Toxicology Letters 240 (2016) 50–59

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Toxicogenomics-based prediction of acetaminophen-induced liver injury using human hepatic cell systems

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HIGHLIGHTS

- hHEP, HepaRG, HepG2 and hSPK-HPC are investigated for their capacity to predict hepatic toxicity.
- Cells exposed to acetaminophen are compared to clinical liver samples of acute liver failure.
- Transcriptomics analysis show comparable hepatotoxic functions in hHEP, HepaRG and hSKP-HPC.
- HepaRG shows the highest prediction of 'damage of liver', followed by hSKP-HPC and hHEP cells.
- HepG2 shows the slightest response to APAP and do not show activation of 'damage of liver' function.

ARTICLE INFO

Article history:
Received 13 August 2015
Received in revised form 15 October 2015
Accepted 15 October 2015
Available online 20 October 2015

Keywords:
DILI
Acetaminophen (APAP)
Acute liver failure
Adult stem cells
HepaRG
HepG2
Human primary hepatocytes
In vitro toxicity testing
Alternative methods

ABSTRACT

Primary human hepatocytes (hHEP), human HepaRG and HepG2 cell lines are the most used human liver-based *in vitro* models for hepatotoxicity testing, including screening of drug-induced liver injury (DILI)—inducing compounds. hHEP are the reference hepatic *in vitro* system, but their availability is limited and the cells available for toxicology studies are often of poor quality. Hepatic cell lines on the other hand are highly proliferative and represent an inexhaustible hepatic cell source. However, these hepatoma-derived cells do not represent the population diversity and display reduced hepatic metabolism. Alternatively, stem cell-derived hepatic cells, which can be produced in high numbers and can differentiate into multiple cell lineages, are also being evaluated as a cell source for *in vitro* hepatotoxicity studies. Human skin-derived precursors (hSKP) are post-natal stem cells that, after conversion towards hepatic cells (hSKP-HPC), respond to hepatotoxic compounds in a comparable way as hHEP. In the current study, four different human hepatic cell systems (hSKP-HPC, hHEP, HepaRG and HepG2) are evaluated for their capacity to predict hepatic toxicity. Their hepatotoxic response to acetaminophen (APAP) exposure is compared to data obtained from patients suffering from APAP-induced acute liver failure (ALF). The results indicate that hHEP, HepaRG and hSKP-HPC identify comparable APAP-induced hepatotoxic functions and that HepG2 cells show the slightest hepatotoxic response. Pathway analyses further points out that HepaRG cells show the highest predicted activation of the functional genes related to 'damage of

Abbreviations: ALF, acute liver failure; APAP, *N*-acetyl-*p*-aminophenol/acetaminophen/paracetamol; DILI, drug-induced liver injury; ESC, embryonic stem cells; hHEP, primary human hepatocytes; hSKP, human skin-derived precursors; hSKP-HPC, hSKP-derived hepatic progenitor cells; IPA, Ingenuity Pathways Analysis; iPSC, induced pluripotent stem cells; NAPQ, *N*-acetyl-*p*-benzoquinoneimine; NCE, new chemical entities.

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http://dx.doi.org/10.1016/j.toxlet.2015.10.014

liver', followed by hSKP-HPC and hHEP cells that generated similar results. HepG2 did not show any activation of this function.

1. Introduction

At least 1200 drugs have been reported to exhibit potential hepatic toxicity (Biour et al., 2004). DILI is responsible for about half of the cases of ALF and is the main cause of liver transplantations (Mindikoglu et al., 2009; Prakash and Vaz, 2009). APAP (*N*-acetyl-*p*-aminophenol; acetaminophen) or paracetamol as it is mostly referred to in Europe, is a widely used over-the-counter analgesic and antipyretic drug. APAP overdose is the most common cause of acute liver injury leading to ALF (James et al., 2003; Blachier et al., 2013; Larson et al., 2005). At therapeutic levels, APAP is detoxified by glucuronidation and sulfatation and metabolised by cytochromes P450 enzymes to *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is subsequently neutralized by conjugation with glutathione. However, when taken in overdose, NAPQI production exceeds the clearance capacity of glutathione, leading to its hepatic accumulation and binding to DNA, lipids and cysteine groups in proteins (James et al., 2003). Ingestion of a single dose of 10 g can already induce levels of hepatic necrosis leading to liver injury (Whitcomb and Block, 1994).

Despite improvements in toxicological research, the overall frequency of drug hepatotoxicity has not decreased in the last 15 years (Blachier et al., 2013). One of the reasons for this problem is the poor detection of hepatotoxicity early during drug development. The discrepancies between preclinical studies with animal species and liver injury observed in humans, point to the fact that the current methodology applied by the pharmaceutical industry, does not allow to efficiently evaluate the potential hepatotoxicity of new chemical entities (NCE). It is believed that a shift from the use of animal testing towards alternative methods, such as application of human cell-based *in vitro* hepatic models, could result in a better evaluation of the hepatotoxic potential of NCE. Especially the relative low cost of *in vitro* experiments *versus* animal testing and the circumvention of interspecies extrapolation, make their use attractive. hHEP are today the gold standard for xenobiotic metabolism and cytotoxicity studies as they are thought to mimic the *in vivo* liver functionality most accurately. However, the availability of fresh human liver samples is very limited. Furthermore, isolated hepatocytes only have a short lifespan in culture (Guguen-Guillouzo and Guillouzo, 2010). Hepatic cell lines, such as HepG2 and HepaRG, are also extensively used in toxicological studies. HepG2 cells were originally isolated from a hepatocellular carcinoma and have shown to display several genotopic features of liver cells (Sassa et al., 1987). However, these cells have a much lower metabolic capacity compared to primary hepatocytes (Xu et al., 2004). HepaRG cells were also isolated from a hepatocellular carcinoma and at their most differentiated state they are composed of biliary-like and hepatocyte-like cells (Gripon et al., 2002). HepaRG display hepatic functions and express liver-specific genes at similar levels of primary hepatocytes. Therefore these cells are frequently used as an alternative to primary hepatocytes (Anthérieu et al., 2012; Guguen-Guillouzo et al., 2010; Guillouzo et al., 2007; Rodrigues et al., 2013). More recently, human stem cells were also considered as potential cell sources for hepatotoxicity testing (Rodrigues and De Kock, 2014; Szkolnicka et al., 2014; Chapin and Stedman, 2009; De Kock et al., 2012). Besides pluripotent embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) also multipotent postnatal stem cells are receiving increasing attention. Our group has previously shown that hSKP have the ability to differentiate into cells expressing relevant hepatic markers (hSKP-HPC) (Rodrigues et al., 2014; De Kock et al., 2009). A combination of both markers of immature and adult hepatocytes is expressed in the differentiated cells at the protein and gene level (Rodrigues et al., 2014; De Kock et al., 2009). Using a toxicogenomics approach, it was demonstrated that these cells could be employed in the evaluation of the hepatotoxicity potential of several hepatotoxic compounds (De Kock et al., 2009; Rodrigues et al., 2015a). In the current study, the response to APAP is evaluated in a set of commonly used human hepatic cell systems *i.e.* hHEP, HepaRG and HepG2 as well as in hSKP-HPC. The capacity to predict APAP-induced ALF is evaluated by comparing the hepatotoxic responses of the different cell types exposed to APAP to human liver samples from patients suffering from APAP-induced ALF.

2. Material and methods

2.1. Isolation, culture and hepatic differentiation of human skin-derived stem cells

hSKP were isolated from small skin segments obtained by circumcision of boys between 1 and 10 year old. Informed consent of the parents of the donors was obtained under the auspices of the Ethics Committee of the '*Vrije Universiteit Brussel*' and the '*Universitair Ziekenhuis Brussel*'. Cell isolation and culture were performed as previously described (De Kock et al., 2009). The cells were seeded at a density of 7.5×10^4 viable cells per mL (3×10^4 viable cells per cm^2) and cultured for 2 weeks in a 5% (v/v) CO_2-humidified air incubator at 37 °C. The culture medium used was composed of DMEM + GLUTAMAX/F12 Nutrient Mixture (3:1; Life Technologies) supplemented with 7.33 IU/mL benzyl penicillin (Continental Pharma), 50 mg/mL streptomycin sulfate (Sigma–Aldrich), 2.5 mg/mL fungizone, 2% (v/v) B27 Supplement (Life Technologies), 40 ng/mL basic fibroblast growth factor (FGF) 2 (Promega) and 20 ng/mL epidermal growth factor (EGF) (Promega). After 2 weeks, hSKP formed three-dimensional spheres that were passaged using 0.2 mg/mL Liberase DH solution (Roche Applied Science) and seeded at a density of 1.3×10^4 cells per cm^2 for further culture as monolayers. At this point, the cells were either differentiated to hepatic cells or cryopreserved for later use. All experiments with hSKP were performed in triplicate. Hepatic differentiation of hSKP was carried out as previously documented (Rodrigues et al., 2015) using a 24-day protocol in which subconfluent cells were exposed in a time-dependent manner to the following growth factors and cytokines: Activin A (Life Technologies), FGF4 (Biosource), bone morphogenetic protein 4 (BMP4), hepatocyte growth factor (HGF) (Life Technologies), insulin-transferin-sodium selenite solution (ITS) (Sigma-Aldrich), dexamethasone (Sigma–Aldrich) and oncostatin M (Life Technologies). The obtained differentiated hepatocyte-like cells are further referred to as hepatic progenitor cells obtained from human skin-derived precursors (hSKP-HPC).

2.2. Cell culture of hepatic cell lines (HepG2 and HepaRG)

HepG2 cells (ATCC; clone HB-8065) were recovered from liquid nitrogen and were cultured in 75 cm^2 tissue culture flasks (Falcon) in a humidified incubator (37 °C, 5% (v/v) CO_2). The cell culture medium was composed of DMEM (Lonza) containing 10% (v/v) bovine calf serum (Gibco). The cells were passaged at sub

confluence at least three times before use in exposure experiments. A seeding density of approximately 13,000 cells/cm^2 was employed each time.

Cryo-preserved differentiated HepaRG cells were obtained from Biopredic International. The cells were cultivated for six days on collagen-coated recipients and using basal hepatic medium (Biopredic International) supplemented with HepaRG Maintenance and Metabolism Supplement (Biopredic International). During the exposure experiments the basal hepatic medium (Biopredic International) was supplemented with HepaRG Induction Supplement (Biopredic International).

2.3. Publically available data sets

Microarray data (Affymetrix GeneChip Human Genome U133 Plus 2.0 Array) from primary human hepatocytes exposed to APAP and respective vehicle controls were obtained from the toxicology database TG-GATEs (Uehara et al., 2010; Urushidani and Nagao, 2005). These datasets were used for comparative gene expression analysis with own data.

2.4. Clinical human liver samples

Patients diagnosed at the *Universitair Ziekenhuis Leuven* (Belgium) between 2011 and 2013 with ALF due to acetaminophen intoxication, were treated by orthotopic liver transplantation. After surgical removal of the explant liver, human liver tissue samples were obtained and stored at −80 °C before processing for microarray analysis. Samples from healthy livers from individuals deceased from brain damage were made available by the *Universitair Ziekenhuis Brussel* (Belgium). Liver samples from three patients suffering from ALF due to APAP intoxication and liver samples from two individuals deceased from brain damage were used. The study was approved by the ethical committee of the corresponding universities.

2.5. MTT assay

APAP cytotoxicity in hSKP-HPC, HepaRG ad HepG2 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay as previously described (Mosmann, 1983). The different cell cultures were exposed for 24 h to APAP concentrations ranging from 0.33 mM to 33 mM (for hSKP-HPC), 1.7 mM to 87.5 mM (for HepaRG) and 1.0 mM to 50.0 mM (for HepG2). All tests were conducted in triplicate. The 24 hour IC_{10} value for each cell type was determined by 4 parameter logistic nonlinear regression analysis of the obtained dose response curves. This analysis was performed with Masterplex Readerfit 2010 software (Hitashi Solutions, USA). The resulting IC_{10} values were used in further exposure experiments, since these values are conventionally accepted as a compromise between inflicted cytotoxicity (only 10% cell death) and maintenance of sufficient ells for read out measurements.

2.6. Exposure experiments

hSKP-HPC, HepG2 and HepaRG cells were exposed to the respective APAP IC_{10} concentrations and vehicle controls for 24 h. All experiments were conducted in triplicate.

2.7. RNA isolation

After exposure to APAP for 24 h, the exposed cells and respective controls were scraped off and pelleted in RNAse-free 1.5 mL tubes. The pellets were subsequently triturated with a RNA protecting solution composed of RNA stabilization reagent (Qiagen) and respective culture media at a 5:1 ratio (v/v) and frozen at −80 °C until further processing. Total RNA was extracted from all samples using the GenElute Mammalian Total RNA Purification Miniprep Kit (Sigma–Aldrich), according to the manufacturer's instructions. The isolated RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific).

2.8. Microarray

Whole genome expression analysis was performed using the Affymetrix microarray technology (including reagents and instrumentation). For each sample, 100 ng total RNA was amplified using the Genechip 3′ IVT Express Kit (Affymetrix). The amplified RNA (aRNA) was purified with magnetic beads, fragmented with the fragmentation reagent and subsequently hybridized to Affymetrix Human Genome U133 plus 2.0 arrays. The arrays were then placed at 45 °C in a Genechip Hybridization Oven-645 (Affymetrix) for 16 h. After incubation, the arrays were washed on a Genechip Fluidics Station-450 (Affymetrix) and stained with the Affymetrix HWS kit following the manufacturer's instructions. The chips were scanned with an Affymetrix Gene-Chip Scanner-3000-7G, and the quality control matrices were confirmed with Affymetrix Gene Chip Operating Software, following the manufacturer's guidelines.

Background correction, summarization, and normalization of all data were done with Robust Multiarray Analysis (RMA software) and Partek Genomics Suite 6.6. Principal component analysis (PCA) plots were produced using Partek Genomics Suite 6.6. All functional toxicological analyses were performed using Ingenuity Pathways Analysis (IPA, version December 2014; Qiagen). The identification of enriched functional gene classes was done using a Fisher's Exact test *p*-value smaller than 0.05 and a fold up or down regulation of at least two times.

The microarray data discussed in this study have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO Series accession number GSE74000) (Rodrigues et al., 2015b).

2.9. Quantitative polymerase chain reaction

mRNA was reverse-transcribed into cDNA using the iScript-cDNA Synthesis Kit (BioRad), followed by cDNA purification with the GenElute PCR Clean-Up Kit (Sigma–Aldrich). The obtained cDNA samples were used for quantitative amplification of the genes of interest using TaqMan® Gene Expression Assays (Life Technologies). The following genes were evaluated: *CYP3A4* (Hs00430021_m1), *CYP3A5* (Hs01070905_m1) and *CYP3A7* (Hs00426361_m1). Four stable reference genes were used to normalize qPCR data: *B2M* (Hs99999907_m1), *HMBS* (Hs00609296_g1), *UBC* (Hs00824723_m1) and *18S* (Hs99999901_s1). Each run was done in duplicate and included per for each run two negative controls (NTC) and a serial dilution of pooled cDNA mix from all cDNA samples to estimate the qPCR efficiency. The qPCR reaction mix consisted of 12.5 mL TaqMan Universal Master Mix (Applied Biosystems), 1.25 mL 20× Assay-on-Demand Mix (Applied Biosystems), and 2 mL of cDNA in a 25-mL volume adjusted with DNase-/RNase-free water. qPCR conditions, using the iQ5™ Bio-Rad system (BioRad) were as follows: incubation for 10 mins at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, and annealing for 1 min at 60 °C (BioRad). The qPCR efficiency was estimated by the iQ5 Optical System Software (Version 2), and only data with PCR efficiency between 0.85 and 1.15 was used. Results are expressed as the fold changes normalized against the geometric means of these 4 reference gene using Qbase PLUS software (Biogazelle). Statistical analyses were performed by a 1-way unpaired ANOVA

with correction for multiple testing. Gene expressions with a fold change (up-regulation or down-regulation) of at least 2-fold and a corrected *P* value of 0.05 were considered to be significantly different.

3. Results

3.1. Intercomparison of hepatic in vitro systems exposed to APAP

Determination of the sub cytotoxic concentrations of APAP showed that the hepatic *in vitro* systems under evaluation display different sensitivities to the drug. The IC_{10} values found for HepG2 cells, hHEP, HepaRG and hSKP-HPC are 2, 5, 13 and 18 mM, respectively. Consequently the exposure experiments for the evaluation of the gene expression modulation by APAP were carried out at the specific IC_{10} concentrations for each cell type.

Principle component analysis (PCA) (Fig. 1) shows a clear clustering per cell type. Exposure to APAP leads to a slight, but reproducible upwards plot shift (Fig. 1). Significant gene modulation occurs for all cell models and accounts for 3621 genes in hSKP-HPC, 2541 genes in HepaRG, 1125 genes in hHEP and 167 genes in HepG2 cells.

When the expression of the main APAP-metabolizing CYP enzymes was analyzed by QPCR, it could be observed that *CYP3A5*, which is a fetal isoform of *CYP3A4*, is 15-fold up regulated when hSKP-HPC are exposed to APAP (Fig. 2). This could be an indication that the metabolic capacities of these cells are increased by exposure to the drug. In the other cell models, there was an up regulation of other CYPs, but *CYP3A5* remained constant. HepaRG displayed a 7-fold up regulation of *CYP3A4* and showed a significant down regulation of *CYP3A7*. In HepG2, *CYP3A4*, *CYP3A5* and *CYP3A7* expression was not influenced by APAP. Microarray data of hHEP exposed to APAP showed that *CYP3A4*, *CYP3A5* and *CYP3A7* expression was not significantly modulated by APAP. In addition, the expression of *CYP2E1* and *CYP2D6* remained constant in all hepatic cell models.

Pathway analysis of the modulated genes shows the significantly enriched toxicological gene classes due to APAP-exposure in all *in vitro* systems (Table 1). Our data shows significant enrichment of the hepatotoxic gene classes 'Liver Proliferation', 'Liver Fibrosis', 'Liver Necrosis' and 'Liver Damage' in all models. The 'Liver Failure' gene class is significantly enriched in all but HepG2 cells. hSKP-HPC show the highest number of modified genes for all the toxicity gene classes, except for one, 'Liver

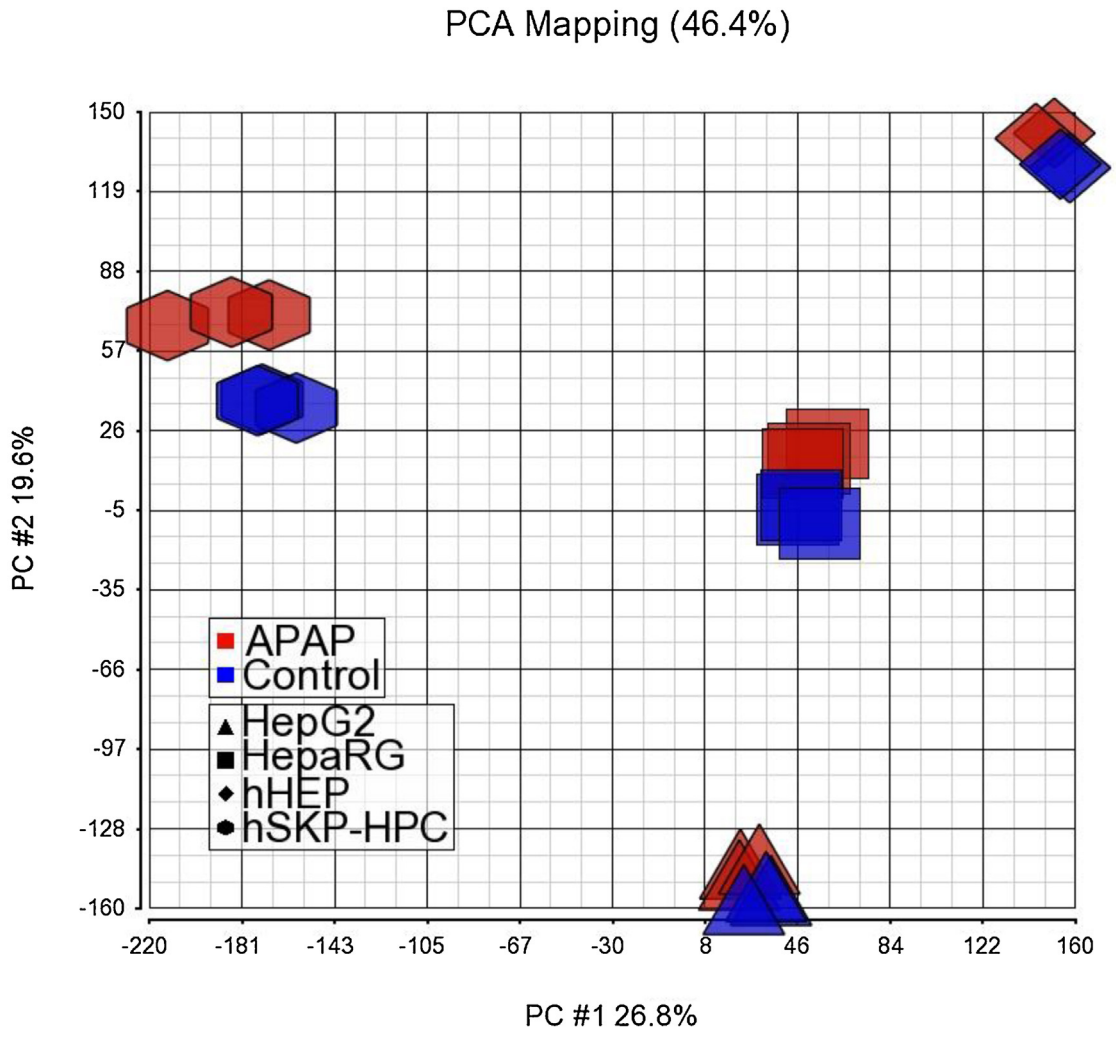

Fig. 1. PCA-plot of microarray data of hepatic *in vitro* cell models HepG2HepaRG, hHEP and hSKP-HPC. All independent cell types group together. Exposure to APAP slightly shifts the plots in all hepatic cell systems upwards (based on analysis of the whole genome (55′000 probesets); the principle variables PC1 and PC2 represent the amount of variance in gene expression between analyzed samples).

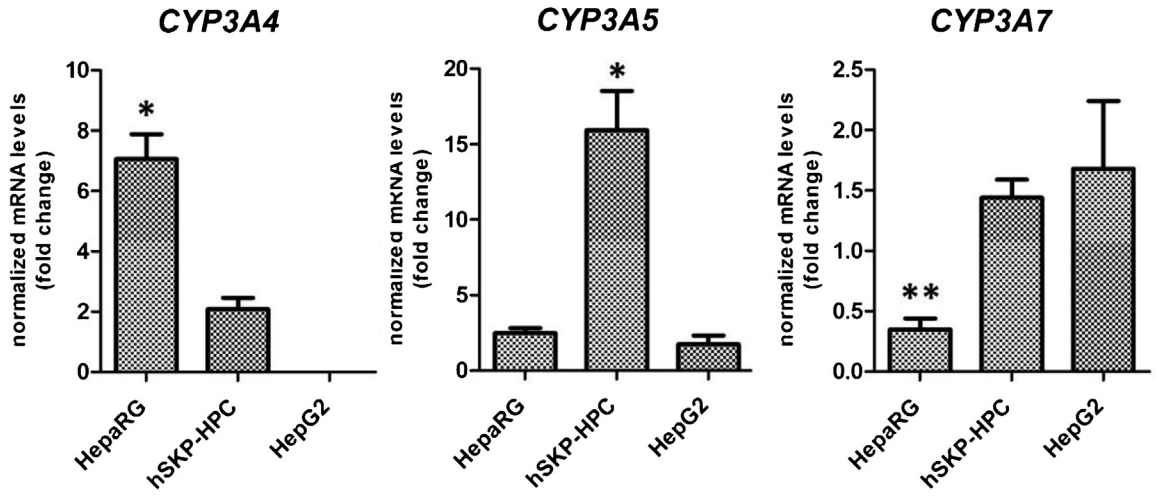

Fig. 2. Modulation of expression of CYP3A4, CYP3A5 and CYP3A7 genes due to exposure to APAP. (* significantly up regulated (one-way ANOVA $P < 0.05$); ** significantly down regulated (one-way ANOVA $P < 0.05$)).

Table 1
Enrichment of APAP-related hepatotoxicity gene classes in human hepatic cell systems exposed to APAP. The selection is based on a gene modulation of at least 2-fold and a Fisher's Exact *p*-value <0.05. (Ratio: identified genes/number of genes in gene class).

	hSKP-HPC		hHEP		HepaRG		HepG2	
	p-value	Ratio (%)	*p*-value	Ratio (%)	*p*-value	Ratio (%)	*p*-value	Ratio (%)
Liver failure	2.42E-02	19.5% (16/82)	3.43E-03	11.0% (9/82)	6.91E-03	17.1% (14/82)	–	–
Liver proliferation	2.18E-03	18.0% (61/339)	2.52E-05	8.8% (30/339)	8.77E-04	13.9% (47/339)	8.93E-03	1.8% (6/339)
Liver fibrosis	7.36E-03	12.8% (51/398)	1.01E-02	6.0% (24/398)	1.31E-03	11.1% (44/398)	3.52E-02	0.8% (3/398)
Liver necrosis	3.17E-03	10.5% (61/583)	7.83E-04	4.3% (25/583)	1.65E-03	9.3% (54/583)	1.78E-02	1.0% (6/583)
Liver damage	1.69E-02	8.4% (55/656)	6.01E-05	5.6% (37/656)	1.33E-05	10.4% (68/656)	4.39E-02	0.8% (5/656)

Damage' which was higher enriched in HepaRG cells. On the contrary, HepG2 has the lowest number of modified genes in every toxicity gene class. The significance of the enrichments of the identified gene classes is more pronounced in hHEP (for 'Liver Proliferation' and 'Liver Necrosis') and HepaRG (for 'Liver Failure', Liver Fibrosis' and Liver Damage').

The overlap of commonly modulated genes due to APAP exposure in hHEP, hSKP-HPC, HepaRG and HepG2 is illustrated in Fig. 3. Fifteen genes are commonly modulated among all investigated *in vitro* systems (Fig. 3A). However, pathway analysis of these genes does not show a specific enrichment of any toxicological gene classes, suggesting that these molecules do not represent hepatotoxicity-specific genes. Yet, by omitting HepG2 cells, 279 common genes between hHEP, hSKP-HPC and HepaRG are obtained (Fig. 3B). Conversely, these genes contribute to the significant enrichment of some of the toxicological categories specific to hepatotoxicity mentioned above (*i.e.* 'Liver Necrosis', 'Liver Proliferation' and 'Liver Fibrosis' with respectively 4, 6 and 6 genes). This low number of commonly modulates genes indicates that every *in vitro* system contributes to the enrichment of the triggered hepatotoxic gene classes (Table 1) in an independent and specific way.

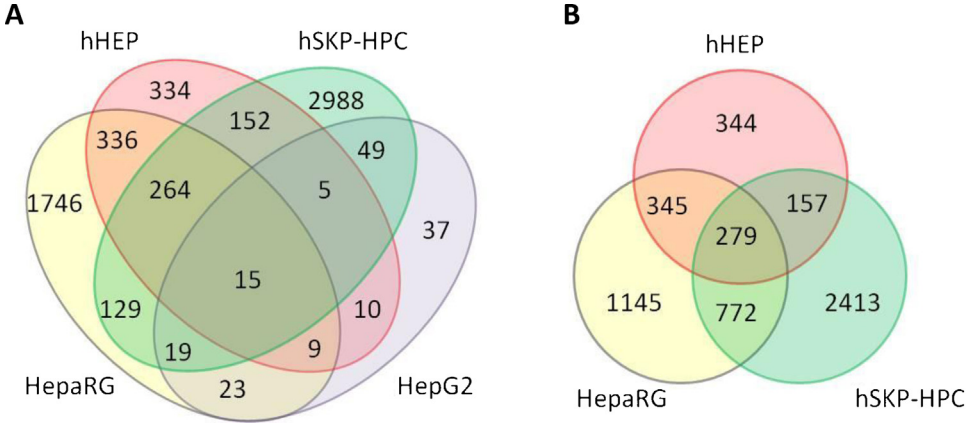

Fig. 3. Overlap of APAP-modulated genes in hHEP, hSKP-HPC, HepaRG and HepG2 exposed to APAP *versus* untreated controls. Selection based on the modulation of at least 2-fold and *p*-value <0.05 (Fisher's Exact).

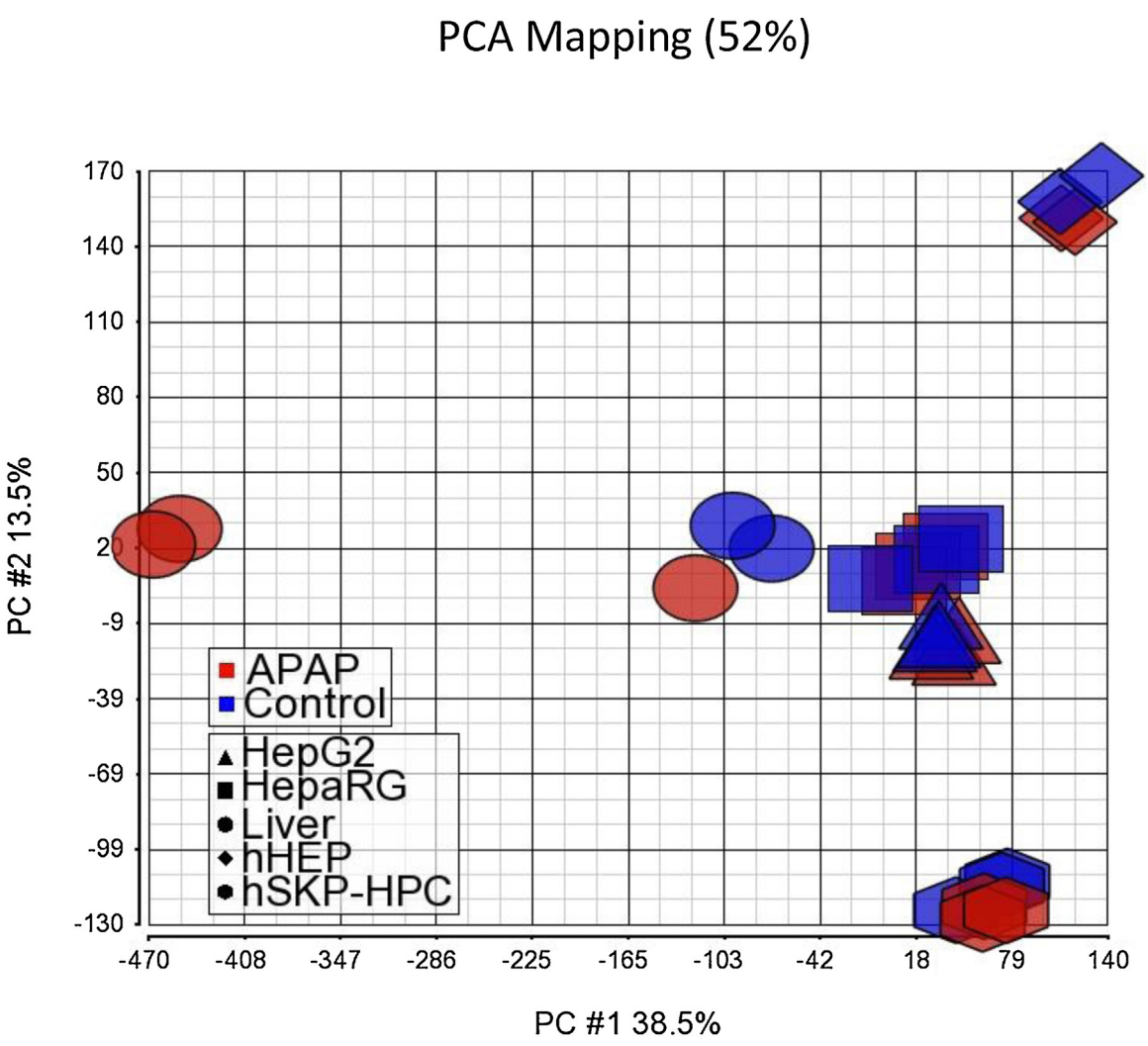

Fig. 4. PCA plot of microarray data of APAP-exposed and non-exposed *in vitro* hepatic cell systems and clinical samples of healthy human livers and of APAP-induced ALF livers (based on analysis of the whole genome (55′000 probesets); the principle variables PC1 and PC2 represent the amount of variance in gene expression between analyzed samples).

3.2. Predictive capacity of hepatic in vitro *systems to APAP-induced ALF*

By analyzing the differences in gene expression between liver samples of patients suffering from APAP-induced ALF and samples from healthy livers, a list of 2754 significant modulated genes was obtained. These genes are considered to relate to changes caused by APAP. In the PCA plot of the microarray data, the highest variance is observed for the clinical ALF-samples (Fig. 4). The control liver samples, on the other hand group nicely together.

Several genes are commonly modulated between the hepatic *in vitro* models exposed to APAP and the APAP-ALF samples (Fig. 5). The highest number of commonly APAP-modulated genes is noticed between the clinical ALF samples and hSKP-HPC, followed by HepaRG and hHEP. Only a very limited number of APAP-modulated genes is common between HepG2 cells and ALF samples. APAP modulation in HepG2 cells shows thus the lowest compliance with the clinical situation. On the contrary, hSKP-HPC and HepaRG have the highest concordance with the clinical samples.

No commonly modulated genes were found among all the *in vitro* models and ALF samples. However, when HepG2 cells are excluded 12 and 17 genes were commonly up and down regulated, respectively (Table 2). These genes could represent potential ALF biomarkers that can be traceable in *in vitro* hepatic cell models.

Based on specific gene expression differences of APAP-induced ALF samples compared to healthy liver, a prediction diagram for the ‘Damage of Liver’ gene class could be established. Fig. 6 illustrates the relevant genes found in ALF that lead to an activation of ‘Damage of Liver’.

In this prediction diagram, the activation of this function is represented by the modulation of 17 genes (4 up regulated and 13 down regulated). Four supplementary genes were added to the diagram, although their modulation in the ALF samples is inconsistent to the activation of the toxicity function. The modulation in expression of the same genes in the different *in vitro* models led to the activation of the ‘Damage of Liver’ function for hHEP, HepaRG and hSKP-HPC, although only one gene *i.e.* IGF1 appeared to be commonly modulated in these *in vitro* systems and ALF samples. HepG2 did not show any activation. HepaRG seems to have the highest similitude with ALF showing the same modulation in 6 out of the 17 genes of the ALF prediction diagram (*i.e. IGF1*, *PAWR*, *TLR3*, *ALB*, *CD44* and *CFH*). Additionally, 2 genes that

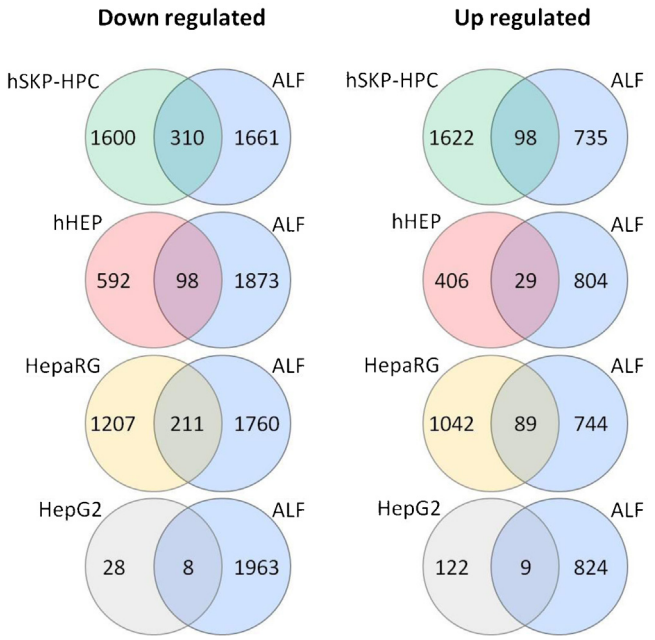

Fig. 5. Commonly modulated genes between *in vitro* systems exposed to APAP and APAP-induced ALF.

were inconsistent with the activation of the gene class in ALF samples, contribute to the activity of the toxicity class in HepaRG (*SH3BP5*, *EGR1*). hSKP-HPC and hHEP modulate both 3 out of the 17 genes correctly (*i.e. IGF1*, *PAWR* and *SAA2* for hSKP-HPC and *IGF1*, *CD44* and *TLR3* for hHEP). Furthermore, 3 (*EGR1*, *IL6ST* and *SH3BP5*) and 2 genes (*EGR1*, *MAPK8*) which are inconsistent with the activation of "Damage of Liver" in ALF samples are correctly modulated in hHEP and hSKP-HPC, respectively. Interestingly, the application of the three *in vitro* systems shows a complementary effect of a total of 11 genes, which lead to the activation of the function. The respective fold changes of these genes were documented in Table 1 of Rodrigues et al. (2015b).

Based on specific gene expression of APAP-induced ALF samples, a prediction diagram for 'Necrosis of Liver' could also be established (See Fig. 2 and Table 2 of Rodrigues et al. (2015b)). All *in vitro* systems showed an activation of this hepatotoxic function base on the same identified genes. HepaRG cells showed the highest number of genes followed by hHEP and hSKP-HPC that identified the same number of genes. In HepG2 cells only one gene contributed to the activation of the function.

4. Discussion

Because of ethical, economic and scientific constraints, the use of experimental animals for toxicological purposes is being increasingly discouraged. Yet, the safety of chemicals including

Table 2
Commonly modulated genes between ALF samples and human hepatic *in vitro* systems (hHEP, HepaRG and hSKP-HPC) exposed to APAP.

Symbol	Gene	hSKP-HPC		hHEP		HepaRG		ALF	
		Fold change	*p*-value	Fold change	*p*-value	Fold change	*p*-value	Fold change	*p*-value
SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	5.7	8.57E-03	2.647	4.47E-02	7.391	4.18E-04	5.826	3.60E-02
GLS	Glutaminase	4.7	6.73E-03	5.186	1.16E-03	4.145	3.10E-04	5.274	2.76E-02
BRD2	Bromodomain containing 2	3.3	5.96E-03	3.228	4.38E-04	2.291	1.50E-03	3.931	4.01E-02
OSBPL8	Oxysterol binding protein-like 8	2.4	7.30E-03	2.395	5.79E-03	2.454	1.26E-03	3.669	2.94E-02
AK9	Adenylate kinase 9	2.5	2.31E-02	2.417	2.69E-02	4.13	1.60E-02	3.174	4.45E-02
MGAT4A	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-*N*-acetylglucosaminyltransferase, isozyme A	4.5	1.38E-05	3.356	2.32E-05	3.403	1.81E-02	2.781	4.20E-03
FNBP1	Formin binding protein 1	3.0	1.82E-03	2.761	1.62E-02	5.483	5.77E-05	2.635	2.06E-02
SLFN5	Schlafen family member 5	3.7	2.54E-02	2.816	3.01E-02	3.673	1.66E-03	2.633	5.29E-03
PGM2L1	Phosphoglucomutase 2-like 1	3.7	4.49E-03	3.067	1.01E-04	5.33	3.23E-03	2.596	3.08E-03
TNPO1	Transportin 1	2.7	2.37E-02	2.013	1.66E-03	2.229	8.78E-05	2.579	4.74E-03
EFR3B	EFR3 homolog B	6.2	9.88E-03	2.415	4.69E-02	3.271	9.39E-03	2.293	4.94E-02
NEAT1	Nuclear paraspeckle assembly transcript 1	3.2	4.41E-02	4.982	4.68E-02	4.256	3.63E-03	2.246	2.33E-02
HSBP1L1	Heat shock factor binding protein 1-like 1	−2.5	2.14E-02	−2.392	2.05E-02	−2.672	8.07E-04	−2.535	1.42E-02
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5	−2.3	9.93E-04	−2.069	3.88E-02	−2.074	1.67E-04	−2.828	2.60E-02
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	−6.2	1.92E-03	−2.121	4.58E-02	−4.852	5.16E-04	−3.269	4.15E-02
NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	−2.2	5.83E-04	−2.515	3.23E-03	−2.63	5.45E-04	−3.312	3.29E-03
GTF2H2B	General transcription factor IIH, polypeptide 2B	−2.5	5.12E-03	−4.061	1.69E-02	−7.914	9.09E-03	−3.622	1.26E-02
KLHL5	Kelch-like family member 5	−3.6	1.05E-04	−2.802	3.80E-04	−3.948	1.32E-05	−3.669	3.81E-02
TM9SF3	Transmembrane 9 superfamily member 3	−2.9	9.71E-04	−2.903	1.76E-03	−4.031	9.97E-05	−4.735	3.29E-02
PARP9	Poly (ADP-ribose) polymerase family, member 9	−4.4	5.36E-05	−2.015	2.48E-02	−3.573	1.05E-03	−4.753	2.36E-02
PDSS2	Prenyl (decaprenyl) diphosphate synthase, subunit 2	−2.1	2.42E-03	−2.078	5.27E-03	−2.057	1.67E-02	−4.875	9.21E-03
APOB	Apolipoprotein B	−10.0	2.77E-03	−2.062	5.92E-03	−2.473	5.05E-04	−5.664	8.73E-03
NNMT	Nicotinamide N-methyltransferase	−2.1	6.54E-03	−5.152	3.04E-04	−3.09	2.50E-04	−6.067	2.77E-02
ELL2	Elongation factor, RNA polymerase II, 2	−3.6	5.71E-04	−2.238	2.28E-02	−6.308	1.24E-03	−6.166	1.56E-03
AGPS	Alkylglycerone phosphate synthase	−10.3	2.90E-03	−2.119	3.44E-03	−3.701	4.35E-06	−6.832	1.49E-03
PAIP1	Poly(A) binding protein interacting protein 1	−2.6	8.87E-04	−2.177	1.99E-02	−3.001	2.94E-04	−7.051	1.16E-03
PSMC2	Proteasome (prosome, macropain) 26S subunit, ATPase, 2	−3.7	6.66E-04	−2.152	3.57E-03	−5.263	3.99E-04	−9.069	1.68E-03
HNRNPD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa)	−4.7	1.31E-02	−2.178	1.25E-02	−7.587	8.26E-03	−9.7	4.64E-02
IGF1	Insulin-like growth factor 1 (somatomedin C)	−48.5	1.24E-03	−6.977	3.52E-02	−15.974	2.07E-02	−104.243	2.81E-02

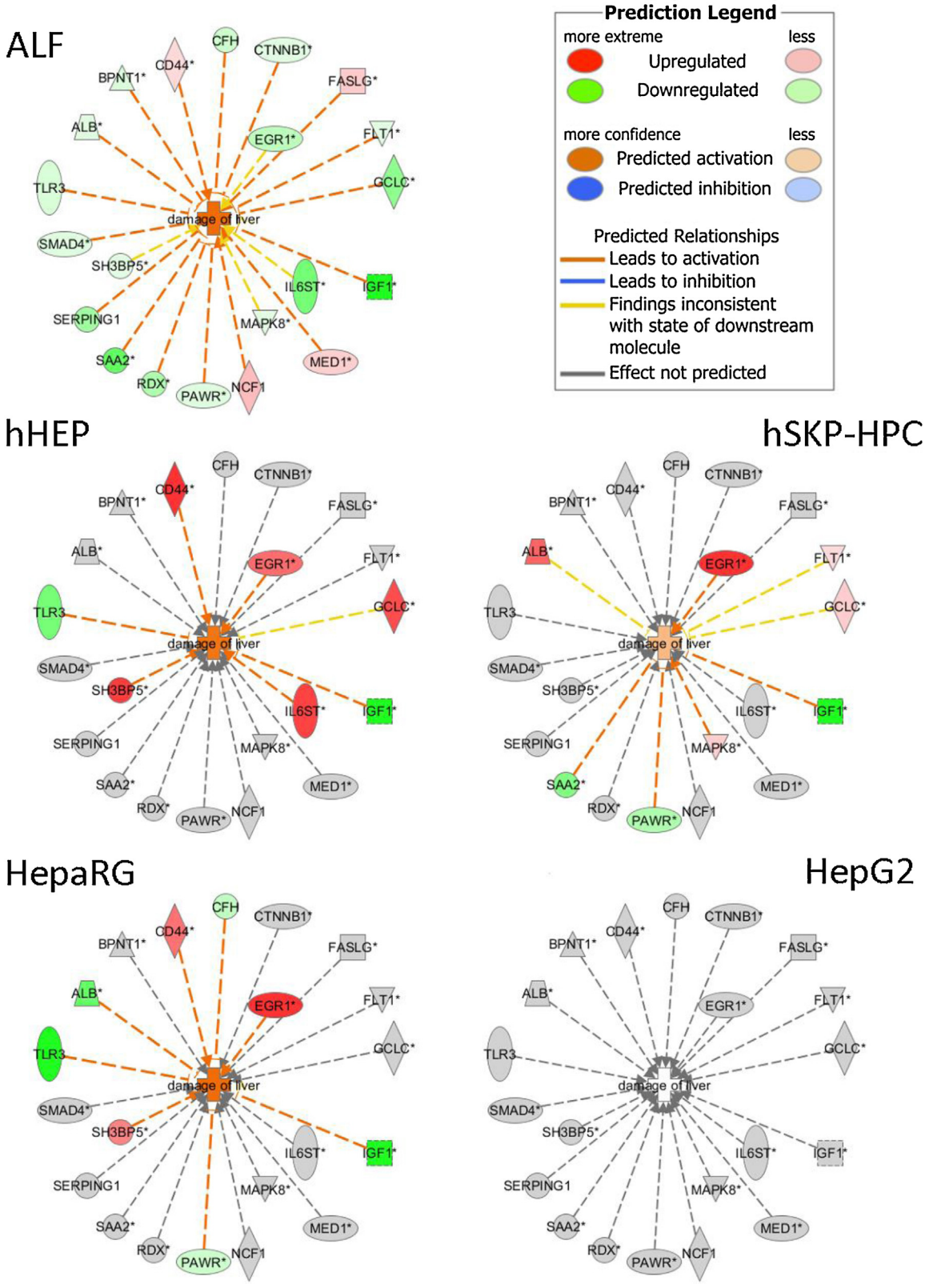

Fig. 6. Prediction diagram for the "Damage of Liver" toxicity gene class after APAP exposure. The selection of genes was based on the significant modulated molecules of this gene class in the APAP-induced ALF samples *versus* control samples. For this selection of genes, the "Damage of Liver" function was activated for hHEP, HepaRG and hSKP-HPC, but not for HepG2 (diagram prepared using IPA). Detailed gene expression fold changes are summarized in Table 1 of Rodrigues et al. (2015b).

novel pharmaceutical molecules need to be assessed to protect human health. Since drug-induced liver injury is the major cause for the discontinuation of the development of NCE, the potential induction of hepatic toxicity must be evaluated during preclinical drug development. Today the most commonly used hepatic *in vitro* models for toxicity testing consist of primary human hepatocytes and hepatic cell lines such as HepaRG and HepG2. Human primary hepatocytes are difficult to obtain and can only be maintained in culture for a limited period of time because of dedifferentiation and loss of specific phenotype. Cell lines are of tumorous origin and do not adequately represent the population diversity. New developments in stem cell research are also creating new cell-based alternatives for hepatotoxicity testing since stem cells represent a virtually inexhaustible cell source and have the ability to differentiate towards multiple cell types, including liver cells. In our research group, multipotent post natal stem cells are isolated from human skin samples (De Kock et al., 2012). These so-called human skin-derived precursors (hSKP) can be differentiated to hepatocyte-like cells following a protocol that mimics liver development *in vivo* (Rodrigues et al., 2014, 2015a; De Kock et al., 2009; Uehara et al., 2010). We previously demonstrated that when these differentiated cells (hSKP-HPC) are exposed to a known hepatotoxic compound, such as APAP of sodium valproate, they trigger important hepatotoxic pathways (Rodrigues et al., 2014; Rodrigues et al., 2015a).

Basal levels of several CYPs were found to be minimal in hSKP-HPC (Rodrigues et al., 2014). However, it must be noticed that the xenobiotic to which the cells are exposed may influence the expression of these biotransformation enzymes. We observed for example, that the expression of *CYP3A5*, which is a fetal isoform of *CYP3A4*, is highly up regulated when hSKP-HPC are exposed to APAP. This could be an indication that the metabolic capacities of hSKP-HPC are induced by this drug and as such accelerate its own metabolism.

In the current study we compared the effects of APAP in hSKP-HPC to those induced in commonly used *in vitro* models of human origin, namely hHEP, HepaRG and HepG2 and evaluated the prediction potential of each model by comparing the results with those generated by the analysis of liver samples of patients suffering from ALF induced by APAP. The availability of these samples is scarce. In addition, the quality of ALF liver samples is per definition poor due to the high amount of necrotic tissue. Therefore, only a limited number of clinical samples could be analyzed. Addition of supplementary samples of APAP-induced ALF can in the future fine-tune the currently obtained results.

Enrichment of hepatotoxicity gene classes related to APAP toxicity, was observed in all the studied hepatic *in vitro* models. HepG2 cells performed less good and did not show a significant enrichment of the 'Liver Failure' toxicity gene class. These cells also consistently modulated a lower number of genes that contributed to significant enrichment of other typical hepatic toxicity gene classes. Even for the gene classes that were unanimously triggered in the four *in vitro* systems under examination, HepG2 showed the lowest enrichment values. The low performance of HepG2 cells can be related to the fact that these cells lost, since their introduction in the late 1980s (Sassa et al., 1987), a substantial set of liver specific functions, including the expression of major CYPs involved in xenobiotic metabolism (Guguen-Guillouzo and Guillouzo, 2010). Another possible reason for the difference between HepG2 and the other cell systems is that HepG2 cells are proliferative and therefore show a lower APAP IC_{10} value than the other three models. In this case the IC_{10} value is calculated based on the combination of inflicted cellular death as well as inhibition of proliferation.

The hSKP-HPC model performed rather well. It consistently showed a higher enrichment of liver toxicity-specific gene classes. hHEP and HepaRG cells modulated less genes, but showed a higher accuracy in the enrichment of the toxicity gene classes. Furthermore, the highest number of commonly modulated genes could be shown between hSKP-HPC and ALF samples, followed by HepaRG, hHEP and HepG2.

HepaRG cells seem to have the highest predictive capacity for ALF. This cell line showed the highest concordance with both 'Damage of Liver' and 'Necrosis of Liver' functions identified by the modulation in clinical APAP-induced ALF samples. hHEP and hSKP-HPC follow closely, also showing a significant activation of these functions. HepG2 on the other hand did not show an activation of 'Damage of Liver'.

Overall, hSKP-HPC performed well when compared to the classical hepatic *in vitro* systems. This recently introduced skin stem cell-derived model shows an accurate response to APAP exposure. Further experiments should confirm these results at the protein and metabolic levels. However, this study embodies already the potential of hSKP-HPC to elucidate hepatotoxic effects that chemical compounds might cause and facilitates its acceptance as an adequate human hepatic *in vitro* model.

Conflict of interest

There is no conflict of interest.

Acknowledgements

This research has received funding from the Fund for Scientific Research in Flanders (FWO), the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 266838 (DETECTIVE) and from ISRIB (Brustem) and BELSPO (IAP-HEPRO).

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