Human Skin-Derived Stem Cells as a Novel Cell Source for In Vitro Hepatotoxicity Screening of Pharmaceuticals

Robim M. Rodrigues,¹ Joery De Kock,¹ Steven Branson,¹ Mathieu Vinken,¹ Kesavan Meganathan,² Umesh Chaudhari,² Agapios Sachinidis,² Olivier Govaere,³ Tania Roskams,³ Veerle De Boe,⁴ Tamara Vanhaecke,¹ and Vera Rogiers¹

Human skin-derived precursors (hSKP) are postnatal stem cells with neural crest properties that reside in the dermis of human skin. These cells can be easily isolated from small (fore) skin segments and have the capacity to differentiate into multiple cell types. In this study, we show that upon exposure to hepatogenic growth factors and cytokines, hSKP acquire sufficient hepatic features that could make these cells suitable in vitro tools for hepatotoxicity screening of new chemical entities and already existing pharmaceutical compounds. Indeed, hepatic differentiated hSKP [hSKP-derived hepatic progenitor cells (hSKP-HPC)] express hepatic progenitor cell markers (*EPCAM*, *NCAM2*, *PROM1*) and adult hepatocyte markers (*ALB*), as well as key biotransformation enzymes (*CYP1B1*, *FMO1*, *GSTA4*, *GSTM3*) and influx and efflux drug transporters (*ABCC4*, *ABCA1*, *SLC2A5*). Using a toxicogenomics approach, we could demonstrate that hSKP-HPC respond to acetaminophen exposure in a comparable way to primary human hepatocytes in culture. The toxicological responses “liver damage”, “liver proliferation”, “liver necrosis” and “liver steatosis” were found to be significantly enriched in both in vitro models. Also genes associated with either cytotoxic responses or induction of apoptosis (*BCL2L11*, *FOS*, *HMOX1*, *TIMP3*, and *AHR*) were commonly upregulated and might represent future molecular biomarkers for hepatotoxicity. In conclusion, our data gives a first indication that hSKP-HPC might represent a suitable pre-clinical model for in vitro screening of hepatotoxicity. To the best of our knowledge, this is the first report in which human postnatal stem cells derived from skin are described as a potentially relevant cell source for in vitro hepatotoxicity testing of pharmaceutical compounds.

Introduction

THE HIGH ATTRITION RATE of new chemical entities (NCEs) represents a major concern for the pharmaceutical industry and considerably increases the cost of developing new drugs [1,2]. One of the major causes for the discontinuation of drug candidates is hepatic toxicity [3] and drug-induced liver injury (DILI) accounts for most cases of acute liver failure (ALF) [4]. Detection of hepatotoxicity, however, often occurs only late during drug development, jeopardizing the potential marketing of these molecules [3]. Therefore, any improvement that accelerates hepatotoxicity detection is extremely valuable. Such improvement could be the implementation of new alternative methods during early drug development. Because of ethical, economical, and scientific constraints, the use of experimental animals for

toxicological purposes is indeed being increasingly discouraged. For instance, it has been documented that animal toxicity data does not necessarily correlate with human results [5,6]. In this respect, 3R (Refinement, Reduction, Replacement) strategies [6] have been introduced not only in the European legislation, but also worldwide, although to a limited extent. In Europe, the recently implemented chemical legislation Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) calls for safety evaluation of chemicals using a minimum of experimental animals [7]. Testing and marketing bans have been put in place, making animal use forbidden for toxicological testing of cosmetics and their ingredients [8,9]. Furthermore, in the pharmaceutical industry, consensus is growing to use alternative methods to animal testing whenever possible [10].

¹Department of Toxicology, Center for Pharmaceutical Research, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel (VUB), Brussels, Belgium.

²Institute of Neurophysiology, Center of Physiology, University of Cologne, Cologne, Germany.

³Department of Morphology and Molecular Pathology, Katholieke Universiteit Leuven (KUL), University Hospital Leuven, Leuven, Belgium.

⁴Department of Urology, UZ Brussels, Brussels, Belgium.

Since the liver is a first-line target organ during toxicant assault, human-based in vitro hepatic cell systems are valuable tools to study, the potential hepatotoxicity of pharmaceuticals and other substances. Freshly isolated human hepatocytes represent the model that most appropriately reflects the in vivo situation and are considered as the gold standard in the area of liver-based in vitro modelling [11]. Human hepatocytes, however, are difficult to obtain because of intensified liver transplantation programmes. In addition, these cells are phenotypically unstable and have a limited life span [11]. Therefore, immortalized human liver cell lines, including HepG2 [12,13], Fa2N-4 [14], and HepaRG [15] have become increasingly popular as in vitro models to study human liver function and toxicity. These cell lines are readily available and can be kept in culture for long periods of time, but they suffer from genotypical instability and decreased or even absent metabolic capacity. In addition, currently available cell lines do not represent population diversity [16]. Stem cells could form a novel and attractive alternative human cell source to develop in vitro systems for predicting liver toxicity. Indeed, the establishment of continuous cell lines from embryonic stem cells [17] and induced pluripotent stem cells [18], the advancements in isolating and culturing adult stem cells (ASC) [19] and in particular the breakthrough in differentiating them into cells with particular functionalities brought huge expectations to the scientific and industrial community.

ASC reside in specialized stem cell niches [20] from where they contribute to tissue renewal and/or regeneration [21]. These cells have the ability to give rise to a range of cells of the tissue within which they reside.

ASC can be isolated from a wide range of tissues and are able to differentiate towards their hepatic progeny. Among others, ASC isolated from bone marrow [22], adipose tissue [23], umbilical cord matrix [24], umbilical cord epithelial cell layer [25], and placenta [26] have been successfully differentiated towards cells with hepatic characteristics. In this study, human skin-derived precursors (hSKP) and their hepatic derivatives are evaluated.

hSKP are a multipotent cell population isolated from human dermal (fore)skin [27–29]. These neural crest related ASC can also be isolated from skin of the abdomen [30], breast [30], arm [31], face [32], and scalp [33] mostly upon aesthetic interventions. These cells have a high self-renewal and high multipotent differentiation capacity. More specifically, it was previously shown by our group that hSKP, besides their ectodermal and mesodermal differentiation potential, can be directed towards the hepatic lineage [34–36] upon sequential exposure to growth factors and cytokines that mimic liver development in vivo [22].

The present study focuses on the actual application of hepatic differentiated hSKP as a potential novel in vitro model for hepatotoxicity screening of chemical substances, in case pharmaceuticals. Acetyl-para-aminophenol, acetaminophen (APAP), which is a common over-the-counter analgesic considered to be safe when used at therapeutic doses, is used as a proof-of-principle reference compound. When taken in overdose, APAP becomes hepatotoxic and can cause ALF, the latter being a leading cause of DILI [37]. The toxic mechanism of APAP in adult hepatocytes is known to involve several toxicity pathways [38,39]. It is acknowledged that APAP is metabolically activated to *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is normally detoxified by glutathione.

Depletion of glutathione after APAP overdose leads to NAPQI accumulation that adversely binds to different cellular proteins causing toxicity. Here we evaluate to what extent APAP exposure modulates the whole genome expression of hepatic differentiated hSKP versus primary human hepatocytes by using a full genome microarray platform.

Materials and Methods

Isolation and cultivation of hSKP

hSKP were isolated from foreskin circumcision samples of 1- to 10-year old boys after informed consent of the parents. The cells were isolated as previously described [36]. After isolation, cells were seeded at a density of 7.5×10^4 viable cells/mL in growth medium composed of Dulbecco's modified Eagle medium (DMEM)+GLUTAMAX/F12 Nutrient Mixture (3:1; Life Technologies) supplemented with 7.3 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, and 20 ng/mL epidermal growth factor (both from Promega). The cells were cultured in suspension at 37°C in a humidified incubator under an atmosphere of 5% (v/v) CO₂. Every 2–3 days the growth medium was replenished. After 2 weeks of culture, the hSKP spheres were passaged using 0.2 mg/mL Liberase DH solution (Roche Applied Science). Obtained single cells were seeded at a density of 1.3×10^4 cells/cm². After a second passage (7 days later), sufficient cells were obtained to establish a small cryopreserved cell bank. hSKP were collected in a cryoprotectant solution composed of 90% (v/v) fetal bovine serum (Hyclone) and 10% (v/v) dimethylsulfoxide (cell culture grade; Sigma-Aldrich) at a density of 1×10^6 cells/mL and preserved in liquid nitrogen. All further experiments were based on these batches to rule out donor variability.

Hepatogenic differentiation of hSKP

hSKP were differentiated after a slightly modified protocol established previously by our group [36]. In essence, cells were seeded on collagen type 1 (BD Biosciences)-coated 24-well plates and T75 culture flasks (both BD Falcon) and cultured until 90% confluence in Basal Medium (BM) was reached. BM consisted 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 (Life Technologies), 0.1 mM L-ascorbic acid (Sigma-Aldrich), 4 mg/L L-nicotinamide (Sigma-Aldrich), 1 mg/mL linoleic acid-albumin (Sigma-Aldrich) and 27.3 mg/mL sodium pyruvate (Sigma-Aldrich). The differentiation protocol was started when the cells reached 90% cell confluence. At day 0, BM was supplemented with 50 ng/mL activin A (Life Technologies). At day 1, BM was supplemented with 25 ng/mL activin A (Life Technologies), 5 ng/mL FGF4 (Biosource) and 10 ng/mL BMP4. At day 3, BM was supplemented with 10 ng/mL FGF4 (Biosource) and 20 ng/mL BMP4. At day 6, BM was supplemented with 5 ng/mL FGF4 (Biosource), 10 ng/mL BMP4, 30 ng/mL hepatocyte growth factor (HGF) (Life Technologies) and 0.5% (v/v) insulin-transferrin-sodium-selenite (ITS) (Sigma-Aldrich) (final). At day 9, BM was supplemented with 30 ng/mL HGF (Life Technologies), 0.25%

(v/v) ITS (Sigma-Aldrich) and 0.02 µg/mL dexamethasone (Sigma-Aldrich). At day 12, BM was supplemented with 30 ng/mL HGF (Life Technologies) and 0.02 µg/mL dexamethasone (Sigma-Aldrich). At day 15, 18, and 21, BM was supplemented with 30 ng/mL HGF (Life Technologies), 0.02 µg/mL dexamethasone (Sigma-Aldrich) and 10 ng/mL oncostatin M (Life Technologies). The differentiation protocol was completed at day 24.

Human liver samples

Human liver biopsy samples from deceased healthy persons were obtained from the UZ Brussel upon informed consent of the family and approval of the Ethics Committee. Small pieces of liver tissue were snap frozen and further stored in liquid nitrogen.

Cytotoxicity assessment and IC₁₀ determination

After 24 days of hepatogenic differentiation, cells were exposed for 24 h to six concentrations of APAP (Sigma-Aldrich) ranging from 5 to 500 µg/mL (corresponding to 33 µM to 3.3 mM, respectively). Subsequently, the effect of APAP on cell viability was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay as previously described [40]. The 24-h IC₁₀ value was determined by a four-parameter logistic nonlinear regression analysis of the obtained dose response curves, using Masterplex Readerfit 2010 software (Hitachi Solutions). The test was conducted in triplicate.

Isolation of total RNA

Treated and untreated cells were collected in a RNA protecting solution composed of RNA stabilization reagent (Qiagen) and BM (5:1). Cell cultures of three 75 cm² culture flasks were used per condition. Total RNA was extracted from all samples using a GenElute Mammalian Total RNA Purification Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions. Purity and quantification of the isolated RNA were determined by spectrophotometric analysis using a Nanodrop spectrophotometer (Thermo Scientific).

Microarray data analysis

Microarray technologies from Affymetrix (including reagents and instrumentation) were used for whole genome expression analysis. One hundred nanogram total RNA per sample was amplified using a Genechip 3' IVT Express Kit, following the manufacturer's instructions (Affymetrix). The amplified RNA (aRNA) was purified with magnetic beads and 15 mg Biotin-aRNA was treated with the fragmentation reagent. 12.5 mg fragmented aRNA was subsequently hybridized to Affymetrix Human Genome U133 plus 2.0 arrays and placed in a Genechip Hybridization Oven-645 (Affymetrix) rotating at 60 rpm at 45°C for 16 h. After incubation, the arrays were washed on a Genechip Fluidics Station-450 (Affymetrix) and stained with an Affymetrix HWS kit as indicated by the manufacturer's protocols. The chips were scanned with an Affymetrix Gene-Chip Scanner-3000-7G, and quality control matrices were confirmed with Affymetrix GCOS software, following the manufacturer's guidelines. Microarray data of human hepatocyte cultures (hHEP) established from human cryopreserved hepatocytes were

obtained from the toxicology database TG-GATEs. The latter being established by a 5-year collaboration between the National Institute of Health Sciences and 17 pharmaceutical companies in Japan [41,42]. Only data from cells exposed for 24 h to 5 mM APAP and the respective vehicle controls was used for comparative gene expression analysis with self obtained data. Background correction, summarization, and normalization of all data were carried out with Robust Multitarray Analysis [43]. For principal component analysis (PCA) and determination of differential gene expression, output data files were analyzed with GeneSpring GX v11.5 software (Agilent Technologies). Functional toxicological analyses were performed using Ingenuity Pathways Analysis (version SEP 2011; Ingenuity Systems) with a Fisher's Exact test (*P* value < 0.05) to identify enriched functional gene classes.

Reverse transcriptase quantitative polymerase chain reaction

After total RNA isolation, as described above, mRNA was reverse-transcribed into cDNA using an iScript-cDNA Synthesis Kit (BioRad), followed by cDNA purification with a GenElute PCR Clean-Up Kit (Sigma-Aldrich). These cDNA products were then quantitatively amplified using Taqman probes and primers specific to the target genes of interest. The gene expression assays used in this study are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). All samples were done in duplicate, and each run included two no template controls and a serial dilution of a pooled cDNA mix from all cDNA samples to estimate the quantitative polymerase chain reaction (qPCR) efficiency. The qPCR reaction mix consisted of 10 µL TaqMan Fast Advanced Master Mix (Life Technologies), 1 µL 20× Assay-on-Demand Mix (Life Technologies), and 2 µL of cDNA in a 20 µL volume adjusted with DNase-/RNase-free water. qPCR conditions, using a StepOne Plus system (Life Technologies) were as follows: incubation for 20 s at 95°C, followed by 40 cycles of 1 s denaturation at 95°C, and annealing for 20 s at 60°C (Life Technologies).

qPCR data analysis

The qPCR efficiency was estimated by StepOne Plus System's Software, and only data with PCR efficiency between 0.85 and 1.15 was used. Four stable reference genes were identified to normalize the qPCR data as determined by geNorm (Biogazelle). These were glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*), hydroxy-methylbilane synthase (*HMBS*), and ubiquitin C (*UBC*). Results are expressed as fold changes normalized against the geometric mean of these four reference genes using qbasePLUS software (Biogazelle). Statistical analyses were performed by a one-way unpaired analysis of variance test with correction for multiple testing. Gene expressions with a fold change (up- or downregulation) of at least two and a corrected *P* value of 0.05 or lower were considered to be significantly different.

Immunocytochemistry

Cells were fixated with 4% (w/v) paraformaldehyde, and then further exposed to 100 mM glycine. After permeabilization with 0.1% (v/v) Triton X, nonspecific sites were blocked

with 10% (v/v) donkey serum. Immunolocalization was obtained by incubation of the cells with the following primary antibodies: antialbumin (Alb; Bethyl Laboratories-A80-129F), antiaryl hydrocarbon receptor (Ahr; Santa Cruz-8089c), antihepatocyte nuclear factor (Hnf) 4 α (Santa Cruz—8987), anti-Hnf1 α (Santa Cruz—10791), anticytokeratin (Ck) 18 (Abcam—ab93741), anti-Ck19 (Abcam—ab76539), anti-GATA motif binding factor (GATA) 6 (Abcam—ab22600) antiepithelial cell adhesion molecule (Epcam; Sigma-Aldrich—SAB3300055), anticytochrome P450 (CYP) 1b1 (Sigma-Aldrich—HPA026863), antilflavin containing monooxygenase (Fmo) 1 (Sigma-Aldrich—HPA023680), antineural cell adhesion molecule (Ncam) 2 (Sigma-Aldrich—HPA030900) and antiprominin (Prom) 1 (Miltenyi Biotec—130-090-851). The secondary antibodies used were: anti-rabbit DyLight 488 (711-485-152), anti-mouse DyLight 488 (715-485-150) and anti-goat DyLight 488 (705-485-147) obtained from Jackson ImmunoResearch Europe. After washing with phosphate buffered saline, the cells were mounted with Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories) for nuclear staining and bleaching protection. Images were obtained by fluorescence microscopy (Nikon Eclipse Ti-S).

Results

Characterization of hSKP-derived hepatic progeny

Hepatogenic differentiation strongly modulates the gene expression of hSKP. After 24 days of sequential exposure to hepatogenic growth factors and cytokines, 6685 and 4245 genes are at least twofold up or downregulated, respectively (Student *t*-test with *P* value < 0.05). A 10-fold upregulation or more is observed for 299 genes, whereas 86 genes are at least 10-fold downregulated. As illustrated by the PCA plots shown in Fig. 1A, samples of both undifferentiated (hSKP) and hepatic differentiated hSKP, further described as hSKP-derived hepatic progenitor cell (hSKP-HPC), group well together. The latter shows the robustness of the cell culture conditions and the high reproducibility of the microarray datasets. These plots also highlight a shift of the hSKP-HPC towards hHEP and human liver samples (LIVER). Further analysis of the hSKP-HPC shows that, compared to undifferentiated hSKP, these cells express significantly higher levels of typical hepatic progenitor cell markers, including *EPCAM*, GATA motif binding protein 6 (*GATA6*), prominin 1 (*PROM1*), *NCAM*, SMAD family member 4 (*SMAD4*), stem cell factor receptor (*KIT*), and thymocyte differentiation antigen 1 (*THY1*) (Fig. 1B). For some of these markers, the observations made are confirmed at the protein level (Fig. 2). Immunocytochemistry analysis also shows that hSKP-HPC express markers typical of more mature hepatic cells, such as albumin, HNF 1 α and 4 α (HNF1 α , HNF4 α) and Ahr (Fig. 2). 27% ($\pm$ 5%) of hSKP-HPC (counted in three independent experiments) are albumin-positive. This value represents the yield of hepatogenic differentiation. Additionally, it can be observed that *Gata6*, *Hnf1 α* , *Hnf4 α* , *C-Kit*, *Ahr*, and *Fmo1* are mainly located in the nucleus. Albumin, *Prom1*, *Ncam2*, and *Cyp1B1* are as expected mainly cytoplasmatic or membrane-bound.

Comparison between hSKP-HPC and hHEP with respect to the expression levels of typical hepatic markers shows that the major cytochrome P450 enzymes (CYP) are expressed at

a lower level in hSKP-HPC than in hHEP (Fig. 1C). *CYP1A1* and *CYP1A2* reach 48% of the hHEP expression levels and *CYP2D6*, *CYP3A4*, *CYP3A7* and *CYP2E1* get to 23%, 16%, 13%, and 2%, respectively. Interestingly, the expression of *CYP1B1* is a 100-fold higher in hSKP-HPC than in hHEP (Fig. 1C). The expression of the flavin-containing monooxygenase 1 (*FMO1*), which is a typical fetal liver phase I enzyme, is ten times higher in hSKP-HPC than in hHEP (Fig. 1D). *FMO2* and *FMO4*, which are in general found in adult human liver, achieve levels comparable to those of hHEP (Fig. 1D). *FMO3* expression on the contrary stays 10 times lower than in hHEP (Fig. 1D). The gene expression of monoamine oxidases A and B (*MAOA*, *MAOB*), another class of phase I oxidation enzymes, stays lower in hSKP-HPC than in hHEP (Fig. 1D). The phase II hepatic enzymes glutathione S-transferase *GSTA4* and *GSTM3* are found to be highly expressed in hSKP-HPC and reach levels that are, respectively, three and ninefold higher than those found in hHEP (Fig. 1E). Other phase II enzymes that is, *GSTM1* and *GSTK1* reach 52% and 72% of the expression levels of hHEP, respectively. UDP glucuronosyltransferase 1A (*UGT1A*) and *GSTA1* expression is dramatically lower in hSKP-HPC than in hHEP (Fig. 1E). Typical phase 0 hepatic uptake drug transporters, including solute carrier family 10A1 (*SLC10A1* or *NTCP*) and *SLC22A1* (*OCT1*) are barely expressed at the gene level in hSKP-HPC. On the contrary, other influx transporters, as for instance *SLC2A5* is up to 50-fold higher expressed in hSKP-HPC than in hHEP (Fig. 1F). Phase III efflux transporters, including ATP-binding cassette G2 (*ABCG2* also referred to as *BCRP*), *ABCC4* (*MRP4*), and *ABCA1*, known to be present in both adult and perinatal liver, are also significantly higher expressed in hSKP-HPC (Fig. 1F).

In general, when we evaluate the downstream effects of the modulated genes in hSKP-HPC, an enrichment of the "Gene Classes" related to "Development of Liver" is observed. The latter is composed of 116 known genes. Compared to undifferentiated hSKP, 56 of these are modulated in hSKP-HPC, out of which 37 are upregulated (Table 1). The Venn-diagram in Fig. 3 shows the liver development class genes that are upregulated in hSKP-HPC, hHEP and LIVER in comparison to undifferentiated hSKP. Eight genes are commonly upregulated in the three systems. *GATA6* is 15-fold upregulated in hSKP-HPC. Other highly upregulated (>10-fold) genes are Toll-like receptor 4 (*TLR4*), suppressor of cytokine signaling 3 (*Socs3*) and protein tyrosine phosphatase 12 (*PTPN12*). The top downregulated genes (>10-fold decrease) are *SLC20A1* and vascular endothelial growth factor A (*VEGFA*).

Predictive capacity of hSKP-HPC for APAP-induced hepatotoxicity

By exposing hSKP-HPC to a range of APAP concentrations for 24 h and applying MTT viability tests, subcytotoxic concentrations of APAP could be determined. From a total of three tests an average IC₁₀ value of 18 mM was calculated. The subcytotoxic concentration of APAP used in hHEP was 5 mM, which corresponds to the value previously described [42]. PCA of the microarray data shows highly reproducible results for the in vitro samples (Fig. 4). hSKP-HPC and hHEP exposed to IC₁₀ of APAP [hSKP-derived hepatic progenitor cells exposed to acetaminophen (hSKP-HPC + APAP)

FIG. 1. Characterization of human skin-derived precursors (hSKP)-hepatic progenitor cells. **(A)** Principal component analysis (PCA)-plots of microarray data of undifferentiated hSKP, differentiated hSKP [hSKP-derived hepatic progenitor cells (hSKP-HPC)], human hepatocyte cultures (hHEP), and human liver samples. **(B)** Gene expression (fold change increase) of hepatic progenitor cell markers in hSKP-HPC versus undifferentiated hSKP. **(C, D)** Gene expression of phase I biotransformation enzymes of hSKP-HPC versus hHEP. **(E)** Gene expression of phase II hepatic enzymes of hSKP-HPC versus hHEP. **(F)** Gene expression of phase 0 hepatic up-take transporters and phase III efflux transporters of hSKP-HPC versus hHEP. [§]Significantly increased gene expression in hSKP-HPC versus undifferentiated hSKP (fold change >2; *t*-test *P* value <0.05). *Significantly increased gene expression in hSKP-HPC versus hHEP (fold change >2; *t*-test *P* value <0.05). **Significantly decreased gene expression versus hHEP (fold change <2; *t*-test *P* value <0.05).

and human hepatocyte cultures exposed to acetaminophen (hHEP+APAP)], as well as the respective control samples cluster individually. From the same PCA plot, it can also be noticed that APAP exposure results in a more pronounced shift of hSKP-HPC as compared to hHEP. Upon APAP exposure, a total of 5,591 genes is significantly (Student *t*-test with *P* value <0.05) modulated (minimal twofold) in hSKP-HPC, out of which 2,646 and 2,945 genes are up or down-regulated, respectively. In hHEP, only 511 and 868 genes are significantly (Student *t*-test with *P* value <0.05) up and down-regulated, respectively (minimal twofold). Functional analysis of the differentially expressed genes reveals the enrichment of gene classes of specific toxicological functions. As such, five liver-related "Toxicological Gene Classes" (Liver Damage, Liver Proliferation, Liver Necrosis/Cell Death, Liver Steatosis and Liver Hepatitis) could be identified with high accuracy (Fisher's Exact *P* value <0.05) in both hSKP-

HPC and hHEP exposed to APAP (Table 2). The hSKP-HPC+APAP data could also identify two additionally relevant toxicological functions (Liver Steatohepatitis and Liver Edema). The percentage of modulated genes in each function was consistently higher in the hSKP-HPC+APAP samples than in the hHEP+APAP samples. The identified toxicological classes can be further divided in subfunctions. Though, these subfunctions do not always correlate among hSKP-HPC+APAP and hHEP+APAP (Table 3). More specifically, the Liver Damage gene class is divided in four subfunctions in hSKP-HPC+APAP and two different subfunctions in hHEP+APAP. The Liver Proliferation Class on the other hand has two common subfunctions in both cell types, but an extra three subfunctions in hHEP+APAP. Six subfunctions of the Liver Necrosis/Cell Death Class are enriched in hSKP-HPC+APAP and four in hHEP+APAP. Only one of these, however, is common to both cell systems.

FIG. 2. hSKP-HPC immunocytochemistry stainings of hepatic progenitor cell markers, as well as specific markers of mature hepatocytes. Color images available online at www.liebertpub.com/scd

Four subfunctions of Liver Hepatitis are identified in hHEP+APAP out of which one is common to hHEP+APAP and hSKP-HPC+APAP. Liver Steatosis is composed of two subfunctions, of which each is identified by a different cell system. The number of modulated genes of each subfunction is consistently higher in hSKP-HPC+APAP compared to hHEP+APAP, resulting in higher subfunction enrichment in the former cell type. The gene lists of the different subfunctions of the toxicological classes have been summarized in the Supplementary Data (Supplementary Tables S2–S6). When looking at the gene level, it is found that a number of genes of the previously identified “Toxicological Classes” are commonly modulated in both hSKP-HPC and hHEP exposed to APAP. Five genes, including Bcl-2 interacting mediator of cell death (*BCL2L1*), cellular oncogene c-fos (*FOS*),

TABLE 1. “DEVELOPMENT OF LIVER” CLASS GENES MODULATED DUE TO HEPATOGENIC DIFFERENTIATION (THRESHOLD SET AT MINIMUM TWOFOLD UP- OR DOWNREGULATION, $P < 0.05$ (FISHER’S DIRECT))

Upregulated genes	Fold change	Downregulated genes	Fold change
<i>GATA6</i>	14.8	<i>MET</i>	–2.0
<i>TLR4</i>	14.7	<i>FGFR2</i>	–2.0
<i>SOCS3</i>	12.5	<i>PKM</i>	–2.2
<i>PTPN12</i>	11.4	<i>TCEB3</i>	–2.2
<i>MAN2A1</i>	6.9	<i>MDM4^a</i>	–2.2
<i>TGFBR3</i>	6.6	<i>KLF6</i>	–2.3
<i>MLL</i>	5.5	<i>CCND3</i>	–2.5
<i>LBP</i>	4.5	<i>NF1</i>	–2.6
<i>SMARCA4</i>	4.3	<i>XPB1^a</i>	–2.8
<i>MECOM</i>	4.2	<i>STAT5B</i>	–3.1
<i>AHR</i>	4.0	<i>EPOR^a</i>	–3.3
<i>CADM1</i>	3.8	<i>HGF</i>	–3.4
<i>SOD2</i>	3.5	<i>JARID2</i>	–4.2
<i>HLX</i>	3.4	<i>SP3</i>	–4.7
<i>NCOA6</i>	3.4	<i>IL1A^a</i>	–5.7
<i>CCND2</i>	3.2	<i>IL6^a</i>	–7.2
<i>RUNX1</i>	3.2	<i>HES1</i>	–7.9
<i>RXRA</i>	3.2	<i>VEGFA</i>	–10.7
<i>RAPGEF2</i>	3.2	<i>SLC20A1</i>	–29.9
<i>KRAS</i>	3.2		
<i>IGF2R</i>	3.1		
<i>JUN</i>	3.0		
<i>ADA</i>	2.8		
<i>MYD88</i>	2.6		
<i>BRD1</i>	2.5		
<i>SPTBN1</i>	2.5		
<i>SMAD3</i>	2.5		
<i>TRAF3</i>	2.4		
<i>ARF6</i>	2.4		
<i>ACADM</i>	2.4		
<i>MED1</i>	2.4		
<i>MBTD1</i>	2.2		
<i>ADAR</i>	2.2		
<i>SP1</i>	2.1		
<i>ASL</i>	2.1		
<i>ICMT</i>	2.1		
<i>RELA</i>	2.0		

^aGenes expected to be upregulated for enrichment of Development of Liver Class.

heme oxygenase 1 (*HMOX1*), TIMP metalloproteinase inhibitor 3 (*TIMP3*) and *AHR*, are significantly upregulated (fold change > 2, Fisher’s Exact P value < 0.05) and three genes, including insulin-like growth factor 1 (*IGF1*), regucalcin (*RGN*) and inhibin beta A (*INHBA*) are significantly downregulated (fold change > 2, Fisher’s Exact P value < 0.05) (Fig. 5). These results were confirmed by qPCR analysis for both hSKP-HPC and hSKP-HPC+APAP samples (Supplementary Fig. S1), validating the obtained microarray data. The upregulation of *AHR* could not be observed in a significant way.

Discussion

DILI is the main cause of ALF in the western world [3,44]. It is also one of the main reasons for post marketing withdrawal of drugs and discontinuation of the development of

FIG. 3. Venn diagram of the genes of the liver development class in hSKP-HPC, hHEP, and LIVER when compared to undifferentiated hSKP (Fisher's Exact *P* value is mentioned between brackets).

NCE, thereby representing a substantial cost for the pharmaceutical industry [3,44]. Current human cell models are scarce or not fully adequate for toxicity screening of pharmaceuticals and other chemical compounds [16,45]. Therefore, novel cell models need to be explored. The ability of ASC to self-renew and to differentiate into cell types of

mesodermal, endodermal, or ectodermal origin makes them attractive as therapeutic agents to replace cell loss due to injury or disease [46]. In addition, ASC represent a potential new cell source for toxicity screening and the use of human ASC in this field could have a pivotal effect on the efficacy and predictability of in vitro screening methods [47].

FIG. 4. PCA-plots of the microarray data of hSKP-HPC and hHEP exposed to acetylpara-aminophenol, acetaminophen (APAP) [respectively hSKP-derived hepatic progenitor cells exposed to acetaminophen (hSKP-HPC+APAP) and human hepatocyte cultures exposed to acetaminophen (hHEP+APAP) samples] and their respective control samples (hSKP-HPC and hHEP).

TABLE 2. ENRICHMENT OF TOXICOLOGICAL GENE CLASSES OF IN VITRO SAMPLES AFTER EXPOSURE TO APAP (hSKP-HPC + APAP, hHEP + APAP)

Toxicological classes	hSKP-HPC + APAP		hHEP + APAP	
	Percentage of modulated genes	P value (Fisher's exact)	Percentage of modulated genes	P value (Fisher's exact)
Liver damage	11% (37/349)	1.78E-03	5% (18/349)	4.96E-03
Liver proliferation	19% (53/286)	9.48E-03	9% (26/286)	4.08E-04
Liver necrosis/cell death	11% (55/483)	1.71E-02	5% (25/483)	3.95E-03
Liver steatosis	16% (55/337)	3.00E-02	7% (23/337)	4.06E-02
Liver hepatitis	7% (25/343)	3.57E-03	5% (16/343)	7.29E-03
Liver steatohepatitis	17% (11/66)	3.00E-02	2% (1/66)	2.30E-01
Liver edema	40% (2/5)	4.13E-02	—	—

APAP, acetyl-para-aminophenol, acetaminophen; hHEP + APAP, human hepatocyte cultures exposed to acetaminophen; hSKP-HPC + APAP, hSKP-derived hepatic progenitor cells exposed to acetaminophen.

In this study, we focused on hSKP and more specifically on their stepwise-differentiated hepatic progeny, as a potential predictive human-based cell system for hepatotoxicity screening of drugs. As illustrated in the results' section, the expression of known hepatic progenitor cell markers was upregulated upon sequential exposure to liver-specific hepatogenic growth factors and cytokines. These markers included *EPCAM*, a surface marker of both human hepatic progenitor cells [48] and newly-derived hepatocytes [49];

NCAM2, known as a liver progenitor cell marker and also expressed in acute and chronic damaged livers [50]; *PROM1* and *KIT*, both having been reported as markers for liver progenitor cells [51]. Markers, such as *SMAD4* and *THY1* have also been shown to be expressed in liver progenitor cells [51–54]. *TLR4* is known to be involved in liver fibrogenesis [55] and *SOC3* in hematopoiesis in fetal liver [56]. Both *GATA6* and *PTPN12* are essential during liver development [57,58]. The expression of all these specific

TABLE 3. ENRICHED SUBFUNCTIONS OF TOXICOLOGICAL GENE CLASSES (MODULATED GENES VERSUS TOTAL GENES INVOLVED IN SUBFUNCTIONS) OF IN VITRO SAMPLES AFTER EXPOSURE TO APAP (hSKP-HPC + APAP, hHEP + APAP)

Subfunctions		hSKP-HPC + APAP	<i>P value</i> (Fisher's exact)	hHEP + APAP	<i>P value</i> (Fisher's exact)
Liver damage	Damage of liver cells	23.3% (10/43)	1.78E-03		
	Damage of hepatocytes	25.0% (7/28)	2.16E-03		
	Injury of liver cells	19.0% (4/21)	7.14E-03		
	Injury of hepatocytes	20.0% (3/15)	2.85E-02		
	Damage of liver			5.9% (18/304)	4.96E-03
Liver proliferation	Injury of liver			5.6% (11/195)	3.49E-02
	Proliferation of hepatocytes	19.4% (39/201)	9.48E-03	9.1% (18/201)	1.94E-03
	Proliferation of liver cells	17.8% (47/264)	1.17E-02	8.7% (23/264)	4.08E-04
	Proliferation of hepatic stellate cells			8.6% (6/70)	3.07E-02
	Quantity of hepatocytes			14.3% (5/35)	8.72E-03
Liver necrosis/ cell death	Arrest in growth of hepatocytes			66.7% (2/3)	1.15E-02
	Apoptosis of liver cells	14.0% (40/285)	1.71E-02		
	Apoptosis of hepatocytes	13.4% (33/247)	2.24E-02		
	Cell death of liver cells	12.4% (49/395)	2.28E-02	5.3% (21/395)	6.26E-03
	Cell death of hepatocytes	12.0% (38/316)	2.80E-02		
Liver hepatitis	Apoptosis of sinusoidal endothelial cells	100.0% (2/2)	4.13E-02		
	Delay in cell death of hepatocytes	50.0% (2/4)	4.13E-02		
	Necrosis of liver			5.3% (25/473)	4.83E-03
	Apoptosis of liver cell lines			14.3% (6/42)	3.95E-03
	Apoptosis of hepatic stellate cells			18.2% (4/22)	1.24E-02
	Alcoholic hepatitis	38.2% (13/34)	3.57E-03	14.7% (5/34)	3.83E-02
	Nonalcoholic steatohepatitis	20.0% (9/45)	3.00E-02		
	Chronic hepatitis C			23.5% (16/68)	2.23E-02
Liver steatosis	Cholestatic hepatitis			9.7% (3/31)	7.29E-03
	Chronic autoimmune hepatitis			33.3% (2/6)	2.20E-02
	Nonalcoholic steatohepatitis	20.0% (9/45)	3.00E-02		
	Hepatic steatosis			6.5% (20/309)	4.06E-02

FIG. 5. Microarray expression of Toxicological Classes genes that are commonly modulated in hSKP-HPC and hHEP treated with APAP. **(A)** Common up-regulated genes. **(B)** Common downregulated genes. **Significantly increased gene expression of hSKP-HPC exposed to APAP versus untreated hSKP-HPC (fold change >2; Fisher's Exact P value <0.05). *Significantly increased gene expression of hHEP exposed to APAP versus untreated hHEP (fold change >2; Fisher's Exact P value <0.05). \$Significantly decreased gene expression of hSKP-HPC exposed to APAP versus untreated hSKP-HPC (fold change >2; Fisher's Exact P value <0.05). \$Significantly decreased gene expression of hHEP exposed to APAP versus untreated hHEP (fold change >2; Fisher's Exact P value <0.05).

hepatic progenitor cell markers, show that hSKP have acquired hepatic progenitor cell-like properties after in vitro differentiation. hSKP-HPC also expressed, both at the protein and gene expression level, more mature hepatic markers, including albumine, Hnf1 α , Hnf4 α , and Ahr. Recently, the latter has been reported to play an important role in hepatocytes responding to a toxic assault [59]. Interestingly, hSKP-HPC also seems to have acquired a metabolic machinery. An increased expression at the gene level is observed for several phase I and phase II biotransformation enzymes, as well as for influx and efflux drug transporters. Indeed, important phase I metabolizing enzymes, such as *FMO1*, *FMO2*, *FMO3* and *FMO4* and *MAOB* are highly expressed in hSKP-HPC. The gene expression of the phase II hepatic enzymes *GSTA4* and *GSTM3* was also increased in the differentiated cells. The drug transporters that were found to be highly expressed in hSKP-HPC are among others the liver basolateral transporters *ABCC4* and *ABCA1*, as well

as the apical transporter *ABCG2*. They are all known to be present during liver development [60]. Although the expression of some liver specific influx transporters, including *SLC22A1* and *SLC10A1*, was not increased in hSKP-HPC, several other influx transporters, such as *SLC2A5* [61] were highly upregulated. It seems, therefore, reasonable to assume that the expression of this metabolizing capacity at the gene level assigns hSKP-HPC adequate cellular and metabolic competences to ultimately serve as a potential novel human-based in vitro cell system useful for screening out liver toxic compounds. Here, we evaluated the toxicological response of hSKP-HPC after exposure to APAP, a hepatotoxic substance accountable for most cases of drug-induced ALF, due to its known hepatotoxicity [62]. Full genome transcriptomics was chosen as the main holistic method to understand hepatotoxic responses. This technology has proven to generate high quality, robust and reproducible data [63]. Because of its high sensitivity, evaluation of early toxicological

responses is possible. Indeed, several *in vivo* transcriptomics studies have reported the predictive character of this technology to detect hepatotoxicity [64–69] in human cell cultures of both primary hepatocytes and hepatic cell lines [64,70,71]. As mentioned before, these cell models have serious drawbacks regarding availability and functionality, respectively.

In this study, functional analysis of the differentially expressed genes elucidates the enrichment of gene classes that are involved in specific toxicological functions. Strikingly, the same hepatotoxic responses could be identified in both hSKP-HPC and hHEP exposed to APAP. Further evidence is found by the observation that a number of genes of the identified “Toxicological Classes” is commonly modulated in both hSKP-HPC and hHEP exposed to APAP. The upregulated genes, *BCL2L11*, *FOS*, *HMOX1*, *TIMP3* and *AHR* are all associated either to cytotoxic responses or induction of apoptosis [72–77] and might be useful molecular biomarkers for hepatotoxicity.

As such, this data gives a first indication that human stem cell-derived hepatic cells could be valuable tools in the early *in vitro* prediction of hepatotoxicity. In addition, it was also found that the percentages of the genes involved in each APAP-triggered “Toxicological Class” are consistently higher in hSKP-HPC than in hHEP. A possible explanation could be the immature status of hSKP-HPC, suggesting an alternative toxicological mode of action in comparison to adult human hepatocytes. This is predominantly evoked by a low expression of CYP2E1 in hSKP-HPC, which is known to be one of the main enzymes involved in the bioactivation of APAP by postnatal parenchymal liver cells. On the other hand, APAP is also known to induce fetal hepatotoxicity [78]. Yet, the presence and activity of CYP2E1 in developing liver remains a topic of debate [79]. Furthermore, it is known that the expression of adult CYP isoforms, including CYP1A2, CYP12C, CYP12D6, and CYP3A4, mainly occurs after birth [80]. This leads to important differences in metabolic capacity between embryonic/fetal liver and adult liver, which could suggest that APAP exhibits its hepatotoxicity through an alternative mode of action within developing hepatocytes. Yet, no specific *in vitro* system is currently available that is able to evaluate human hepatotoxicity of compounds during the ontogeny of parenchymal liver cells. As such, due to their similar characteristics with *in vivo* developing hepatocytes, hSKP-HPC could be a particularly interesting human screening model with focus on for developmental hepatotoxicity.

Acknowledgments

The authors would like to thank Ms. Margit Henry and Ms. Susan Rohani for their excellent technical assistance.

This research has received funding from grants of the Research Council (OZR) of the Vrije Universiteit Brussel, the Fund for Scientific Research in Flanders (FWO-Vlaanderen, GO16312N), the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 20161 (ESNATS), no. 266838 (DETECTIVE), no. 266777 (HEMIBIO), and from ISIRIB (Brustem) and BELSPO (IAP-HEPRO).

Author Disclosure Statement

No competing financial interests exist.

References

1. Pammolli F, L Magazzini and M Riccaboni. (2011). The productivity crisis in pharmaceutical R&D. *Nat Rev Drug Discov* 10:428–438.
2. Tamimi NAM and P Ellis. (2009). Drug development: from concept to marketing! *Nephron Clin Pract* 113:c125–c131.
3. Schuster D, C Laggner and T Langer. (2005). Why drugs fail - A study on side effects in new chemical entities. *Curr Pharm Des* 11:3545–3559.
4. Holt MP and C Ju. (2006). Mechanisms of drug-induced liver injury. *AAPS J* 8:E48–E54.
5. Olson H, G Betton, D Robinson, K Thomas, A Monro, G Kolaja, P Lilly, J Sanders, G Sipes, et al. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 32:56–67.
6. Shanks N, R Greek and J Greek. (2009). Are animal models predictive for humans? *Philos Ethics Humanit Med* 4:2.
7. EU. (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 200/21/EC. *Off J Eur Union* L396:849.
8. EU. (2003). Directive 2003/15/EC of the European Parliament and the Council of 27 February amending Council Directive 76/768/EEC on the approximation of the laws of the Members States relating to cosmetic products. *Off J Eur Union* L396:849.
9. EU. (2009). Report from the Commission to the Council and the European Parliament—Report on the Development, Validation and Legal Acceptance of Alternative Methods to Animal Tests in the Field of Cosmetics—COM 416 final; http://ec.europa.eu/enterprise/cosmetics/html/cosm_animal_test.
10. Vanparys P. (2002). ECVAM and pharmaceuticals. *Altern Lab Anim* 30 Suppl 2:221–223.
11. Guguen-Guillouzo C and A Guillouzo. (2010). General review on *in vitro* hepatocyte models and their applications. *Methods Mol Biol* 640:1–40.
12. Schoonen WGEJ, WMA Westerink, JADM de Roos and E Débiton. (2005). Cytotoxic effects of 100 reference compounds on Hep G2 and HeLa cells and of 60 compounds on ECC-1 and CHO cells. I mechanistic assays on ROS, glutathione depletion and calcein uptake. *Toxicol In Vitro* 19:505–516.
13. Jennen DGJ, C Magkouloupoulou, HB Ketelslegers, MHM van Herwijnen, JCS Kleinjans and JHM van Delft. (2010). Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol Sci* 115:66–79.
14. Mills JB, KA Rose, N Sadagopan, J Sahi and SMF de Morais. (2004). Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharmacol Exp Ther* 309:303–309.
15. Guillouzo A, A Corlu, C Aninat, D Glaize, F Morel and C Guguen-Guillouzo. (2007). The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact* 168:66–73.
16. LeCluyse EL, RP Witek, ME Andersen and MJ Powers. (2012). Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol* 42:501–548.
17. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic

- stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
18. Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
  19. Jiang Y, BN Jahagirdar, RL Reinhardt, RE Schwartz, CD Keene, XR Ortiz-Gonzalez, M Reyes, T Lenvik, T Lund, et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:41–49.
  20. Jones DL and AJ Wagers. (2008). No place like home: anatomy and function of the stem cell niche. *Nat Rev Mol Cell Biol* 9:11–21.
  21. Li L and H Clevers. (2010). Coexistence of quiescent and active adult stem cells in mammals. *Science* 327:542–545.
  22. Snykers S, T Vanhaecke, P Papeleu, A Luttun, Y Jiang, Y Vander Heyden, C Verfaillie and V Rogiers. (2006). Sequential exposure to cytokines reflecting embryogenesis: the key for *in vitro* differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. *Toxicol Sci* 94:330–341; discussion 235.
  23. Banas A, T Teratani, Y Yamamoto, M Tokuhara, F Takeshita, G Quinn, H Okochi and T Ochiya. (2007). Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* 46:219–228.
  24. Anzalone R, M Lo Iacono, S Corrao, F Magno, T Loria, F Cappello, G Zummo, F Farina and G La Rocca. (2010). New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. *Stem Cells Dev* 19:423–438.
  25. Tee JY, V Vaghjiani, YH Liu, P Murthi, J Chan and U Manuelpillai. (2013). Immunogenicity and immunomodulatory properties of hepatocyte-like cells derived from human amniotic epithelial cells. *Curr Stem Cell Res Ther* 8:91–99.
  26. Vaghjiani V, V Vaithilingam, B Tuch, W Sievert and U Manuelpillai. (2013). Deriving hepatocyte-like cells from placental cells for transplantation. *Curr Stem Cell Res Ther* 8:15–24.
  27. Toma JG, M Akhavan, KJ Fernandes, F Barnabé-Heider, A Sadikot, DR Kaplan and FD Miller. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3:778–784.
  28. Biernaskie JA, IA McKenzie, JG Toma and FD Miller. (2006). Isolation of skin-derived precursors (SKPs) and differentiation and enrichment of their Schwann cell progeny. *Nat Protoc* 1:2803–2812.
  29. Jinno H, O Morozova, KL Jones, JA Biernaskie, M Paris, R Hosokawa, MA Rudnicki, Y Chai, F Rossi, MA Marra and FD Miller. (2010). Convergent genesis of an adult neural crest-like dermal stem cell from distinct developmental origins. *Stem Cells* 28:2027–2040.
  30. Gago N, V Pérez-López, JP Sanz-Jaka, P Cormenzana, I Ezaguirre, A Bernad and A Izeta. (2009). Age-dependent depletion of human skin-derived progenitor cells. *Stem Cells* 27:1164–1172.
  31. Buranasinsup S, M Sila-Asna, N Bunyaratvej and A Bunyaratvej. (2006). *In vitro* osteogenesis from human skin-derived precursor cells. *Dev Growth Differ* 48:263–269.
  32. Hunt DPJ, PN Morris, J Sterling, JA Anderson, A Joannides, C Jahoda, A Compston and S Chandran. (2008). A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin. *Stem Cells* 26:163–172.
  33. Shih DTB, DC Lee, SC Chen, RY Tsai, CT Huang, CC Tsai, EY Shen and WT Chiu. (2005). Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. *Stem Cells* 23:1012–1020.
  34. De Kock J, T Vanhaecke, J Biernaskie, V Rogiers and S Snykers. (2009). Characterization and hepatic differentiation of skin-derived precursors from adult foreskin by sequential exposure to hepatogenic cytokines and growth factors reflecting liver development. *Toxicol In Vitro* 23:1522–1527.
  35. De Kock J, S Snykers, E Ramboer, S Demeester, A Heymans, S Branson, T Vanhaecke and V Rogiers. (2011). Evaluation of the multipotent character of human foreskin-derived precursor cells. *Toxicol In Vitro* 25:1191–1202.
  36. De Kock J, M Najar, J Bolleyn, F Al Battah, RM Rodrigues, K Buyl, G Raicevic, O Govaere, S Branson, et al. (2012). Mesoderm-derived stem cells: the link between the transcriptome and their differentiation potential. *Stem Cells Dev* 21:3309–3323.
  37. James LP, PR Mayeux and JA Hinson. (2003). Acetaminophen-induced hepatotoxicity. *Drug Metab Dispos* 31:1499–1506.
  38. McGill MR, MR Sharpe, CD Williams, M Taha, SC Curry and H Jaeschke. (2012). The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 122:1574–1583.
  39. Zimmerman HJ and WC Maddrey. (1995). Acetaminophen (paracetamol) hepatotoxicity with regular intake of alcohol: analysis of instances of therapeutic misadventure. *Hepatology* 22:767–773.
  40. Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63.
  41. Urushidani T. (2005). Toxicogenomics: Japanese initiative. In: *Handbook of Toxicogenomics*. Borlak J. Wiley GmbH Co KGaA, Weinheim, Germany, pp. 623–631.
  42. Uehara T, A Ono, T Maruyama, I Kato, H Yamada, Y Ohno and T Urushidani. (2010). The Japanese toxicogenomics project: application of toxicogenomics. *Mol Nutr Food Res* 54:218–227.
  43. Irizarry RA, BM Bolstad, F Collin, LM Cope, B Hobbs and TP Speed. (2003). Summaries of affymetrix GeneChip probe level data. *Nucleic Acids Res* 31:e15.
  44. Verma S and N Kaplowitz. (2009). Diagnosis, management and prevention of drug-induced liver injury. *Gut* 58:1555–1564.
  45. Guillouzo A. (1998). Liver cell models in *in vitro* toxicology. *Environ Health Perspect* 106 Suppl 2:511–532.
  46. Ilic D and JM Polak. (2011). Stem cells in regenerative medicine: introduction. *Br Med Bull* 98:117–126.
  47. Joery DK, MR Robim, B Jennifer, V Tamara and R Vera. (2012). Focus on stem cells as sources of human target cell for *in vitro* research and testing. *ALTEX* 29:3–10.
  48. Schmelzer E, E Wauthier and LM Reid. (2006). The phenotypes of pluripotent human hepatic progenitors. *Stem Cells* 24:1852–1858.
  49. Yoon SM, D Gerasimidou, R Kuwahara, P Hytioglou, JE Yoo, YN Park and ND Theise. (2011). Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 53:964–973.
  50. Zhou H. (2007). Identification of hepatocytic and bile ductular cell lineages and candidate stem cells in bipolar ductular reactions in cirrhotic human liver. *Hepatology* 45:716–724.
  51. Crosby HA, DA Kelly and AJ Strain. (2001). Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. *Gastroenterology* 120:534–544.

52. Mishra L, T Banker, J Murray, S Byers, A Thenappan, AR He, K Shetty, L Johnson and EP Reddy. (2009). Liver stem cells and hepatocellular carcinoma. *Hepatology* 49:318–329.
53. Kitisin K, N Ganesan, Y Tang, W Jogunoori, EA Volpe, SS Kim, V Katuri, B Kallakury, M Pishvaian, et al. (2007). Disruption of transforming growth factor-beta signaling through beta-spectrin ELF leads to hepatocellular cancer through cyclin D1 activation. *Oncogene* 26:7103–7110.
54. Herrera MB, S Bruno, S Buttiglieri, C Tetta, S Gatti, MC Deregibus, B Bussolati and G Camussi. (2006). Isolation and characterization of a stem cell population from adult human liver. *Stem Cells* 24:2840–2850.
55. Seki E and DA Brenner. (2008). Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* 48: 322–335.
56. Takeda K and S Akira. (2000). STAT family of transcription factors in cytokine-mediated biological responses. *Cytokine Growth Factor Rev* 11:199–207.
57. Zhao R, AJ Watt, J Li, J Luebke-Wheeler, EE Morrissey and SA Duncan. (2005). GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. *Mol Cell Biol* 25:2622–2631.
58. Sirois J, J-F Cote, A Charest, N Uetani, A Bourdeau, SA Duncan, E Daniels and ML Tremblay. (2006). Essential function of PTP-PEST during mouse embryonic vascularization, mesenchyme formation, neurogenesis and early liver development. *Mech Dev* 123:869–880.
59. Nukaya M, JA Walisser, SM Moran, GD Kennedy and CA Bradfield. (2010). Aryl hydrocarbon receptor nuclear translocator in hepatocytes is required for aryl hydrocarbon receptor-mediated adaptive and toxic responses in liver. *Toxicol Sci* 118:554–563.
60. Klaassen CD and LM Aleksunes. (2010). Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev* 62:1–96.
61. Rand EB, AM Depaoli, NO Davidson, GI Bell and CF Burant. (1993). Sequence, tissue distribution, and functional characterization of the rat fructose transporter GLUT5. *Am J Physiol* 264:G1169–G1176.
62. Larson AM, J Polson, RJ Fontana, TJ Davern, E Lalani, LS Hynan, JS Reisch, FV Schiødt, G Ostapowicz, et al. (2005). Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 42:1364–1372.
63. Scott DJ, AS Devonshire, YA Adeleye, ME Schutte, MR Rodrigues, TM Wilkes, MG Sacco, L Gribaldo, M Fabbri, et al. (2011). Inter- and intra-laboratory study to determine the reproducibility of toxicogenomics datasets. *Toxicology* 290:50–58.
64. Cheng F, D Theodorescu, IG Schulman and JK Lee. (2011). *In vitro* transcriptomic prediction of hepatotoxicity for early drug discovery. *J Theor Biol* 290:27–36.
65. Hirode M, A Ono, T Miyagishima, T Nagao, Y Ohno and T Urushidani. (2008). Gene expression profiling in rat liver treated with compounds inducing phospholipidosis. *Toxicol Appl Pharmacol* 229:290–299.
66. Hirode M, A Horinouchi, T Uehara, A Ono, T Miyagishima, H Yamada, T Nagao, Y Ohno and T Urushidani. (2009). Gene expression profiling in rat liver treated with compounds inducing elevation of bilirubin. *Hum Exp Toxicol* 28:231–244.
67. Hirode M, K Omura, N Kiyosawa, T Uehara, T Shimizu, A Ono, T Miyagishima, T Nagao, Y Ohno and T Urushidani. (2009). Gene expression profiling in rat liver treated with various hepatotoxic-compounds inducing coagulopathy. *J Toxicol Sci* 34:281–293.
68. Uehara T, M Hirode, A Ono, N Kiyosawa, K Omura, T Shimizu, Y Mizukawa, T Miyagishima, T Nagao and T Urushidani. (2008). A toxicogenomics approach for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals in rats. *Toxicology* 250:15–26.
69. Uehara T, N Kiyosawa, M Hirode, K Omura, T Shimizu, A Ono, Y Mizukawa, T Miyagishima, T Nagao and T Urushidani. (2008). Gene expression profiling of methapyrilene-induced hepatotoxicity in rat. *J Toxicol Sci* 33:37–50.
70. Blomme EAG, Y Yang and JF Waring. (2009). Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. *Toxicol Lett* 186:22–31.
71. Foster WR, SJ Chen, A He, A Truong, V Bhaskaran, DM Nelson, DM Dambach, LD Lehman-McKeeman and BD Car. (2007). A retrospective analysis of toxicogenomics in the safety assessment of drug candidates. *Toxicol Pathol* 35:621–635.
72. O'Connor L, A Strasser, LA O'Reilly, G Hausmann, JM Adams, S Cory and DC Huang. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 17:384–395.
73. Yang JY, W Xia and MCT Hu. (2006). Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. *Int J Oncol* 29:643–648.
74. Sarma SN, Y-J Kim, M Song and J-C Ryu. (2011). Induction of apoptosis in human leukemia cells through the production of reactive oxygen species and activation of HMOX1 and Noxa by benzene, toluene, and o-xylene. *Toxicology* 280:109–117.
75. Wagner EF. (2002). Functions of AP1 (Fos/Jun) in bone development. *Ann Rheum Dis* 61 Suppl 2:ii40–ii42.
76. Kallio JP, S Hopkins-Donaldson, AH Baker and VM Kähäri. (2011). TIMP-3 promotes apoptosis in nonadherent small cell lung carcinoma cells lacking functional death receptor pathway. *Int J Cancer* 128:991–996.
77. Nebert DW, TP Dalton, AB Okey and FJ Gonzalez. (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* 279:23847–23850.
78. Thiele K, T Kessler, P Arck, A Erhardt and G Tiegs. (2013). Acetaminophen and pregnancy: short- and long-term consequences for mother and child. *J Reprod Immunol* 97:128–139.
79. Ring JA, H Ghabrial, MS Ching, RA Smallwood and DJ Morgan. (1999). Fetal hepatic drug elimination. *Pharmacol Ther* 84:429–445.
80. Gow PJ, H Ghabrial, RA Smallwood, DJ Morgan and MS Ching. (2001). Neonatal hepatic drug elimination. *Pharmacol Toxicol* 88:3–15.

Address correspondence to:

Robim M. Rodrigues

Department of Toxicology

Center for Pharmaceutical Research

Faculty of Medicine and Pharmacy

Vrije Universiteit Brussel (VUB)

Laarbeeklaan 103

Brussels 1090

Belgium

E-mail: robim.marcelino.rodrigues@vub.ac.be

Received for publication March 25, 2013

Accepted after revision August 14, 2013

Prepublished on Liebert Instant Online August 17, 2013