 Cyp450 enzyme activities and gene expression in primary mouse hepatocytes after 0, 42 and 90 h in culture. Values are represented as percentages from activities and gene expression 0 h and as means ± SD of 3 animals for each time point.

*Significant (P value < 0.05) compared to 0 h.

[#]Significant (P value < 0.05) compared to 0 and 42 h.

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Tables

Table 1 Substrates in the CYP450 AL cocktail for measurement of CYP450 activities.

CYP	Substrate	Concentration (μM)	Metabolite
1A2	Phenacetin	10	Acetaminophen
2A4	Coumarin	5	7-HO-Coumarin
2B9/10/13/19/23	Bupropion	10	HO-Bupropion
2C37	Mephenytoin	50	4'-HO-Mephenytoin
2C39-40/44/50/54-55/65-70	Diclofenac	10	4'-HO-Diclofenac
2D9-13/22/26/34/40	Bufuralol	10	Hydroxybufuralol
2E1	Chlorzoxazone	50	6-HO-Chlorzoxazone
3A11	Midazolam	5	1'-HO-Midazolam

Table 2 Expression of Cyp450 genes in primary mouse hepatocytes at 42 and 90 h as percentages of the expression at 0 h.

GENENAME	42h	90h
Cyp17a1*	2659	107
Cyp1a1	119	117
Cyp1a2*	29	7
Cyp1b1	109	104
Cyp20a1	155	213
Cyp21a1	114	108
Cyp26a1*	24	10
Cyp27a1*	66	56
Cyp2a12*	65	128
Cyp2a4-5	42	71
Cyp2b10	1546	1460
Cyp2b13	102	95
Cyp2b19	90	86
Cyp2b9	126	110
Cyp2c29*	32	13
Cyp2c37	24	1
Cyp2c37-50-54*	19	1
Cyp2c38	54	65
Cyp2c44*	27	2
Cyp2c55*	50	16
Cyp2c70*	75	39
Cyp2d10*	88	59
Cyp2d13*	40	20
Cyp2d22*	74	33
Cyp2d26*	69	56
Cyp2d9*	59	22
Cyp2e1*	56	3
Cyp2f2*	22	4
Cyp2g1*	40	37
Cyp2j5*	10	6
Cyp2j6*	99	83

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Cyp2r1	103	76
Cyp2s1*	266	83
Cyp2u1	76	80
Cyp39a1*	197	421
Cyp3a11*	77	76
Cyp3a13*	203	175
Cyp3a25*	21	18
Cyp3a41*	66	65
Cyp3a44*	82	77
Cyp46a1*	83	67
Cyp4a10*	32	38
Cyp4a12*	16	1
Cyp4a14*	19	7
Cyp4b1	202	100
Cyp4f13	70	58
Cyp4f14*	19	5
Cyp4f15*	26	9
Cyp4f16*	381	341
Cyp4v3*	41	22
Cyp51	115	108
Cyp7a1	76	42
Cyp7b1*	17	12
Cyp8b1*	109	7
	69	57

** Significant ($p<0.05$) in multiclass SAM*

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Table 3 Expression of phase II genes in primary mouse hepatocytes at 42 and 90 h as percentages of the expression at 0 h.

GENENAME	42h	90h
Aldo-keto reductases		
Akr1a4	92	101
Akr1b3*	208	321
Akr1b7*	267	285
Akr1b8	131	127
Akr1c12	96	85
Akr1c13	103	107
Akr1c14*	29	47
Akr1c19	125	84
Akr1c20*	27	22
Akr1c21*	86	71
Akr1c6*	4	0
Akr1d1*	20	13
Akr1e1	112	103
Akr7a5	93	83
Sulfotransferases		
Chst10	81	72
Chst11	92	81
Chst4	83	80
Chst9	98	95
D4st1*	151	239
Gal3st1	175	128
Gal3st4	96	81
Hs1bp3*	120	205
Hs2st1*	167	229
Hs3st1	93	88
Hs3st2	88	75
Hs3st3b1*	88	76
Hs3st6	83	73
Hs6st1*	86	59
Hs6st2	94	89

Ndst1	99	100
Ndst2	165	113
Ndst3	94	92
Ndst4	93	84
Sult1a1	110	119
Sult1b1*	67	18
Sult1c2	76	50
Sult1d1*	457	86
Sult1e1*	1231	397
Sult5a1*	67	60
Epoxide hydrolases		
Ephx1	140	133
Ephx2*	47	35
Flavin-Containing Monooxygenase		
Fmo1*	31	19
Fmo2	102	96
Fmo3	99	84
Fmo4	133	108
Fmo5	369	96
Glutathione reductases		
Gsr	146	129
Gss*	153	329
Glutathione peroxidases		
Gpx1*	70	47
Gpx4*	139	161
Gpx6*	58	44
Gpx7*	113	222
Glutathione transferases		
Gsta1-2*	526	409
Gsta2*	194	142
Gsta3*	39	42
Gsta4*	82	47
Gstm1	113	127
Gstm2*	364	383
Gstm3*	378	593

Gstm4	127	125
Gstm5*	197	205
Gstm6	110	85
Gstm7*	64	51
Gsto1	117	97
Gsto2	115	115
Gstp1	98	107
Gstt1*	49	38
Gstt2	78	105
Gstt3	69	55
Gstz1*	94	92
Mgst1*	63	60
Mgst2*	94	76
Mgst3	141	150
N-acetyltransferases		
Gcnt1	110	107
Gcnt2	76	63
Nat10*	153	239
Nat11	114	174
Nat12	145	196
Nat2*	179	122
Nat5	240	332
Nat6	82	114
Nat9	134	135
Glucuronosyl transferases		
Ugt1a1-2-5-6a-6b-7c-9-10*	59	38
Ugt2a3*	30	4
Ugt2b1*	13	1
Ugt2b34	127	112
Ugt2b35	99	147
Ugt2b36*	42	59
Ugt2b37*	40	28
Ugt2b38*	11	5

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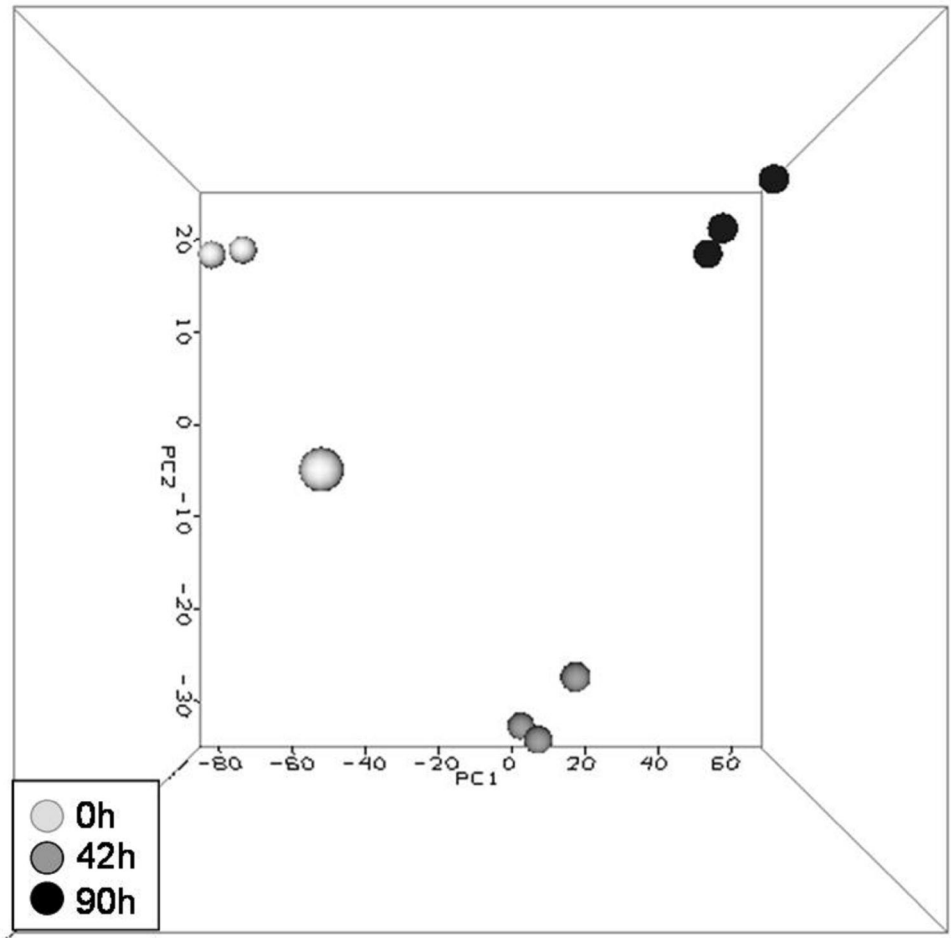
Ugt2b5*	35	39
Ugt3a1*	20	6
Ugt3a2*	26	4
	96	92

** Significant ($p<0.05$) in multiclass SAM*

Figure 1



	PC1	PC2	PC3
% of variance	73.07	15.15	5.17

Figure 2

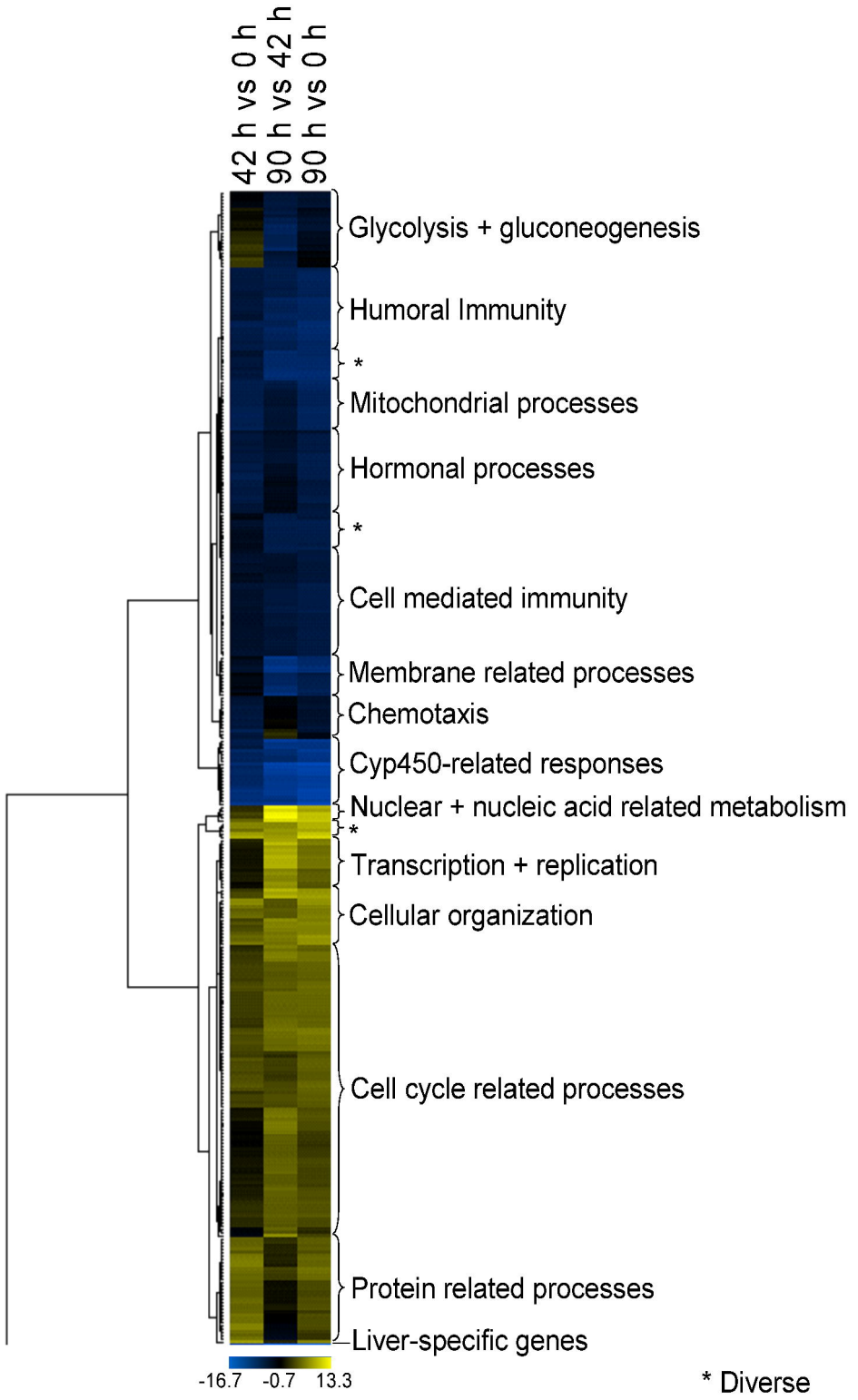
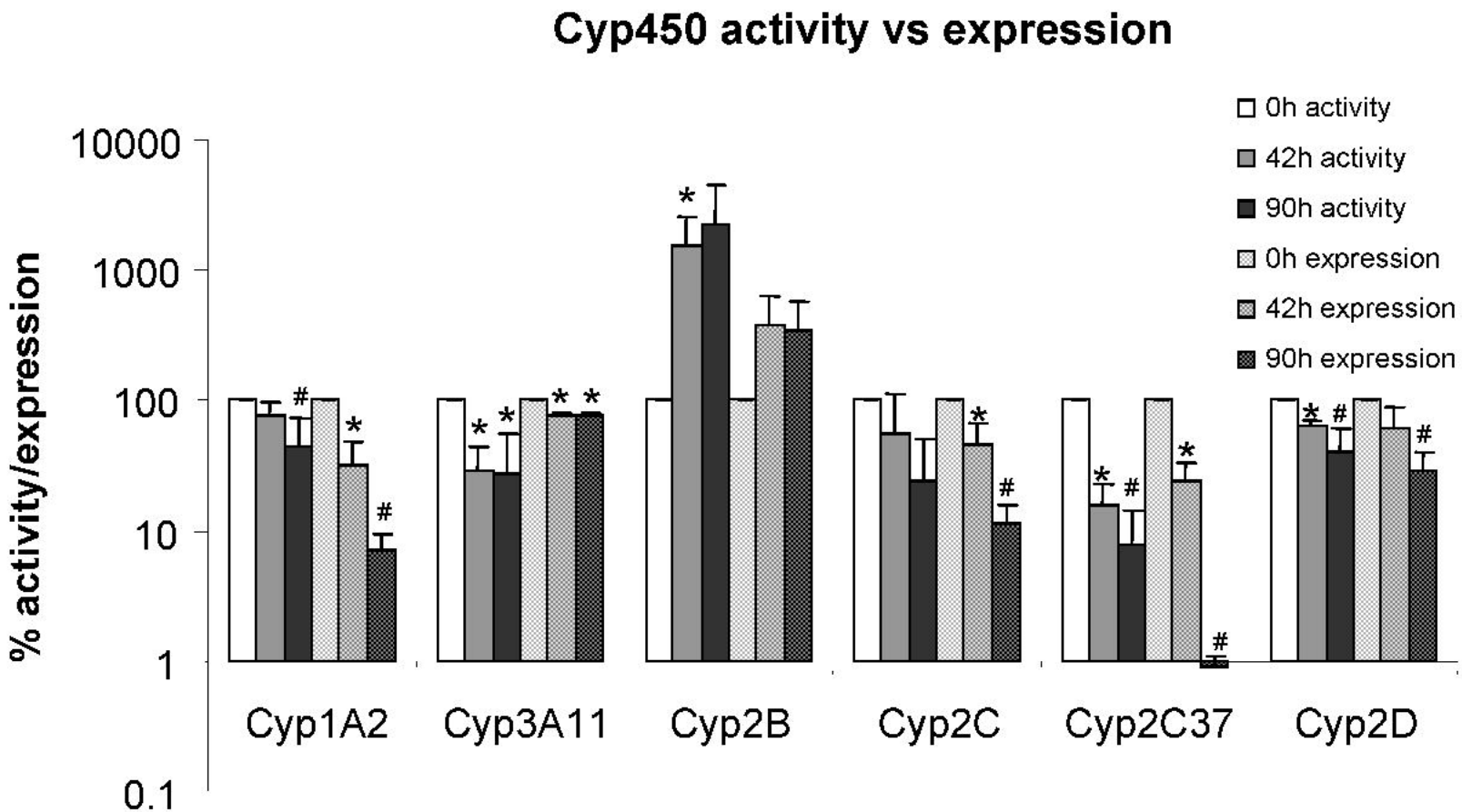

42 h vs 0 h
90 h vs 42 h
90 h vs 0 h
Glycolysis + gluconeogenesis
Humoral Immunity
*
Mitochondrial processes
Hormonal processes
*
Cell mediated immunity
Membrane related processes
Chemotaxis
Cyp450-related responses
Nuclear + nucleic acid related metabolism
*
Transcription + replication
Cellular organization
Cell cycle related processes
Protein related processes
Liver-specific genes
-16.7
-0.7
13.3
* Diverse

Figure 3


Cyp450 activity vs expression
% activity/expression
10000
1000
100
10
1
0.1
0h activity
42h activity
90h activity
0h expression
42h expression
90h expression
Cyp1A2
Cyp3A11
Cyp2B
Cyp2C
Cyp2C37
Cyp2D