Generation of expanded primary hepatocytes for cell based toxicity and metabolism screenings

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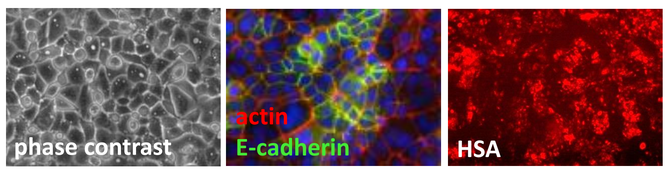
INTRODUCTION

Isolated liver cells, such as **hepatocytes**, **liver sinusoidal endothelial cells** (LSECs), **Kupffer Cells** and **hepatic stellate cells**, are frequently used to study hepatic metabolism, toxicity and disease pathogenesis. However, the current *in vitro* models exhibit several disadvantages, e.g. **short culture longevity** and **artificial culture conditions** focusing on a **single cell type** in 2D culture. The use of primary cells *in vitro* is compromised by the **limited quantity of cells** that can be isolated from one donor, a lack of or very **restricted proliferation capacity** (hepatocytes and LSECs) and/or the change from a quiescent to an activated state (hepatic stellate cells). To overcome this, we have developed a technique which causes primary **human hepatocytes to proliferate up to 40 population doublings** whilst retaining an adult and metabolically competent phenotype with phase I (Cytochrome P450) and phase II activities when cultured at confluence. The resulting cells are called "upcyte® hepatocytes" and combine proliferation with drug metabolizing activity, a feature which makes them uniquely applicable to metabolism and toxicity studies.

RESULTS

metabolism & characteristics



Specific activity (pmol/min/mg protein)				
Cells	**CYP1A2**	**CYP2B6**	**CYP2C9**	**CYP3A4**
#10-03	3.3 ± 0.4	40.3 ± 6.5	91.8 ± 5.5	21.4 ± 9.6
#151-03	0.7 ± 1.4	71.1 ± 11.3	29.1 ± 21.4	77.8 ± 22.6
#422A-03	2.3 ± 0.1	33.6 ± 11.4	4.8 ± 3.1	42.9 ± 6.3
#653-03*	17.1 ± 0.5	68.4 ± 18.4	16.2 ± 0.9	178.3 ± 17.0
HepaRG	10.0 ± 1.5	6.45 ± 0.97	4.57 ± 2.93	48.5 ± 13.9

**Donor 653-03-2D6 available:*
CYP2D6 recombinantly expressed, specific activity for 2D6 ***1627.5 ± 22.4***

upcyte® hepatocytes display an adult phenotype

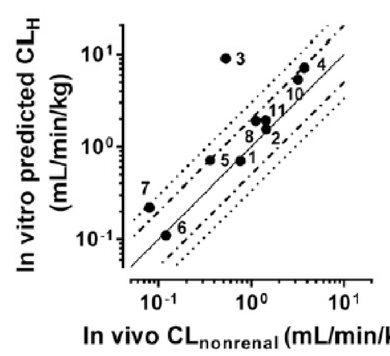
upcyte® hepatocytes express **characteristic adult marker** proteins (CK8, CK18, HSA, AAT), but lack embryonic markers such as AFP. They further show capability for **glycogen storage**, **urea secretion** and **albumin synthesis** (data not shown).

The cells exhibit basal activity of **CYP1A2, 2B6, 2C9, 3A4** and other CYPs in a donor-dependent manner. All enzymes can be induced an inhibited.

upcyte® hepatocytes have similar phase II activities compared to primary hepatocytes

Major hepatic phase II enzymes in humans are **UDP-glucuronosyltransferase (UGT)**, **sulfotransferase (SULT)** and **glutathione S-transferase (GST).** Phase II enzyme activities in upcyte® hepatocytes generated from different donors were similar to those of freshly isolated primary human hepatocytes (data not shown).

clearance prediction



Data represent mean 6±S.D. of triplicate incubations per compound (n = 3) determined at day 7 in sandwich culture. Solid line represents conformity, and dashed lines two- and threefold error range.
The set of reference drugs was subdivided into low and intermediate-cleared compounds (shown here: low): alprazolam (1), prednisolone (2), diazepam (3), voriconazole (4), tolbutamide (5), meloxicam (6), warfarin (7), glimepiride (8), riluzole (10), oxazepam (11).

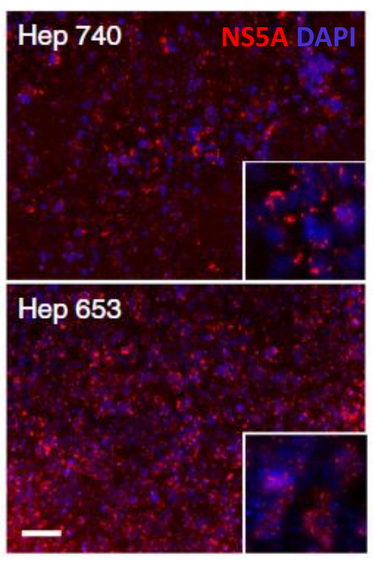
upcyte® hepatocytes are a potent *in vitro* tool for the prediction of hepatic clearance (CL_H)

Correlation between *in vitro* predicted and *in vivo* $CL_{nonrenal}$ was demonstrated using **the well-stirred model disregarding plasma protein binding** for low and intermediate clearance compounds (donor 151-03).

Good correlation between predicted CL_H and observed *in vivo* CL values was observed for the subset of low CL drugs (shown here). **CL_H for 73%** (8 of 11 compounds) were predicted **within twofold** of *in vivo* $CL_{nonrenal}$ and **within threefold for 82%** (9 of 11 compounds) with a trend for overpredicting the actual *in vivo* rate.

Schaefer *et al.*, (2015) - Drug Metabolism & Disposition
upcyte® human hepatocytes: a potent in vitro tool for the prediction of hepatic clearance of metabolically stable compounds
Boehringer Ingelheim Pharma GmbH & Co. KG, DMPK, Biberach an der Riss, Germany

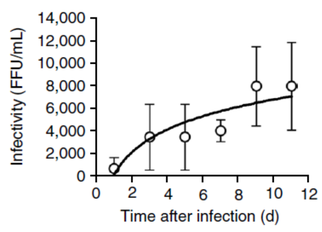
viral infection: HCV



upcyte® hepatocytes support HCVcc infection

HCV infects hepatocytes *in vivo*, but infectivity of primary human hepatocytes *in vitro* is minimal. To assess whether our upcyte® hepatocytes support the full lifecycle of the HCV cell culture variant **HCVcc**, we exposed differentiated hepatocytes (donors 740 and 653-03) to culture medium containing infectious particles of the JC1 genotype expressing an NS5A-RFP fusion protein. Hepatocytes from both donors showed **strong NS5A staining**. More than 80% of the cells were infected in both cultures.

NS5A-RFP levels significantly increased over time, reaching a 20-26-fold induction at day 9 post infection. **Production of infectious particles** showed a similar trend, stabilizing after day 9 and reaching 8,000 focus-forming unit (FFU) per milliliter for donor 653-03.

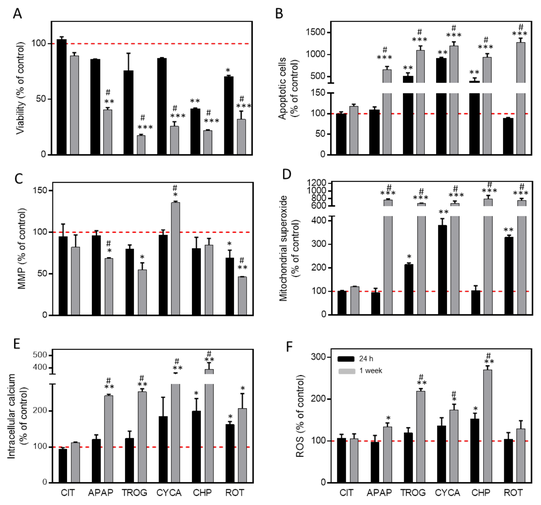


Levy *et al.*, (2015) – Nature Biotechnology
long term culture and expansion of primary human hepatocytes The Hebrew University of Jerusalem, Israel / upcyte technologies GmbH

cytotoxicity - acute and repeated-dose studies

acute and repeated-dose toxicity using sub-cytotoxic concentrations

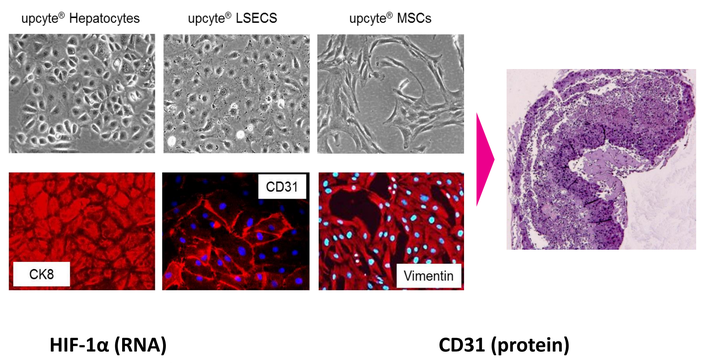
Exposure time length had dramatic effects on the toxicity profile of a compound. For **APAP, no effect was observed after 24 h, whereas 1-week** treatment significantly induced apoptosis, mitochondrial depolarization, ROS production and intracellular Ca^{2+} levels. Other tested compounds caused some effects after 24 h, although a significant difference was detected between the two incubation periods at the lowest concentration. CIT (non hepatotoxic control) did not produce any significant effects after 24 h or 1 week treatment.



*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).*

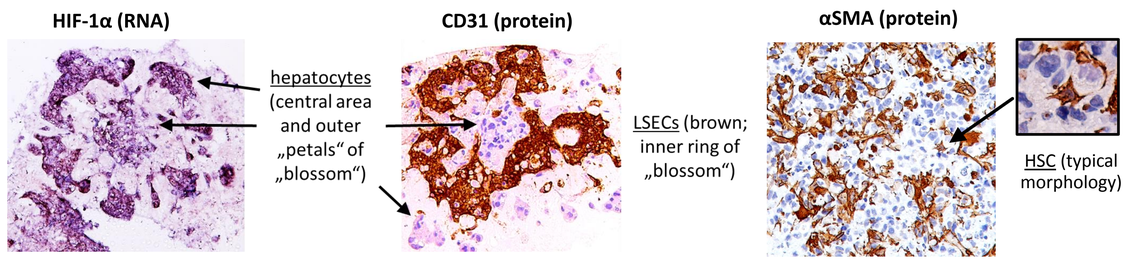
Tolosa *et al.*, (2016) – Toxicological Sciences
human upcyte® hepatocytes: characterization of the hepatic phenotype and evaluation for acute and long-term hepatotoxicity routine testing - University of Valencia, Spain

3D format and co-cultures



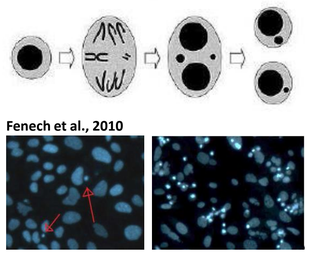
self-assembly in Matrigel™-coated 24-well plates

After 72h, cells showed formation of **structures resembling a liver blossom**. **Hepatocytes** were located in the center and formed the outer petals (HIF-1α). **LSECs** formed an inner ring (CD31), whereas **HSCs** revealed a typical, „star-shaped" morphology (α-SMC).



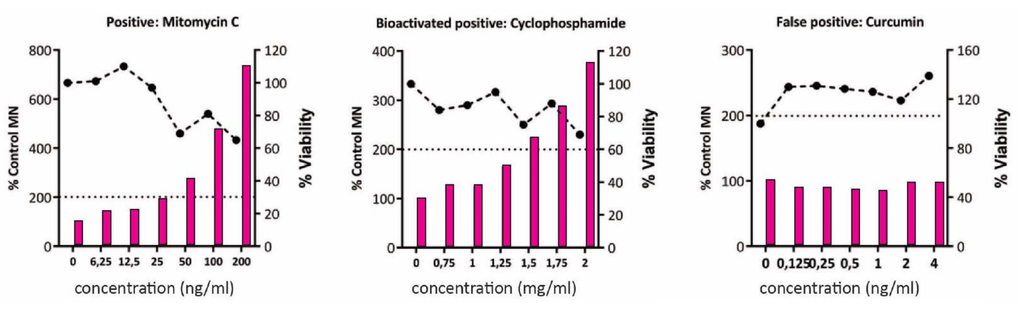
Ramachandran *et al.*, (2016) – Plos One
***In vitro* generation of functional liver organoid-like structures using adult human cells**

genotoxicity: micronuclei (MN) assay



upcyte® hepatocytes can discriminate compounds of 3 classes

The requirements for the MN assay are **proliferating** and **metabolically competent cells**. While pHH lack proliferation, cells lines lack expression of metabolizing enzymes. The use of S9 extract as exogenous metabolizing system involves a high "false positive" rate. Incubation for 96 h (long term treatment for 2 population doublings) allowed upcyte® cells to **discriminate correctly between true positives** (direct and bio-activated), **true negatives and false positives**.



Nörenberg *et al.*, (2013) - Mutation Research
optimization of the metabolically competent & proliferating human upcyte hepatocytes for the in vitro micronuclei assay

SUMMARY & CONCLUSION

In conclusion, upcyte® hepatocyte cultures are characterized by a **differentiated phenotype** and exhibit **functional phase I, phase II and transporter activities**. These data support the use of upcyte® hepatocytes for various applications, such as as metabolism & toxicity screening assays, viral infection and 3D culture. Moreover, this technology allows for the generation of **large batches** of upcyte® hepatocytes (up to 12 x 10^9 cells per donor), enabling a **reproducible** and **standardized** experimental setting.

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