Characterization of proliferating human hepatocytes as a model system for drug interaction studies and toxicity screenings

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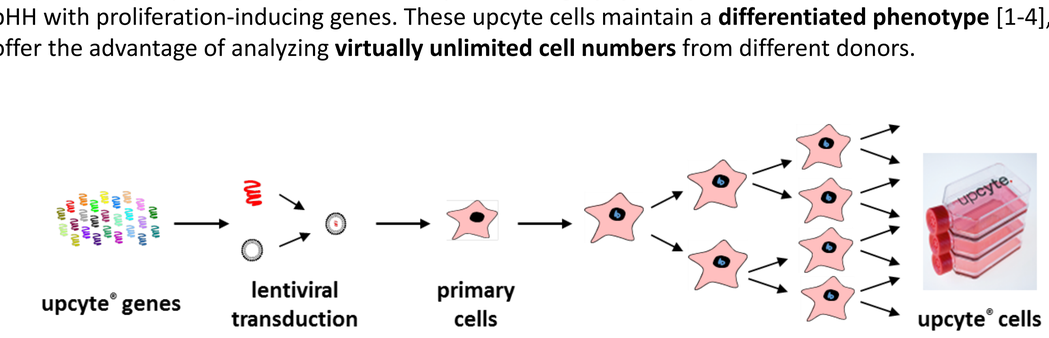
Introduction

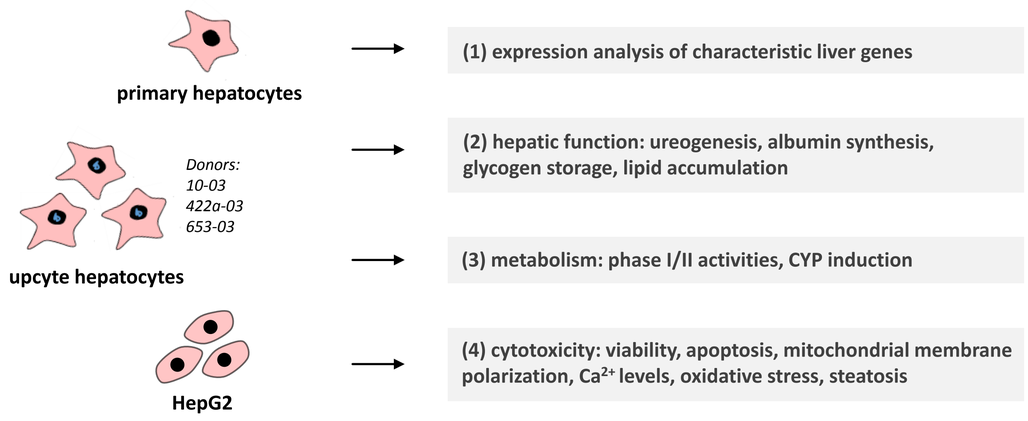
Liver toxicity represents a major cause of adverse effects and drug withdrawal. To efficiently predict the safety profile of a drug candidate, there is a constant need for **reliable preclinical test systems**. While **primary human hepatocytes** (pHH) remain the gold standard for prediction of drug responses, **restricted availability and rapid loss of the hepatic phenotype** limit their use.

We previously reported the generation of proliferating **upcyte hepatocytes** by lentiviral transduction of pHH with proliferation-inducing genes. These upcyte cells maintain a **differentiated phenotype** [1-4], but offer the advantage of analyzing **virtually unlimited cell numbers** from different donors.



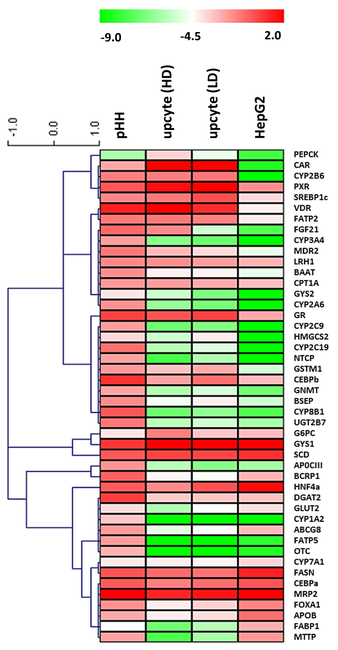
Methods

To evaluate the **suitability of upcyte hepatocytes for drug metabolism and hepatotoxicity** studies, we compared cells generated from three different donors (10-03, 422a-03, 653-03) with HepG2 cells and primary human hepatocytes on a molecular and functional level [5]:

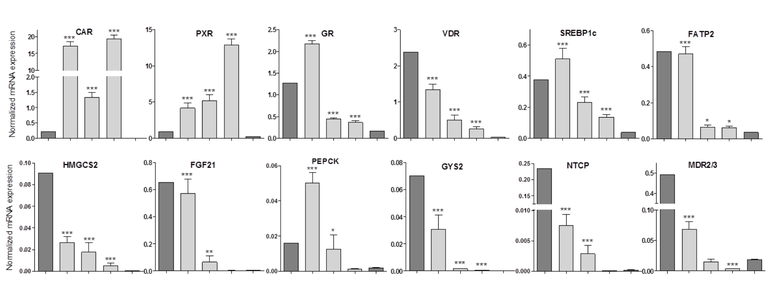


Results

(1) Expression of characteristic liver genes



The mRNA expression of 46 liver genes in donor 653-03 at low (LD) and high densities (HD) was analyzed by qRT-PCR and compared to pHH and HepG2 cells.



*Transcripts from 18 representative genes showing a high expression in donor 653-03 were further analyzed in donors 10-03 and 422a-03, pHH and HepG2 cells (not all genes shown). Data are expressed as mean ± SEM and are normalized on expression levels observed in healthy human liver samples. Statistical analysis was performed using ANOVA and Tukey HSD post hoc test (*p<0.05, **p<0.01, ***p< 0.001).*

↑ Differences in gene expression among upcyte hepatocytes derived from different donors.
The results demonstrate differences among the donors. Higher expression levels were observed in upcyte cells 653-03, whereas the lowest expression usually corresponded to upcyte cells 422a-03 (neonatal origin of these hepatocytes). upcyte hepatocytes showed a significant expression for the genes related to hepatic lipid (eg, SREP1c, FATP2, HMGCS2), carbohydrate (eg, FGF21, PEPCK, GYS2), and bile acid metabolisms (eg, NTCP, MDR2/3), which suggests that upcyte cells can perform most of the major specific functions of differentiated hepatocytes.

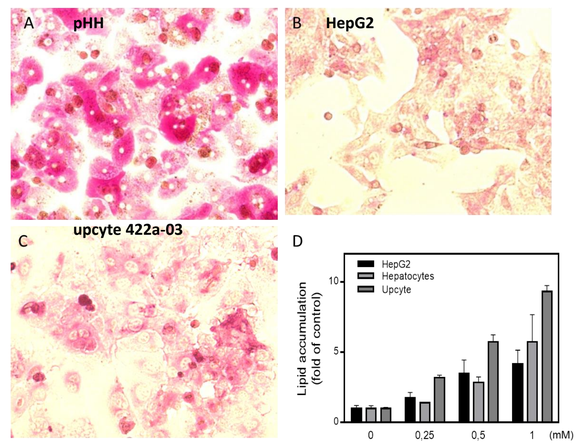
← Heatmap analysis of upcyte cells.
When compared with pHH, 37% of the genes showed a lower basal level in upcytes (fold change < 0.2), whereas a higher percentage of the genes (57%) were poorly expressed in HepG2 cells. The hierarchical classification confirmed that the upcyte expression profile comes closer to the pHH than the HepG2 profile, whereas PCA analysis indicated that, in relation to pHH, the upcyte phenotype is less different (data not shown).

(2) Preservation of hepatic function

	GSH [nmol/mg]	ureogenesis [nmol/mg/h]	albumin synthesis [ng/mg/h]
upcyte 653-03	n.d.	22.7 ± 15.0	21.6 ± 4.1
upcyte 10-03	13.5 ± 2.9	79.3 ± 22.6	18.4 ± 4.4
upcyte 422a-03	4.7 ± 0.3	6.7 ± 4.8	11.8 ± 1.5
HepG2	n.d.	17.7 ± 3.2	8.9 ± 2.3
pHH	14.7 ± 1.1	75.7 ± 10.1	49.1 ± 2.3

Basal glutathione (GSH) levels in cultured hepatocytes were measured by HPLC-MS/MS. For quantification of ureogenesis, hepatocytes were incubated with 2 mM NH_4Cl for 24 h followed by measurement of urea release into the culture medium by HPLC. Secreted albumin levels were determined after 24 h of culture by ELISA.

↑ GSH levels and urea/albumin synthesis in upcyte hepatocytes, pHH and HepG2 cells.
The greatest urea production was shown by the cells from the adult donor (upcyte 10-03) and the ureogenic rates were similar to that of pHH. Regarding albumin synthesis, all upcyte hepatocytes were able to synthesize and secrete albumin, although the production rates were lower than in pHH. Levels of GSH, a key molecule in cell protection against reactive electrophiles and oxidants, from donor 10-03 was comparable to that in pHH, whereas lower GSH levels were found in upcytes from donor 422A-03.



*After incubation with 0.1 µM insulin, cells were subjected to PAS staining and analyzed by microscopy. **(D)** Lipid accumulation in different hepatocyte cell types. Cells were exposed to indicated concentrations of a fatty acids (oleate:palmitate mixture (2:1)) followed by Nile red staining to determine intracellular triglyceride levels. Results are expressed as mean ± SEM and are depicted as fold induction over untreated control cells.*

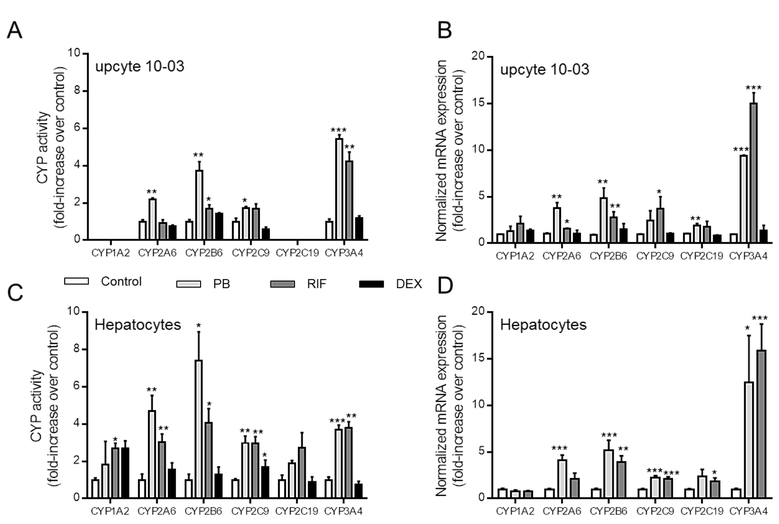
↑ Lipid and glycogen accumulation in upcyte hepatocytes.
Glycogen storages was determined in **(A)** pHH, **(B)** HepG2 cells and **(C)** upcyte hepatocytes (422a-03). Glycogen storage was evident in the upcyte cells stimulated with insulin. Stimulation of upcyte cells with fatty acids resulted in the dose-dependent accumulation of intracellular lipids, similarly to that observed in pHH and HepG2 cells.

(3) CYP metabolism and conjugating activities

→ CYP and conjugating activities in upcyte hepatocytes, pHH and HepG2 cells.
High inter-donor variability was observed among the upcyte cell preparations. In this study no measurable CYP1A2 and CYP2C19 activities were found in upcyte cells, although activity has been reported before [6]. CYP2E1 was activity detected in only one donor. The CYP2D6 and CYP2C9 activities in upcyte cells were lower than in pHH, whereas the CYP2B6, CYP2A6, and CYP3A4 activity levels were similar. The CPR activity values in upcytes were comparable to those of pHH. Regarding phase II enzymes, the UGT1A1 activity levels in upcytes and pHH were similar, but the UGT2B7 and GST activities were lower in upcytes. Overall, the activity levels of the phases I and II enzymes in upcyte hepatocytes were markedly higher than in HepG2 cells.

	upcyte 653-03	Upcyte 10-03	upcyte 422a-03	HepG2	pHH (4-6 donors)
CYP1A2	0	0	0	0.02 ± 0.03	3.9 ± 2.8
CYP2C19	0	0	0	0	2.5 ± 2.1
CYP2B6	2.02 ± 0.18	7.92 ± 0.78	0.62 ± 0.08	0	2.2 ± 3.4
CYP2D6	0.07 ± 0.01	0.12 ± 0.01	0.20 ± 0.02	0.02	5.5 ± 7.9
CYP2C9	3.01 ± 0.39	2.85 ± 0.59	2.02 ± 0.09	0.02 ± 0.02	29.3 ± 14.3
CYP3A4	4.47 ± 0.17	13.4 ± 2.4	14.3 ± 0.9	0.10 ± 0.07	10.1 ± 5.2
CYP2A6	0.44 ± 0.13	17.0 ± 3.1	1.86 ± 0.48	0	16.3 ± 9.2
CYP2E1	0	4.16	0	0	12.5 ± 7.1
UGT1A1	70 ± 17	685 ± 21	217 ± 3	1.68	185 ± 93
UGT2B7	7.3 ± 1.0	54.1 ± 0.5	13.2 ± 1.2	3.9 ± 1.3	169 ± 56
GST	119 ± 13	125 ± 14	218 ± 23	33.3 ± 8.9	358 ± 106
CPR	16.4	23.8	5.4	7.1 ± 1.2	13.0 ± 2.7

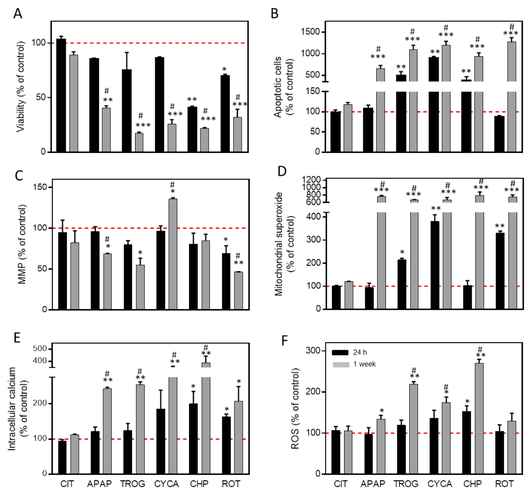
Cells were incubated with a cocktail of selective substrates for individual CYP enzymes. Formation of corresponding metabolites was detected by HPLC-MS/MS. CYP and UGT activities are depicted as pmol/mg/min and CRP activities are expressed as nmol/mg/min. Selective substrates: 10µM phenacetin, 5µM coumarin, 10µM bupropion, 10µM diclofenac, 10µM mephenytoin, 10µM bufuralol, 50µM chlorzoxazone, 5µM midazolam.



← CYP induction in upcyte hepatocytes using prototypical inducers.
A high response to inducers was obtained for the adult donor (10-03), whereas lower effects were observed for the neonatal donor (422a-03) (not shown). Both PB and RIF induced CYP2B6 and CYP3A4 (increases in activity and mRNA values) in upcyte hepatocytes, and less marked effects were observed in CYP2C9. PB-treatment also increased CYP2A6 activity and mRNA levels. In contrast, 1 mM DEX did not induce any effects on CYP enzymes. Overall, the response of upcyte cells to induction was similar to that of pHH.

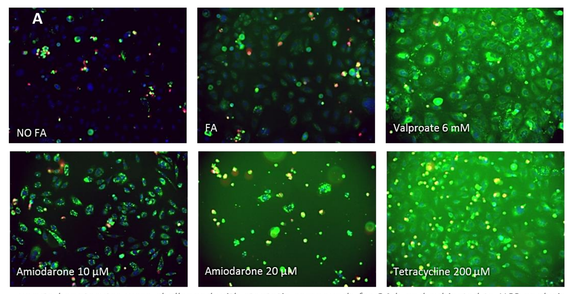
*upcyte hepatocytes (10-03) were incubated with indicated CYP inducers for 48 h (PB= phenobarbital (1mM), RIF= .rifampicin (50µM), DEX= dexamethasone (1µM)). Induction was subsequently evaluated by detection of corresponding transcript levels and respective activities in **(A/B)** upcyte cells and **(C/D)** pHH (from three independent donors. Data are expressed as mean ± SEM and indicate fold inductions over vehicle-treated cells. Statistical analysis was performed using Student t-test (*p<0.05, **p<0.01, ***p< 0.001)*

(4) Suitability for acute and repeated-dose hepatotoxicity studies



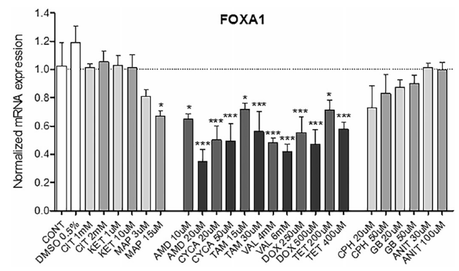
*upcyte hepatocytes (422a-03) were exposed to test compounds for 24 h or 1 week. Fluorescent probes were subsequently employed to evaluate **(A)** viability, **(B)** apoptosis, **(C)** changes in mitochondrial membrane potential (MMP), **(D)** production of mitochondrial superoxide, **(E)** ROS **(F)** intracellular Ca^{2+} levels using HCS. Data are expressed as mean ± SEM as percentages normalized on untreated control cells. Statistical analysis was performed using Student t-test (*p<0.05, **p<0.01, ***p< 0.001 vs. untreated; #p<0.05 vs. 24 h).*

↑Acute and repeated-dose toxicity using a multiparametric HCS approach at sub-cytotoxic concentrations.
Exposure time length had dramatic effects on the toxicity profile for a compound. With APAP, no effect was observed after the 24 h, whereas after 1-week, the number of apoptotic cells, mitochondrial depolarization, the production of ROS and mitochondrial superoxide increased, and intracellular Ca^{2+} levels at the lowest concentrations rose. The other tested compounds caused some effects after 24 h, although a significant difference was detected between the two incubation periods at the lowest concentration. CIT (non hepatotoxic) did not produce any significant effects after the 24 h or 1 week treatments.



upcyte hepatocytes were challenged with steatotic compounds for 24 h and subjected to HCS analysis. Representative images from the HCS analysis are shown. Lipid content was estimated using BODIPY493/503 (green).

↑ Concentration-dependent steatosis in upcyte hepatocytes.
All steatotic compounds produced a dose-dependent increase in lipid accumulation, even at subcytotoxic concentrations.



*upcytes were exposed to test compounds for 24 h. FOXA1 mRNA levels were quantified by RT-Q-PCR. Bars represent mean ± SEM of three upcyte cultures from the different donors, and are expressed as a fold change versus the control cells. ***P<.001, *P<.05, ANOVA and Tukey HSD post hoc test.*

↑ Steatotic drugs down-regulate FOXA1 in upcyte cells.
Steatotic drugs trigger the down-regulation of FOXA1 mRNA in HepG2 and pHH [7]. In agreement with classic hepatic cell models, steatotic drugs also down-regulated FOXA1 expression in upcytes of three donors, whereas non steatotic drugs had a minor effect or none at all . To further validate the specificity, we also assayed the effect of three cholestatic compounds (CHP, GB, ANIT) as controls, which did not repress FOXA1.

Employed hepatotoxic model compounds: ***CIT**: sodium citrate (1-2 mM), **APAP**: paracetamol (0.5-2 mM), **TROG**: troglitazone (50-100 µM), **CYC(A)**: cyclosporin A 20-50 µM, **CHP**: cumene hydroperoxide (100-500 µM), **ROT**: rotenone (0.05-1 µM), **KET**: ketotifen (1-10 µM), **MAP**: maprotiline (3-15 µM), **AMD**: amiodarone (10-20 µM), **DOX**: doxycycline (250-500 µM), **TAM**: tamoxifen (15-30 µM), **TET**: tetracycline (200-400 µM), **VAL**: sodium valproate (4-6 mM).* Cholestatic compounds: ***CHP**: chlorpromazine, **GB**: glyburide*

Summary & conclusion

- mRNA expression profile of upcyte hepatocyte comes **closer to primary hepatocytes** than HepG2 cells
- upcyte hepatocytes retain **key hepatic functions**
- upcyte hepatocytes show **detectable CYP activity** and **respond to prototypical CYP inducers**
- upcyte hepatocytes **predict both acute and long-term toxicity** of hepatotoxic model compounds

Combining the phenotype of primary human hepatocytes and the ease of handling of HepG2 cells, upcyte hepatocytes offer suitable properties to represent a promising hepatic model in preclinical toxicological safety assessments.

References

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