Toxicity assays using expanded liver cells promote the reduction of animal use in pre-clinical research

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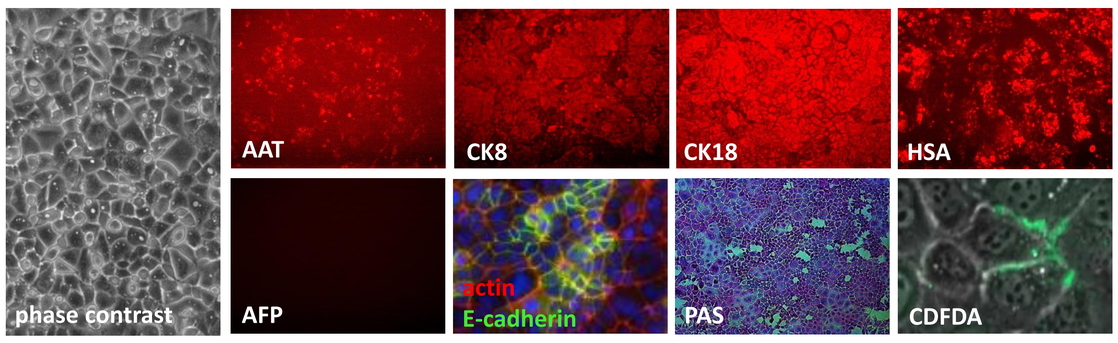
INTRODUCTION

Alternatives to animal testing in toxicity studies such as *in vitro* models were proposed to overcome some of the drawbacks associated with animal experiments and to avoid the unethical procedures. Various alternatives to the use of animals have been suggested, such as *in vitro* models. However, current *in vitro* culture models exhibit several disadvantages such as short culture longevity and artificial conditions focusing on a single cell type in 2D culture. The use of primary cells *in vitro* is further compromised by the limited quantity of cells that can be isolated from one donor, a restricted proliferation capacity (hepatocytes and liver sinusoidal endothelial cells (LSECs)) and/or the change from a quiescent to an activated state (stellate cells). Recently developed **human upcyte® hepatocytes and LSECs** offer the advantage of combining many **features of primary cells** with **large quantities of cells derived from a single donor** as well as an **expanded but finite lifespan**. Here we describe the most recent characterization data of upcyte® hepatocytes and LSECs alone as well as in co-culture to demonstrate their suitability **to replace animal models in pre-clinical studies**.

RESULTS

upcyte® hepatocytes



upcyte® hepatocytes display an adult phenotype

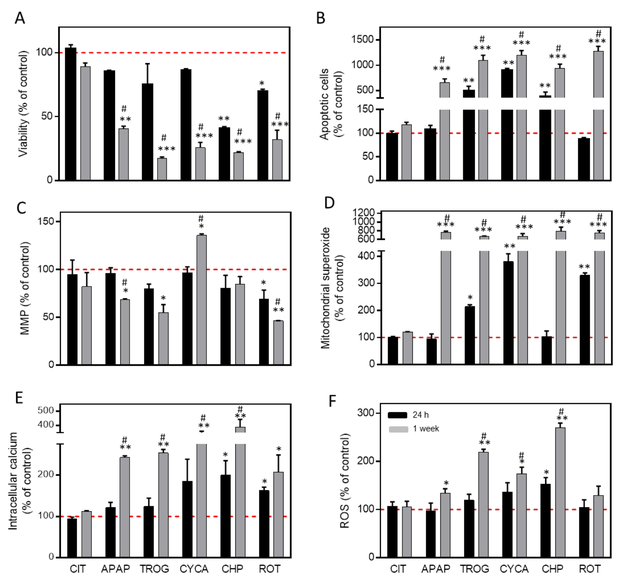
upcyte® hepatocytes expressed the characteristic adult marker proteins **cytokeratin 8 (CK8)**, **cytokeratin 18 (CK18)**, **human serum albumin (HSA)**, **α-anti-trypsin (AAT)**, but lack embryonic markers such as α-fetoprotein (AFP). The cells further expressed E-cadherin and demonstrated marked capability for **glycogen storage (PAS staining)** and **bile secretion (CDFDA staining)**.

phase I activity [pmol/min/mg]	Donor 10-03	Donor 151-03	Donor 422A-03	Donor 653-03
CYP1A2	3.3 ± 0.4	0.7 ± 1.4	2.3 ± 0.1	17.1 ± 0.5
CYP2B6	40.3 ± 6.5	71.1 ± 11.3	33.6 ± 11.4	68.4 ± 18.4
CYP2C9	91.8 ± 5.5	29.1 ± 21.4	4.8 ± 3.1	16.2 ± 0.9
CYP3A4	21.4 ± 9.6	77.8 ± 22.6	42.9 ± 6.3	178.3 ± 17.0

phase II activity [pmol/min/mg]	upcyte® hepatocytes	primary hepatocytes
SULT (Hydroxycoumarin)	6-16	5-98
UGT (Hydroxycoumarin)	32-345	15-496
GST (CDNB)	15-88	21-35

upcyte® hepatocytes maintain metabolic activity

upcyte® hepatocytes (Donors #10-03, #151-03, #422a0-3 and #653-03) expressed metabolizing enzymes of **phase I (e.g. CYP 1A2, 2B6, 2C9 and 3A4)**. Cells further exhibited **phase II activities** (UDP-glucuronosyltransferase **(UGT)**, sulfotransferase **(SULT)** & glutathione-S-transferase **(GST)**) close to primary hepatocytes as well as **functional transporters** (e.g. OATB1B3, OCT1 and NCTP, data not shown).



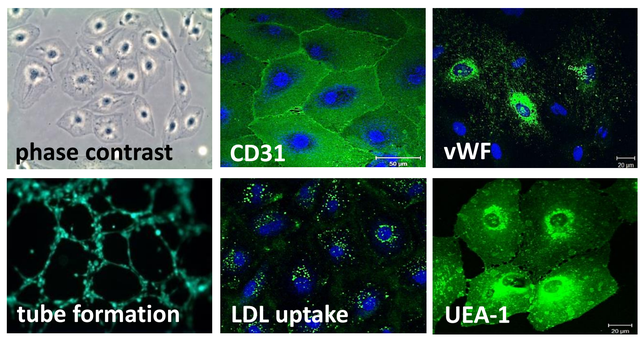
Employed compounds: ***CIT***: *sodium citrate (1-2 mM),* ***APAP***: *acetaminophen (0.5-2 mM),* ***TROG***: *troglitazone (50-100 µM),* ***CYC(A)***: *cyclosporin A 20-50 µM,* ***CHP***: *cumene hydroperoxide (100-500 µM),* ***ROT***: *rotenone (0.05-1 µM),* ***KET***: *ketotifen (1-10 µM)*

Acute and repeated-dose toxicity as determined by high content screening

upcyte® hepatocytes (donor 422a-03) were challenged with hepatotoxic compounds for 24 h or 1 week. Fluorescent probes were employed to evaluate **(A) viability**, **(B) apoptosis**, **(C) mitochondrial membrane potential (MMP)**, as well as levels of **(D) mitochondrial superoxide**, **(E) intracellular Ca^{2+} and (F) reactive oxygen species (ROS)**. Regarding **acetaminophen (APAP)**, no effect was observed after 24 h treatment, whereas **after 1 week, apoptosis and levels of intracellular Ca^{2+}, ROS and mitochondrial superoxide were significantly increased**. In general, repeated dosing over one week markedly increased the sensitivity towards hepatotoxic model compounds when compared to acute treatment. 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).

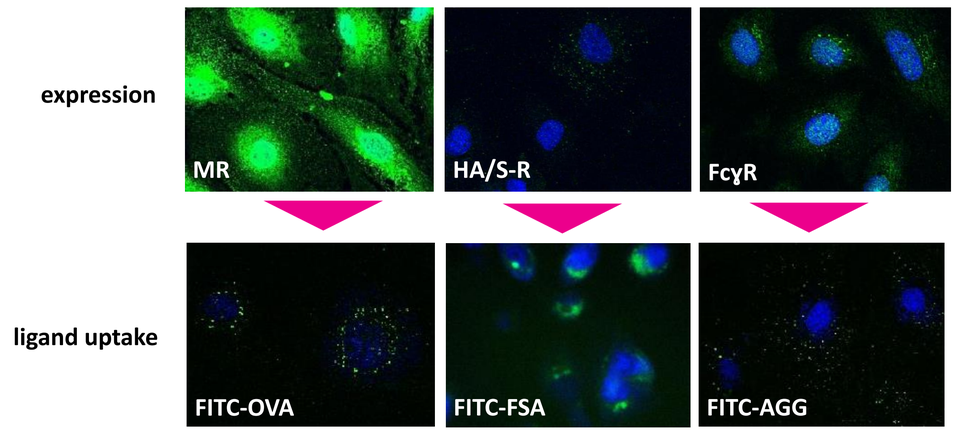
Tolosa *et al. 2016; Toxicological Sciences* 125 (1): 214-29

upcyte® LSECs



upcyte® LSECs express typical endothelial cell markers

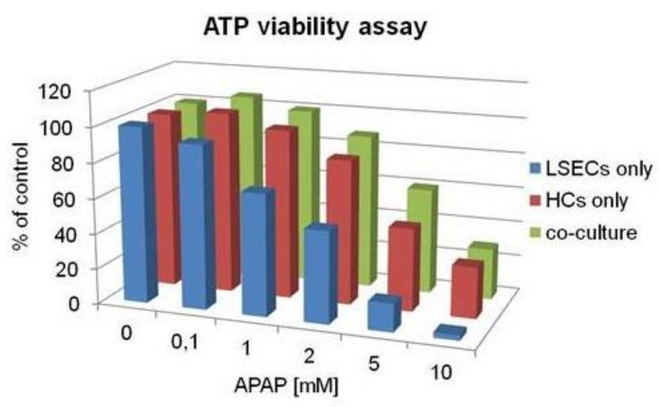
Morphology was comparable to primary cell LSECs. **CD31** was present in all cells investigated and showed a typical membrane localization. **vWF (von-Willebrand-factor)** was evident as perinuclear punctate structures, indicating its presence in the ER. upcyte® LSECs showed pronounced **tube formation** in Matrigel™ from single cells stained with Calcein-AM. **Uptake of Ac-LDL** (AF 488-AcLDL), indicating presence of SR-A (scavenger receptor A), a common endothelial cell receptor, was evident. In addition, we found strong expression of **UEA-1 (Ulex Europaeus Lectin 1)**.



LSEC-specific receptor expression and receptor-mediated endocytosis

We observed **high expression of the mannose receptor (MR) and FcγR in upcyte® LSECs**. The staining of **HA/S-receptor was visible**, but weak and not in all cells. The functional test of receptor-mediated endocytosis was performed by adding fluorescent-labeled ligands to the medium. The following ligands were used: FITC-FSA (HA/S-R), FITC-AGG (FcγR2BII) and FITC-mannan, DTAF-collagen-α-chains and FITC-ovalbumin (all three for MR, just one shown). **Uptake of ligands could be shown for the MR and the FcγR with the ligands FITC Ovalbumin and FITC-AGG** (aggregated gamma globulin). **Uptake of FITC-FSA** (formaldehyde-treated serum albumin**) for the HA/S-R could not be detected in all donors** (2/3).

Co-culture with hepatocytes



upcyte® hepatocytes (HC) and LSECs were cultured in mono- and co-cultures and treated with different concentrations of acetaminophen (APAP) for 72h.

LSECs are direct targets for early toxicity to APAP.

30 min after the administration LSECs become swollen and begin to lose their ability to endocytose FITC-FSA. After 2 h, prior to any effect on hepatocytes, fenestrae are lost [McCuskey, 2006/2008].

upcyte® LSEC alone were considerably more sensitive to APAP than when they were co-cultured with upcyte® hepatocytes (either in 2D or in Matrigel). APAP is detoxified to glucuronide and sulfate metabolites by hepatocytes [Hewitt *et al.*, 2007]. Therefore, this detoxification mechanism may have a protective effect for both hepatocytes and LSECs.

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SUMMARY & CONCLUSION

Overall, **upcyte® hepatocytes** and **LSECs** combine many features of primary cells with large availability to **replace the use of animals in pre-clinical studies**. Furthermore, co-culture of these cells in toxicity studies could boost the predictability of such assays and thereby reduce subsequent animal in vivo studies. Thus, thousands of animal lives could be saved that would otherwise would be used in toxicology studies. Consequently, upcyte® technologies actively contributes to the 3R strategy through the replacement of animal assays with highly predictive in vitro cytotoxicity & genotoxicity assays.