Advanced in vitro models for studying drug induced toxicity

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CHAPTER 3

3D CELL CULTURE IMPROVES LIVER-SPECIFIC CHARACTERISTICS OF HEPG2 CELLS: A GENE EXPRESSION ANALYSIS-BASED COMPARISON OF DIFFERENT *IN VITRO* HEPATOCYTE MODELS

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ABSTRACT

Hepatocytes rapidly de-differentiate when isolated from their natural tissue environment. Three dimensional cell cultures provide physical and chemical cues that improve and preserve the differentiated status of hepatocytes for extended periods. Our recent findings have shown that HepG2 cells differentiate in 3D matrix hydrogels, more closely recapitulating the polarized morphology and functions of *in vivo* hepatocytes compared to conventional monolayer cultures. Here we report the findings from whole genome expression analysis of 2D and 3D HepG2 cell models and also a comparative analysis of these models together with other hepatocyte models including HepaRG and primary human hepatocytes. With increasing duration in 3D culture up to 28 days, HepG2 cells showed coordinated regulation of various signaling pathways associated with cellular differentiation, development, and metabolism reminiscent of *in vivo* hepatocytes. Pathway analysis was used to identify canonical pathways that are differentially changed in HepG2 spheroids in 3D culture during differentiation. Comparative pathway analysis of various *in vitro* models with human liver highlighted the similarities and differences that are inherently associated with specific cell lines. PCA analysis of genes associated with some important biological pathways such as cell cycle regulation and xenobiotic metabolism showed a different expression profile of HepG2 cells in 3D culture than 2D and a similarity with human liver and primary human hepatocytes and/or HepaRG cells. In conclusion, the gene expression of 3D HepG2 spheroids was significantly different from 2D cultures; some important physiological pathways that are absent in monolayer cultures were induced in 3D HepG2 cultures and showed similarity to primary hepatocytes and human liver. Though the expression profile is not similar to current 'gold standard' primary human hepatocytes, the presence of active xenobiotic metabolism pathways, anti-oxidant response pathways and pathways involved in maintaining normal physiology of liver and possibility for a long term culture makes our 3D HepG2 model a powerful tool to detect and understand the mechanisms of drug-induced toxicity.

INTRODUCTION

Various test models are used for assessing toxicity of new chemical entities, mostly, relying on animal models for short and long-term effects of compounds. With the potential risks in species-specific variation in toxic response with animal studies, human cell models are a preferred choice. But it is a major challenge to maintain the differentiated status of human cells in an *in vitro* culture condition. In the absence of a physiological niche, cells rapidly lose their tissue specific properties leading to poor biological responses and a failure to predict the toxicity of the compounds in humans. Different hepatocyte cell lines have been evaluated for their competence in drug screening assays, but an approved cell model that is efficient in accurately predicting the toxic effects of chemicals is still lacking. Currently, human primary hepatocyte cell lines are considered as gold standards for safety assessment studies but these cells rapidly lose their differentiated status in two-dimensional (2D) monolayer cultures [1].

Cells can be cultured as three-dimensional (3D) tissues using extra cellular matrix hydrogels including collagen, matrigel, peptide nanofiber gels and using hanging drop methods, all of which have shown an improvement in hepatocyte function. Although primary hepatocytes cultured as two-dimensional (2D) monolayer cultures or on a single layer of collagen rapidly lose liver tissue properties, sandwich culturing was shown to improve the maintenance of specialized functions [1]. The limited availability of human primary cells, donor-specific variability and cost, drives a demand for the models that use cell lines that are functionally stable and metabolically competent. HepaRG, a cell line derived from a hepatocarcinoma patient [2] are 'bi-potent' progenitor cells which, when cultured in the presence of DMSO differentiate into biliary and hepatocyte like cells and have drug-metabolizing enzymes similar to primary human hepatocytes [2-4]. However, culture of HepaRG cells in DMSO lead to a 3-4 fold increase in LDH and AST release, reduction in proliferation and decreased hepatic functions [5], which may adversely affect assessment of chemical-induced cytotoxicity. Nonetheless, the drug metabolism enzymes and metabolic capacity were found to be similar to or higher than PHH in HepaRG cells [6, 7].

The other widely used cell line for hepatotoxic studies is HepG2. These cells are also of carcinoma origin but have low levels of cytochrome P450 enzymes compared to primary human hepatocytes [8, 9]. Despite their carcinoma origin, these cells have functionally active p53 and an active Nrf2 system, which is an advantage for cytotoxic studies [10, 11]. A high content screening assay using HepG2 cells, measuring calcium levels, mitochondrial membrane potential, DNA content and plasma membrane potential was shown to be 93% sensitive in identifying DILI com-

pounds [12]. A comparative toxicogenomics profiling of HepaRG and HepG2 for their ability to discriminate genotoxic and non-genotoxic compounds showed that HepG2 could better predict chemical carcinogens [13]. However, the low-levels of CYP450 enzymes and nuclear xenobiotic receptors [14, 15] have been a major drawback for their use in drug safety testing. Our previous studies showed that HepG2 cells cultured in 3D form differentiated polarized spheroids that re-acquire many of the properties of hepatocytes *in vivo* [16]. These specialized functions could be maintained for at least 28 days in 3D culture allowing long-term assessment of toxic effects. Functions such as bile acid transport, glycogen storage were present in 3D HepG2 spheroids and levels of phase I, II and III enzymes were also higher increasing its metabolic competency and the capacity to identify hepatotoxic compounds.

In the present study we analyzed the gene expression profiles of HepG2 cells at different stages of spheroid development and differentiation in 3D culture, comparing these with two-dimensional monolayer HepG2 culture, HepaRG, primary human hepatocytes (PHH) and human liver. We observed that HepG2 spheroids showed upregulation of genes associated with hepatocyte development, differentiation and metabolism, which stabilized after 21 days in 3D culture. Ingenuity Pathway Analysis (IPA) was used to examine changes in the core signaling pathways upon culture in 3D and to compare these with other hepatocyte models. Many functional pathways associated with *in vivo* hepatocytes were significantly enriched in HepG2 spheroids. Cell cycle regulation, xenobiotic metabolism pathways such as PXR/RXR, complement system, bile-acid biosynthesis and coagulation system were significantly upregulated and showed a close similarity to the human liver expression compared to other hepatocyte cells. Overall, the gene expression analysis of 3D spheroids showed robust improvement in the physiological and metabolic profile of HepG2 cells, indicating that this model may represent a powerful *in vitro* tool for studying liver biology.

MATERIALS AND METHODS

Cell line and 3D cell culturing

Human hepatoma HepG2 cell line was obtained from American type tissue culture (ATCC, Wesel, Germany), cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, The Netherlands), 25 U/mL penicillin, and 25 μg/mL streptomycin (PSA, Invitrogen). The cells were cultured at 37°C with 5% CO2. Matrigel (Erembodegem,BD Biosciences) was used to culture 3D spheroids as previously described [16].

RNA isolation and microarray analysis of 2D and 3D HepG2 cells

RNA was extracted from 3 day cultured 2D HepG2 cells and 3D HepG2 cells cul-

tured at day 3, 7 14, 21 and 28. Total RNA was extracted from 2D/3D cultured cells using Trizol reagent (Invitrogen) followed by clean up using RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity quality and integrity was determined using the Agilent bioanalyser (Agilent Technologies Inc, Santa Clara, USA). Biotinylated cRNA was prepared using the Affymetrix 3' IVT-Express Labeling Kit (Affymetrix, Santa Clara, USA) and hybridization steps were performed by Service XS B.V (Leiden, The Netherlands) on Affymetrix HT Human Genome U133 plus PM plate. Array plates were scanned using the Affymetrix GeneTitan scanner. BRB Array Tools software (developed by Dr. Richard Simon and BRB-ArrayTools Development Team) was used to normalize the .cel data using the Robust Multichip Average (RMA) method. Differentially expressed genes (p-value < 0.001) between the various experimental conditions were identified with an ANOVA test followed by calculation of the false discovery rate according to Benjamini and Hochber [17]. Classification of the selected genes according to their biological and toxicological functions was performed using the Ingenuity Pathway Analysis IPA® software (Ingenuity Systems, Redwood, USA). Heatmap representations and hierarchical clustering (using Pearson correlation) were performed using the Multi Experiment Viewer software [18].

Re-annotation, normalization, and data filtering for comparative gene expression profiling

To compare basal gene expression data from 2D and 3D HepG2 cells with other cell models, different data sources were combined. Raw data files from untreated HepaRG and HepG2 cells were obtained from the department of Toxicogenomics, Maastricht University [13, 19]. Primary cryopreserved human hepatocyte data was downloaded from TG-GATEs [20]; 16 microarrays of untreated conditions were randomly selected from this database. Post-mortem liver data was obtained through GEO, accession numbers GSE13471 and GSE3526 respectively. Raw data files were loaded into R version 2.15.2 for Windows (64-bit) [21], re-annotated to Entrez Gene using Brainarray's custom CDF version 15.1.0 [22]. The R-packages used was obtained from BioConductor version 2.11 [23] Since the combined data set was originating from the Affymetrix Human Genome U133 Plus 2.0 and Affymetrix HT Human Genome U133 plus PM (GeneTitan) platforms, normalization was performed in a multi-step procedure. Data from different chip types were merged based on 18909 overlapping Entrez Gene ID's, followed by scaling of the GeneTitan data and quantile normalization on the merged set.

Principal component analysis

Principal Component Analysis (PCA) was applied to identify data patterns and to

highlight data similarity and differences between the treated and untreated cell lines at different time points. Therefore, the normalized intensities of the combined data sets were uploaded into the PCA module of ArrayTrack [24]. PCA analysis was performed on whole genome expression and filtered gene sets of hepatocyte-specific canonical pathways. PCA data were visualized using Tibco Silver Spotfire (Paulo Alto, CA, USA).

Ingenuity Pathway analysis

To better understand the biological processes and canonical pathways that changed significantly and to make a comparative overview of gene expression profiles in the different cell models, differentially expressed genes (DEG's) with a p value <0.001 were uploaded onto ingenuity pathway analysis (Ingenuity® systems, www.ingenuity.com).

RESULTS

Gene expression profile of HepG2 cells in 3D culture correlates with differentiation of hepatocytes

When HepG2 cells are cultured in an extracellular matrix (ECM) protein-rich hydrogel that simulates a tissue microenvironment, cells form spheroids and exhibit many features of hepatocytes *in vivo* [16]. A significant change in the gene expression was observed during the 3D culture period with a gradual increase in DEG's (P<0.001, Fold change>1.5) until day 14. Thereafter, the change in gene expression follows a downward trend with a steady gene expression between day 21 and 28 (Fig. 1A). Spheroid development in the 3D culture seems to recapitulate ontogeny of a developing liver. Genes that are associated with the fetal liver CYP3A7, CYP1A1 [25, 26] are highly expressed in the initial culture period until 7 days and their expression was reduced later during the culture (Fig. 2C). Similarly genes that are expressed by adult liver such as Flavin containing monooxygenase 5 (FMO5), haptoglobin (HP) [27, 28] were induced after 7-days in 3D culture and highly expressed from 14–days (Fig. 2C). The terminal hepatocyte differentiation marker glucose-6-phosphate (G6PC) was 150-fold higher after 14 days and 230-fold at both 21 and 28 day cultures, indicating that the differentiation process stabilizes between day 14 and 21 days. Differentiated liver marker genes albumin, transferrin, fibronectin, aldolase-b, apolipoprotein, IGF2, fibrinogen beta-chain and fibrinogen gamma chain, which previous studies showed were either absent in HepG2 cells or weakly expressed (Yu *et al*. 2001) were increased in HepG2 spheroid cultures (Fig.1B). Recently, human iPSC-LB were shown to form vascularized and functional human liver [29] with upregulation of a set of 83-genes that are involved in liver development. Almost all of

these genes were also serially upregulated in the HepG2 spheroid cultures (Fig. 2B). Hepatocyte specific gene expression is controlled by liver-enriched transcriptional factors belonging to HNF, C/EBP family members, which act synergistically to maintain tissue functions [30]. The transcript levels of HNF4α, C/EBPβ were also increased with time in HepG2 spheroids (Supplementary data S1). Taken together, these results indicate that HepG2 cells display a trend toward liver-like differentiation when cultured as 3D spheroids.

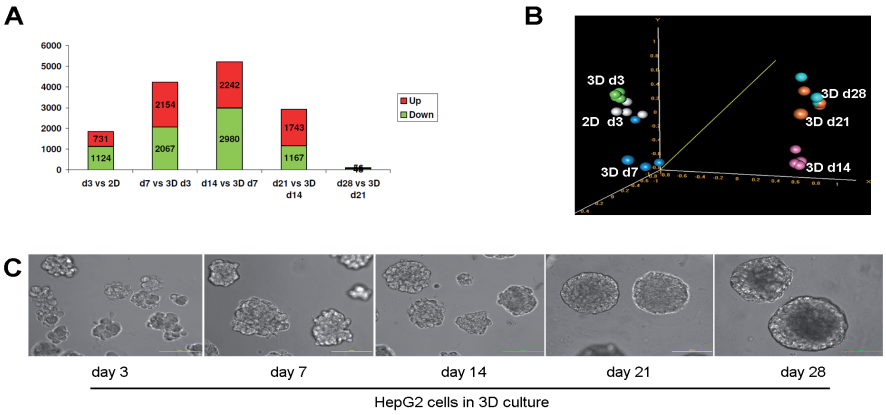

Figure 1. *Gene expression changes in differentiated HepG2 spheroids.* Differentially expressed genes which are significantly changed ($p<0.001$ and FC >1.5 fold) in time during 3D culture (A) Green represents number of genes downregulated / Red represents the upregulated genes. PCA plot showing the distribution of genes in 2D HepG2 cells and at different times in 3D culture where HepG2 cells differentiate into a spheroid (B). Phase contrast images of HepG2 cells in 3D culture with time (C) scale bars 100 μm.

Drug detoxifying enzyme gene expression coordinately increases with (spheroid) differentiation

One of the major limitations of HepG2 cells in drug screening assays is their low level of cytochrome P450 enzymes, phase II conjugating enzymes and drug transporters. Furthermore, the key transcription factors that regulate the drug metabolizing enzymes are also poorly expressed in HepG2 cultures [31]. Xenobiotic metabolism involves various phase I and phase II drug metabolism as well as phase III mediated excretion processes. Expression of these drug-metabolizing enzymes and transporters is higher in differentiated HepG2 spheroids compared to 2D monolayer cultured HepG2 cells. Xenobiotic CYP450 enzymes CYP 2C18, 3A5, 7A1, 4F2 and others (Fig. 2A) were upregulated upon 3D culturing. Also other CYP450 enzymes mainly involved in sterol and fatty acid metabolism, including CYP 7A1, 8B1, 17A1, 19A1, 51A1, 2J2, 4B1, 4F12, had a significantly higher expression in 3D HepG2 spheroids. Phase II enzymes, which are involved in glutathione, glucuronidation and sulfation conjugation reactions were also upregulated in HepG2 spheroids. Glutathione- S- transferases GSTA1, K1, M3, M4 and sulfotransferases SULT2A1, 1C2,

1E1; UDP-glucoronosyltransferases UGT1A1, 1A6, 1A7, 1A8, 1A9,1A10, 2A3, 2B4, 2B28 were significantly upregulated in 3D HepG2 spheroids (Fig. 2A). Besides these, alcohol dehydrogenases and aldehyde dehydrogenases showed an increased expression with time in 3D culture.

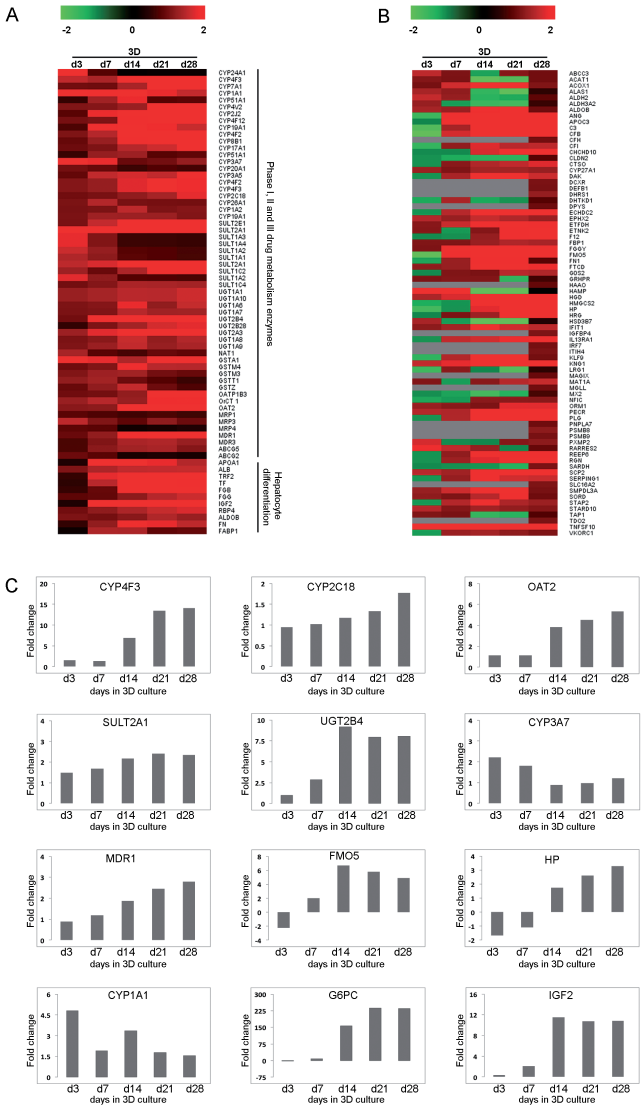

Figure 2. *Expression of genes associated with differentiated hepatocytes in 3D culture*. Heat map showing fold change gene expression changes in Phase I, II and III drug metabolism enzymes (A). Fold change expression of genes involved in differentiation and development of the liver (B) in HepG2 spheroids compared to 2D monolayer cultures, data is average (4 experiments) fold change compared to 2D HepG2 gene expression. Fold change gene expression of individual genes corresponding to xenobiotic metabolism, differentiation markers, fetal and adult liver markers over time in 3D culture (C).

Figure 3. *Canonical pathways that are significantly induced in HepG2 cells cultured as spheroids.* Significantly enriched pathways in day 28 differentiated HepG2 spheroids compared to 2D cultured HepG2 cells from Ingenuity Pathway Analysis (IPA), upregulated pathways (A), downregulated pathways (B).

Liver-specific functions are enriched in 3D HepG2 spheroids

Besides the metabolic competence it is important for an in vitro system to emulate liver specific functions in order to accurately predict a human stress response. Ingenuity Pathway Analysis was used to identify the canonical pathways that are significantly enriched in differentiated HepG2 spheroids compared to HepG2 cells grown as monolayer cultures. The principal upregulated pathways included many xenobiotic metabolism pathways (FXR/RXR, LXR/RXR, PXR/RXR activation) and hepatocyte specific pathways related to liver physiology (coagulation system, complement system, extrinisic prothrombin activation, bile acid biosynthesis) and many pathways related to functional hepatocytes are significantly upregulated (Fig. 3A). The principal downregulated pathways mostly belonged to cell cycle regulation with canonical pathway 'cell cycle control of chromosomal replication' being most strongly downregulated pathway with 80% of downregulated genes (Fig 3B). This indicates that HepG2 spheroids are functionally and metabolically differentiated at the pathway level.

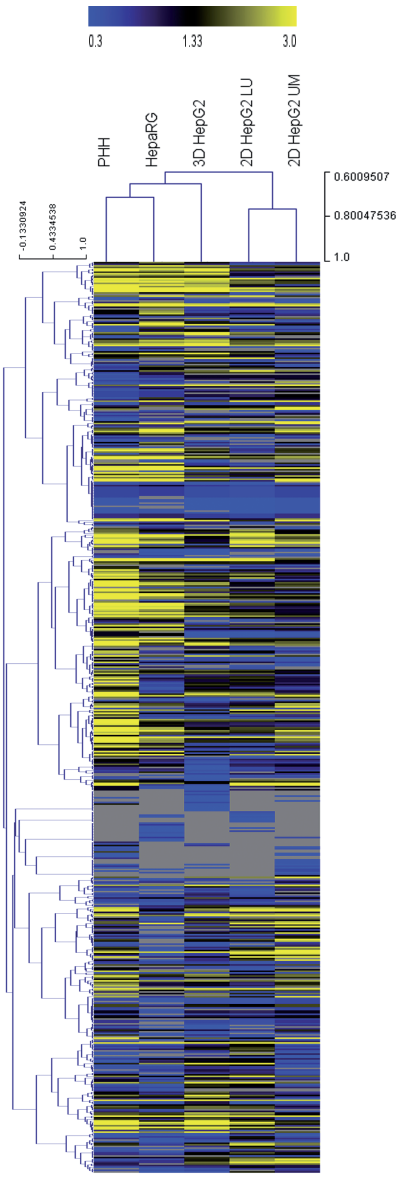

Figure 4. *Heat map showing regulation of canonical pathways across hepatocyte models in comparison to human liver*. Scale is – log (p-value); -log (pvalue) 1.3 (p=0.05) represent pathways that are significantly different from human liver. 2D HepG2 LU/2D HepG2 UM: Two sources of HepG2 cells from Leiden University (LU); Maastricht University (UM).

Pathway analysis of *in vitro* cellular models compared to human liver

It is anticipated that *in vitro* cultured hepatocytes derived from liver tissue exhibit a different expression profile to intact liver. Ingenuity Pathway Analysis was used to examine the similarities and overall changes in the expression profiles of *in vitro* cellular models compared to human liver. HepG2 cells in 2D culture from 2 different sources showed a close association, but there was also a difference in several molecular pathways, highlighting the effects of source and culture conditions of the HepG2 cells as observed in earlier studies [32]. A close similarity in regulation of molecular pathway was observed between HepaRG and cryopreserved PHH. 3D HepG2 gene expression was different from monolayer cultured HepG2 cells and an association with PHH and HepaRG was observed in hierarchical clustering (Fig. 4). A detailed heat map showing the changes in individual molecular canonical pathways compared to human liver is in supplementary figure S3.

Pathways that are differentially regulated or that are similar to human liver, specific to a cell type are listed in Supplementary data S4. Canonical pathways related to cytokine signaling, MAPK signaling, xenobiotic receptor signaling were similar in PHH and human liver. In HepaRG cells apoptosis signaling, hepatocyte functional pathways such as coagulation system, gluconeogenesis, cell cycle regulation etc. are some of the hepatocyte specific canonical pathways that are not differentially regulated compared to hu-

man liver expression. Pathways related to protein biosynthesis, cytokine signaling, aryl hydrocarbon signaling in xenobiotic metabolism were some of the pathways that were unaltered in HepG2 monolayer cultures compared to human liver. Cell cycle regulation, coagulation system, complement system, PXR/RXR, FXR/RXR xenobiotic signaling, epithelial adherens junction signaling etc. were similar in 3D HepG2 cells and human liver.

To further understand the relationship between different liver models, important functional pathways of hepatocytes were selected and their distribution was

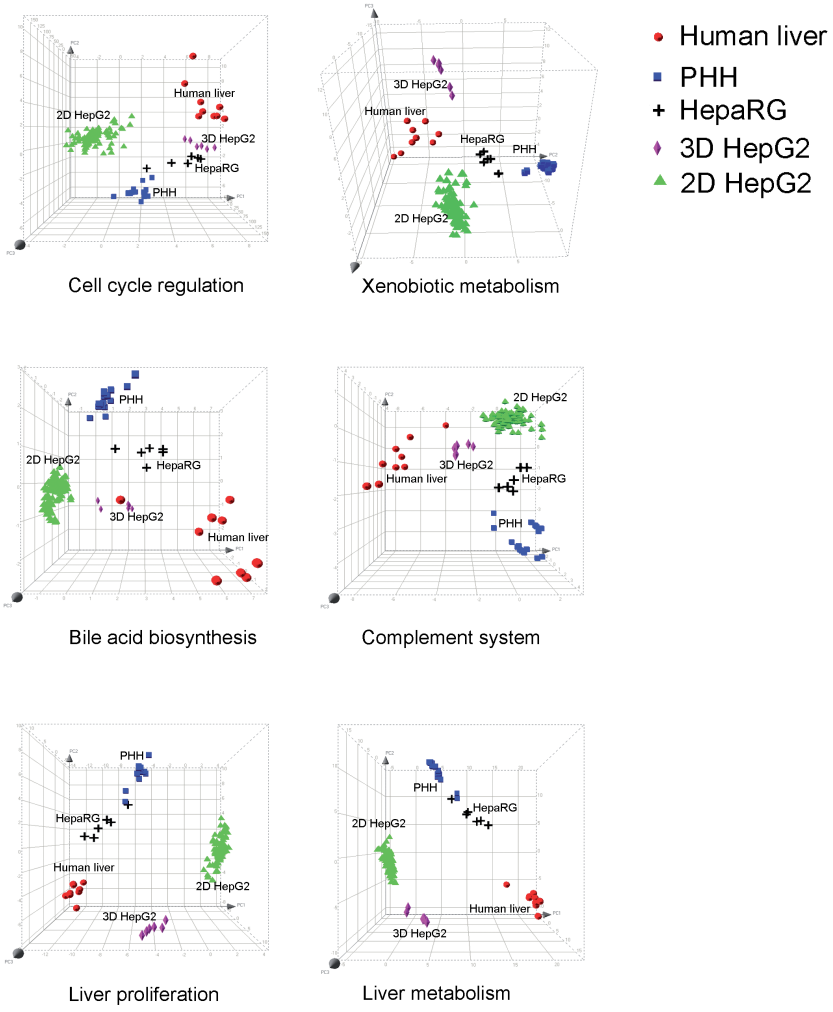

Figure 5. *PCA of physiologically relevant molecular pathways*. PCA of selected pathways related to hepatocyte function and differentiation, cell cycle regulation, xenobiotic metabolism, bile acid biosynthesis, complement system, liver proliferation and liver metabolism, in 2D/ 3D HepG2 cells, primary human hepatocytes, HepaRG and human liver.

analyzed using PCA. Pathways related to liver proliferation, liver metabolism, bile acid biosynthesis, complement system, xenobiotic metabolism and cell cycle regulation were analyzed by plotting the normalized log2 intensities of the genes from the groups on PCA (Fig. 5). As observed in IPA analysis, 3D HepG2 cells showed close similarity with human liver expression for cell cycle pathway, supporting the previous observations that the proliferation is ceased in HepG2 spheroids [16]. The expression of cell cycle regulation genes were closely associated in HepaRG and 3D HepG2 spheroids. For other pathways 3D HepG2 spheroid expression was different from 2D HepG2 cells with a trend moving towards human liver. Primary human hepatocytes and HepaRG had a close association both at whole genome level and for selected pathways in this study. Bile acid biosynthesis and complement system pathways in 3D HepG2 spheroids showed a close similarity to human liver than other models, as observed in ingenuity pathway analysis. Together, this data suggests that HepG2 cells in 3D culture transformed into differentiated hepatocytes acquiring various specialized functions of a liver tissue.

DISCUSSION

Hepatocytes extracted from fresh liver tissue are considered as 'gold standards' for assessing liver toxicity, but their rapid deterioration in culture and high variability is a major limitation for their reliability in drug screening assays. In the wake of high-drug attrition rates due to liver injury, there is a significant interest in developing a robust model that can predict hepatotoxicity in humans. We previously showed that 3D HepG2 spheroids show many of the morphological and functional properties of human liver [16]. In this study we made a detailed investigation of the transcriptomic profile of HepG2 spheroids comparing these with those of HepG2 monolayer cultures, primary human hepatocytes, HepaRG cell line and human liver tissue

Our findings suggest that the differentiation of HepG2 cells in 3D culture recapitulate many of the early ontogenetic events of a developing liver. After 21 days in 3D culture, HepG2 spheroids showed a steady state gene expression profile, after which there was no significant change in the gene expression up to day 28. Genes expressed by human fetal liver (CYP3A7, CYP1A1) were highly expressed during the first 7 days in 3D culture whereas genes associated with differentiated adult liver (Flavin containing mono-oxygenase 5 and haptoglobin) were expressed after day 7 [27, 33].

Under optimal conditions hepatocyte cell lines also have the capacity to generate well-differentiated functional hepatocytes and also may have the capacity to form a liver tissue. Similarly iPSC co-cultures in 3D ECM matrigel allowed the formation of liver buds, and upon transplantation into immuno-deficient mice developed vasculature resembling adult liver, [29] opening new avenues to develop organs for

liver transplantation. In these experiments a set of 83 genes that were serially up-regulated in liver development were analyzed in iPSC's. Interestingly, almost all of these genes were also up regulated in 3D HepG2 spheroids over time, suggestive of a similar differentiation process in our hydrogel cultures.

Differentiation of HepG2 in 3D spheroids also led to induction of various drug-metabolizing enzymes that are typically poorly expressed in 2D HepG2 cell cultures. Phase I enzymes belonging to CYP3A CYP2C, CYP1A, CYP2D families are major enzymes involved in metabolism of 90% of prescribed drugs [34-36]. Most of the members belonging to these families are upregulated in differentiated HepG2 spheroids. Also various enzymes belonging to phase II drug metabolism and transporters were also highly expressed in HepG2 spheroids compared to its native origin. The increased expression was observed after 14 days in 3D culture and a stable expression remained up to 28 days, thus providing a window for studying more chronic/repeated dose effects of novel drugs or other chemical entities.

The higher expression of drug metabolic enzymes does not imply that a cellular model is sufficiently robust to detect a toxic stress response. Though, the presence of Phase I, II and drug metabolizing enzymes is promising for making an accurate estimation of toxicity, stress signaling pathways need various other co-regulatory genes in order to show an actual biological response. Ingenuity pathway analysis (IPA) on differentiated 3D HepG2 spheroids showed significantly enriched xenobiotic metabolism pathways. The top upregulated pathway in the list was 'acute response singling pathway' which is required for a proper inflammatory response. Other xenobiotic signaling pathways PXR/RXR, PPARα/RXR, FXR/RXR, LXR/RXR, were also significantly enriched during 3D spheroid culture, suggesting that 3D HepG2 spheroids might be more sensitive for a xenobiotic response. PXR together with RXR plays an important role in drug metabolism, most importantly they activate the CY3A4 gene, which is involved in the metabolism of 50% of current drugs. Upon activation PXR/RXR induces the expression of various phase II and drug metabolizing enzymes [37, 38]. It also plays a major role in regulating bile acid synthesis, gluconeogenesis and lipid metabolism [39, 40]. PPARα is also an important target for various pharmacological agents and play a major role in xenobiotic metabolism [41]. FXR/RXR and LXR/RXR pathways are also involved in regulating both endogenous and xenobiotic responses [39, 42], suggesting 3D HepG2 spheroids might be more responsive to xenobiotics and would serve as a useful model to help investigate the mechanisms of toxicity.

Principal component analysis on whole genome gene expression profiles of different hepatocyte models was in agreement with previous studies for PHH and HepaRG [4, 6]. A close similarity of HepaRG gene expression profile with cryopreserved PHH suggest that HepaRG may offer a suitable alternative to PHH in toxicity

assays. This still needs to be investigated, but until now there were no large scale toxicity screens comparing these two models. However, in a comparative toxicogenomics analysis both HepG2 and HepaRG cells performed similar compared to PHH [43] and in another transcriptomic study, HepG2 cells performed better in identifying genotoxic compounds than HepaRG cells [13]. The gene expression profile of 3D HepG2 spheroids was different from 2D HepG2 cells and was not closely associated with any other models at the global gene expression level, but showed a close similarity with human liver for important xenobiotic metabolism pathways such as PXR/RXR. Furthermore, the reduced expression of cell cycle genes and the absence of cell cycle, which might represent a physiologically relevant tissue level toxicity assessment and therefore may be advantageous in studying mechanistic toxic responses. Further toxicogenomics studies on HepG2 spheroids would validate their xenobiotic response and similarity to higher models, which may be more similar to an *in vivo* response.

In this study we did not make a direct comparison of drug metabolizing enzymes in various models due to the source of transcriptomic data from primary human hepatocytes and HepaRG. These data sets were from 0.5% DMSO treated controls from another study. Though DMSO will not have an effect on overall gene expression, it is known that DMSO induces expression of various phase I, II xenobiotic metabolizing enzymes and transporters, which therefore might overestimate the comparison with untreated 2D and 3D HepG2 cells. Instead a comparative pathway analysis was performed across different models to understand the similarity and association of different data sets.

In conclusion, HepG2 spheroids in 3D culture showed transcriptional features recapitulating liver development and differentiation. Phase I, II drug metabolic enzymes and drug transporters are highly upregulated in spheroid cultures. Canonical pathways of functional hepatocytes are highly enriched and had a close similarity to human liver. With a higher complexity and amenability to high-throughput assays, 3D HepG2 spheroid model could prove to be promising tool for future drug discovery and development research.

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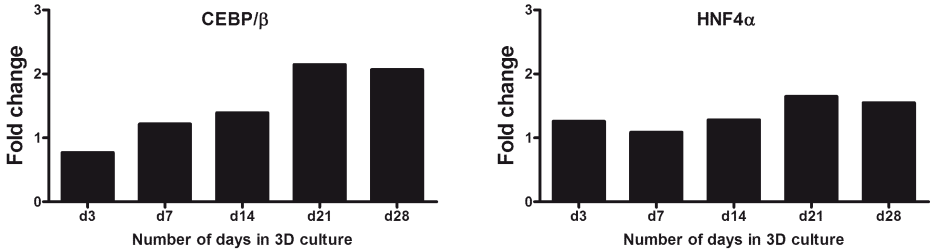
SUPPLEMENTARY DATA



Supplementary S1. Transcript level of HNF4α and C/EBPβ in HepG2 spheroids with time in 3D culture.

Supplementary S2. Entrez ID of genes used in PCA for figure 5.

Xenobiotic metabolism pathway

5243	124	126	127	130	196	8644	1109	126133	5832
216	160428	8659	501	405	472	1066	9469	56548	113189
51363	1387	1562	1573	66002	9451	2098	2330	2938	3265
9653	3845	3990	4128	5608	6885	5594	5599	5601	5603
5469	4257	22808	8648	4780	4790	1728	4835	8856	4893
5289	5287	8503	10891	5515	5520	5522	5578	5581	9978
6256	6819	6783	6822	7363					

Cyclins and cell cycle regulation

472	545	8945	890	891	9133	595	898	9134	993
983	1017	1019	1026	1031	1869	1870	1871	1876	2932
8841	9759	10014	55869	5515	5520	5522	5925	25942	
6500l728622		6502	6839	7027	7040				

Bile acid biosynthesis

8644	1109	570	1581	1582	80270	6342

Complement system

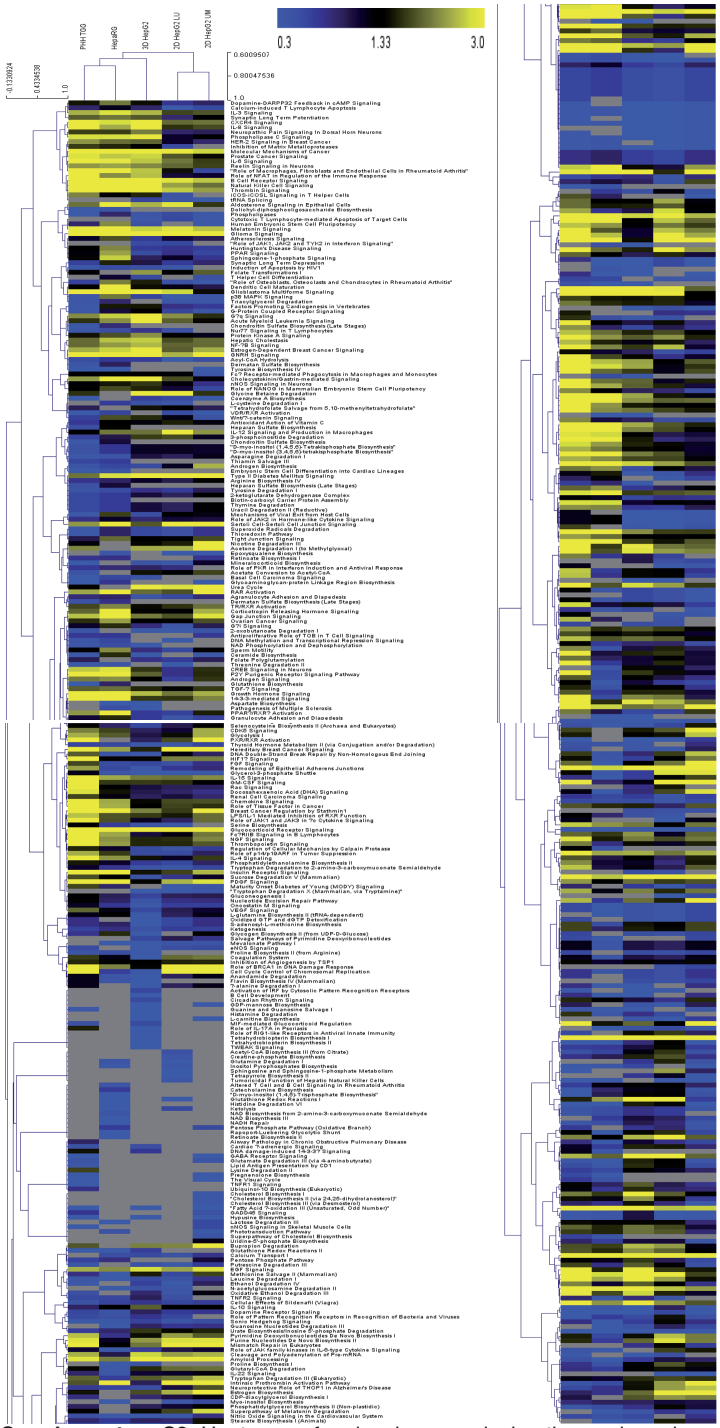
717	718	727	728	731	732	733	1604	629	5648
4153									

Liver proliferation

100	185	196	405	718	727	595	898	1017	1026
1050	1051	11113	1490	23405	10395	1869	1906	1956	957
2056	2147	355	2305	10468	2932	3172	3265	3480	3484
3557	3570	3643	3725	4254	3953	3956	3958	5594	5599
5601	4192	5469	4493	4609	4780	4790	9971	8856	113791
5340	5465	5467	5468	5562	5925	6256	6342	6502	4088
4092	9021	6696	6772	7039	7040	7048	7057	7076	7132
8743	8805	7422							

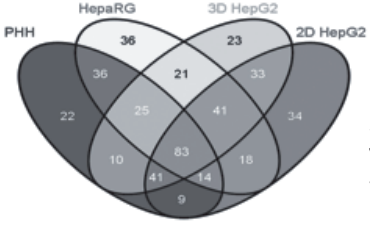
Liver metabolism

100	185	196	405	718	727	595	898	1017	1026
1050	1051	11113	1490	23405	10395	1869	1906	1956	957
2056	2147	355	2305	10468	2932	3172	3265	3480	3484
3557	3570	3643	3725	4254	3953	3956	3958	5594	5599
5601	4192	5469	4493	4609	4780	4790	9971	8856	113791
5340	5465	5467	5468	5562	5925	6256	6342	6502	4088
4092	9021	6696	6772	7039	7040	7048	7057	7076	7132
8743	8805	7422	2	2152	2153	2155	2159	2160	2161
2165	2243	2244	3818	3827	5624	3053	5054	5345	7035
28976	39	51	51703	124	126	127	130	216	8659
501	1374	1562	1573	66002	3033	3028	3712	717	728
731	732	733	1604	629	5648	4153	80781	1280	8644
1109	570	1581	1582	80270	10797	441024	4548	6470	226
229	2027	2597	5223	5230	5313	5315	10841	3034	



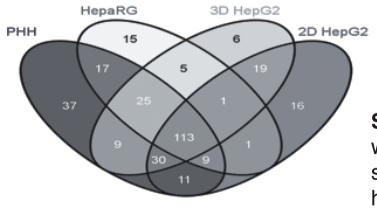
Supplementary S3. Heat map of molecular canonical pathways in various *in vitro* models compared to human liver gene expression. Scale is – log (p-value); -log (pvalue) 1.3 (p=0.05) represent pathways that are significantly different from human liver.

PHH	HepaRG	3D HepG2	2D HepG2
The Visual Cycle	Lactose Degradation III	L-carnitine Biosynthesis	Tetrapyrrole Biosynthesis II
TNFR1 Signaling	NAD Biosynthesis III	Tetrahydrobiopterin Biosynthesis I	Sphingosine and Sphingosine-1-phosphate Metabolism
GABA Receptor Signaling	Rapoport-Luebering Glycolytic Shunt	Tetrahydrobiopterin Biosynthesis II	Vitamin-C Transport
DNA Double-Strand Break Repair by Homologous Recombination	Retinoate Biosynthesis II	Role of IL-17A in Psoriasis	Oleate Biosynthesis II (Animals)
nNOS Signaling in Skeletal Muscle Cells	Histidine Degradation VI	GDP-mannose Biosynthesis	Tumoricidal Function of Hepatic Natural Killer Cells
Pregnenolone Biosynthesis	NADH Repair	TWEAK Signaling	Creatine-phosphate Biosynthesis
Lipid Antigen Presentation by CD1	D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	Histamine Degradation	Acetyl-CoA Biosynthesis III (from Citrate)
Acetate Conversion to Acetyl-CoA	IL-17A Signaling in Fibroblasts	B Cell Development	Methylthiopropionate Biosynthesis
Glutamate Degradation III (via 4-aminobutyrate)	Ethanol Degradation II	Role of RIG1-like Receptors in Antiviral Innate Immunity	Inhibition of Matrix Metalloproteases
Heparan Sulfate Biosynthesis	Glutaryl-CoA Degradation	Circadian Rhythm Signaling	Androgen Signaling
Phenylalanine Degradation IV (Mammalian, via Side Chain)	Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	Activation of IRF by Cytosolic Pattern Recognition Receptors	Dopamine-DARPP32 Feedback in cAMP Signaling
GADD45 Signaling	NAD Biosynthesis from 2-amino-3-carboxymuconate Semialdehyde	DNA damage-induced 14-3-3? Signaling	CREB Signaling in Neurons
Ubiquinol-10 Biosynthesis (Eukaryotic)	RhoA Signaling	1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)	Macropinocytosis Signaling
Airway Pathology in Chronic Obstructive Pulmonary Disease	Phototransduction Pathway	Choline Biosynthesis III	P2Y Purigenic Receptor Signaling Pathway
Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	CMP-N-acetylneuraminate Biosynthesis I (Eukaryotes)	Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	Axonal Guidance Signaling
Fatty Acid ?-oxidation I	IL-17A Signaling in Airway Cells	PXR/RXR Activation	Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Nicotine Degradation III	Role of IL-17A in Arthritis	Intrinsic Prothrombin Activation Pathway	Neurotrophin/TRK Signaling
Folate Transformations I	Pyridoxal 5'-phosphate Salvage Pathway	Glycolysis I	Synaptic Long Term Potentiation
IL-12 Signaling and Production in Macrophages	Tryptophan Degradation III (Eukaryotic)	Cell Cycle: G1/S Checkpoint Regulation	G Beta Gamma Signaling
	Catecholamine Biosynthesis	FXR/RXR Activation	Neuropathic Pain Signaling In Dorsal Horn Neurons
	Pentose Phosphate Pathway (Oxidative Branch)	Complement System	Endothelin-1 Signaling
	Toll-like Receptor Signaling	PI3K/AKT Signaling	Hepatic Fibrosis / Hepatic Stellate Cell Activation
	Salvage Pathways of Pyrimidine Ribonucleotides	Prolactin Signaling	Neuregulin Signaling
	tRNA Charging	Ovarian Cancer Signaling	?-Adrenergic Signaling
	Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	Inhibition of Angiogenesis by TSP1	Role of NFAT in Regulation of the Immune Response
	Actin Cytoskeleton Signaling		Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency
	5-aminoimidazole Ribonucleotide Biosynthesis I		HER-2 Signaling in Breast Cancer
	G Protein Signaling Mediated by Tubby		Phospholipase C Signaling
	Cleavage and Polyadenylation of Pre-mRNA		NF-?B Activation by Viruses
	IL-17 Signaling		IL-4 Signaling
	Small Cell Lung Cancer Signaling		Non-Small Cell Lung Cancer Signaling
	Purine Nucleotides De Novo Biosynthesis II		Antiproliferative Role of Somatostatin Receptor 2
	Assembly of RNA Polymerase II Complex		fMLP Signaling in Neutrophils
	G?12/13 Signaling		Corticotropin Releasing Hormone Signaling
	HIF1a Signaling		
	Ceramide Signaling		



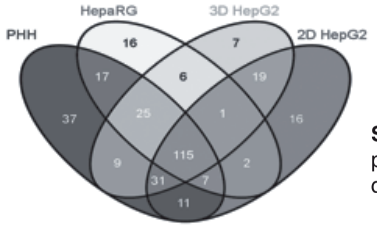
Supplementary S4 (A). List of pathways (generated from IPA) that have not significantly changed when compared to human liver expression and that are unique to specific model. Venn diagram showing the overlap of all the pathways in various models that are not significantly changed with human liver.

PHH	HepaRG	3D HepG2	2D HepG2
Gluconeogenesis I	Heparan Sulfate Biosynthesis	G-Protein Coupled Receptor Signaling	D-myo-inositol (1,4,5)-trisphosphate Degradation
Estrogen Receptor Signaling	Fc? Receptor-mediated Phagocytosis in Macrophages and Monocytes	Type I Diabetes Mellitus Signaling	D-myo-inositol-5-phosphate Metabolism
Apoptosis Signaling	Methionine Degradation I (to Homocysteine)	OX40 Signaling Pathway	Crosstalk between Dendritic Cells and Natural Killer Cells
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	Synaptic Long Term Depression	Notch Signaling	D-glucuronate Degradation I
Nucleotide Excision Repair Pathway	Granulocyte Adhesion and Diapedesis	Methionine Salvage II (Mammalian)	Inosine-5'-phosphate Biosynthesis II
Adenine and Adenosine Salvage III	Ceramide Biosynthesis	mTOR Signaling	Fatty Acid Activation
Selenocysteine Biosynthesis II (Archaea and Eukaryotes)	Glycogen Biosynthesis II (from UDP-D-Glucose)		Choline Biosynthesis III
Superpathway of Serine and Glycine Biosynthesis I	G?i Signaling		Telomere Extension by Telomerase
Agrin Interactions at Neuromuscular Junction	Mechanisms of Viral Exit from Host Cells		Adenosine Nucleotides Degradation II
ERK5 Signaling	Atherosclerosis Signaling		Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism
DNA damage-induced 14-3-3? Signaling	Tight Junction Signaling		?-linolenate Biosynthesis II (Animals)
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	p38 MAPK Signaling		1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)
Phosphatidylethanolamine Biosynthesis II	Human Embryonic Stem Cell Pluripotency		Histidine Degradation III
Role of IL-17A in Psoriasis	Sperm Motility		Extrinsic Prothrombin Activation Pathway
Regulation of Cellular Mechanics by Calpain Protease	PPAR Signaling		Valine Degradation I
Cyclins and Cell Cycle Regulation			DNA Double-Strand Break Repair by Homologous Recombination
CTLA4 Signaling in Cytotoxic T Lymphocytes			
Lysine Degradation II			
Lysine Degradation V			
Renal Cell Carcinoma Signaling			
Maturity Onset Diabetes of Young (MODY) Signaling			
FGF Signaling			
G Protein Signaling Mediated by Tubby			
IL-17A Signaling in Gastric Cells			
DNA Double-Strand Break Repair by Non-Homologous End Joining			
VEGF Signaling			
RAN Signaling			
AMPK Signaling			
CD28 Signaling in T Helper Cells			
T Cell Receptor Signaling			
RhoGDI Signaling			
Thyroid Cancer Signaling			
Purine Ribonucleosides Degradation to Ribose-1-phosphate			
Remodeling of Epithelial Adherens Junctions			
Bladder Cancer Signaling			
Aryl Hydrocarbon Receptor Signaling			
Rac Signaling			



Supplementary S4 (B). List of pathways (generated from IPA) that have significantly changed when compared to human liver expression and that are unique to specific model. Venn diagram showing the overlap of all the pathways in various models that are not significantly changed with human liver.

Common pathways in "PHH" and "3D HepG2" (9)	Common pathways in "PHH" and "HepaRG" (17)	Common pathways in "PHH" and "2D HepG2" (11)
Glycine Cleavage Complex	Huntington's Disease Signaling	Methylglyoxal Degradation III
Lymphotoxin Receptor Signaling	Sphingosine-1-phosphate Signaling	Serotonin Degradation
Regulation of Actin-based Motility by Rho	Superpathway of Methionine Degradation	Coagulation System
CNTF Signaling	VEGF Family Ligand-Receptor Interactions	Noradrenaline and Adrenaline Degradation
IL-17A Signaling in Gastric Cells	Sucrose Degradation V (Mammalian)	MSP-RON Signaling Pathway
Angiopoietin Signaling	Leptin Signaling in Obesity	Clathrin-mediated Endocytosis Signaling
iCOS-iCOSL Signaling in T Helper Cells	Epithelial Adherens Junction Signaling	Protein Ubiquitination Pathway
iNOS Signaling	Melanocyte Development and Pigmentation Signaling	Bile Acid Biosynthesis, Neutral Pathway
Ephrin A Signaling	CDK5 Signaling	Role of CHK Proteins in Cell Cycle Checkpoint Control
	CCR5 Signaling in Macrophages	PXR/RXR Activation
	PPAR/RXR Activation	Cell Cycle Control of Chromosomal Replication
	PTEN Signaling	
	Role of p14/p19ARF in Tumor Suppression	
	Leukocyte Extravasation Signaling	
	Docosahexaenoic Acid (DHA) Signaling	
	Role of JAK1 and JAK3 in Cytokine Signaling	
	FcRIIB Signaling in B Lymphocytes	



Supplementary S5. List of pathways (generated from IPA) that have significantly changed compared to human liver expression and that are common in primary human hepatocytes. Venn diagram showing the overlap of the pathways .