Development of a next-generation hepatocyte medium that increases the viability and functionality of human primary and iPS cell-derived hepatocytes for long-term studies

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Abstract

Human induced pluripotent stem (hiPS) cell-derived hepatocytes as well as human primary hepatocytes have the potential to serve as predictive human *in vitro* model systems for liver-disease and drug development studies, provided they possess relevant hepatocyte functions. Some studies, like chronic toxicity testing, demand that hepatocytes maintain functionality throughout a 2-week culture period, but standard culturing practices fall far short of this desired usage window.

To address this problem, we developed a novel hepatocyte maintenance medium that allows culturing of hiPS cell-derived hepatocytes for 14 days. We performed multiple analyses, including RT-qPCR, immunostainings, and functional assays, to investigate if our hepatocyte differentiation and maintenance system could generate mature hepatocytes and support their functionality during an extended culture time. Importantly, the hiPS cell-derived hepatocytes expressed important genes of the drug metabolizing machinery such as CYPs, phase II enzymes, and transporters during the entire culture time. Next, we exposed these hiPS cell-derived hepatocytes to known hepatotoxins for up to 14 days and found they respond correctly to these toxic compounds, demonstrating their utility for chronic toxicology studies. The hiPS cell-derived hepatocytes also respond to insulin, and they can take up and store low-density lipoproteins and fatty acids.

Since our new maintenance medium substantially extended the lifespan of hiPS cell-derived hepatocytes, we tested if it also could extend the lifespan of human primary hepatocytes (hphep cells). Cryopreserved hphep cells cultured in the medium maintained viability and displayed healthy morphology for up to 4 weeks. We also evaluated if the new culture conditions could enable intrinsic clearance (CL_{int}) studies by culturing hphep cells in the new medium for 10 days without medium change. During the timespan, no dedifferentiation or major cell loss was observed. ATP content was maintained, and CYP activities remained stable. In a proof-of-concept study, we showed that hphep cells cultured in the new medium could be used to accurately predict the *in vivo* CL_{int} of quinidine.

Taken together, our novel medium extends the functionality and viability of both hiPS cell-derived hepatocytes and hphep cells in conventional 2D cultures. This increased longevity and functionality significantly advances the applications of *in vitro* hepatocyte models.

1 Enhanced hiPS-HEP v2 cells display hepatic markers and functional characteristics of mature hepatocytes

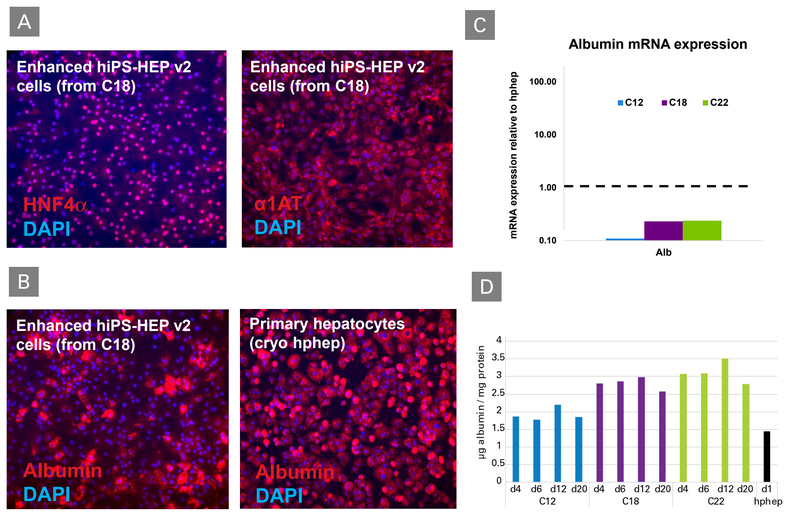

Figure 1. Characterization of hiPS cell-derived hepatocytes grown in improved maintenance medium. Panel A. hiPS cell-derived hepatocytes (hereafter referred to as "enhanced hiPS-HEP v2 cells") derived from the hiPS cell line ChiPSC18 (C18) grown in improved maintenance medium until Day 12 post-thawing show homogeneous staining for markers of adult hepatocyte cell fate: hepatocyte nuclear factor 4α (HNF4α), a transcription factor that regulates hepatic genes, as well as the hepatocyte-specific α1-antitrypsin (α1AT). **Panel B.** Representative images of enhanced hiPS-HEP v2 cells derived from C18 (on Day 12 post-thawing) and cryopreserved human primary hepatocytes (hphep cells; 24 hr post-thawing) stained for albumin and DAPI. Note that in both cultures, only a subset of hepatocytes is strongly immuno-positive for albumin, in agreement with the metabolic zonation observed in the liver lobe. Enhanced hiPS-HEP v2 derived from ChiPSC12 (C12) and ChiPSC22 (C22) display similar staining patterns (data not shown). **Panel C.** mRNA expression of albumin (Alb) after 20 days in culture as compared to hphep cells (dashed line) after 24 hr in culture (n = 2). **Panel D.** Albumin secretion as measured by ELISA; n = 2 for enhanced hiPS-HEP v2 cells, and n = 3 donors for hphep cells. Enhanced hiPS-HEP v2 cells display similar or higher albumin secretion levels than hphep cells.

2 Enhanced hiPS-HEP v2 cells show functional metabolic characteristics

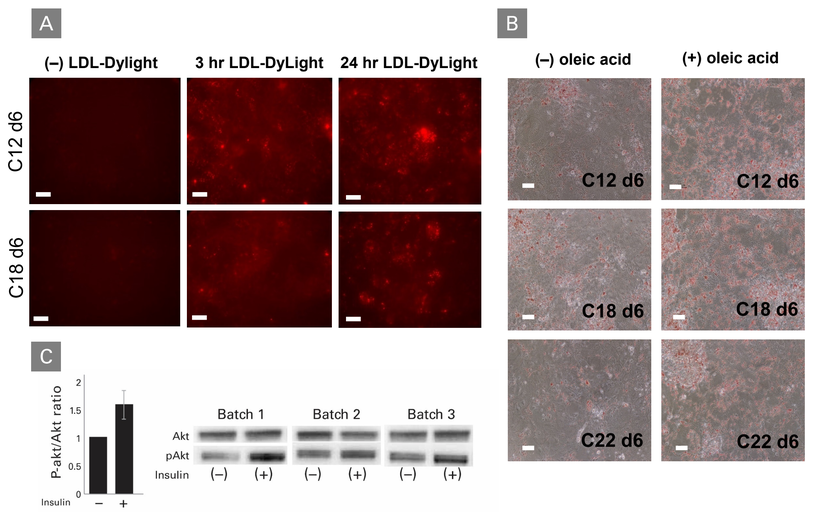

Figure 2. Analysis of metabolic features of enhanced hiPS-HEP v2 cells. Analyses were performed to assess the uptake of fatty acids and LDL, and response to insulin with phosphorylation of AKT. **Panel A.** Enhanced hiPS-HEP v2 cells take up fluorescently labeled LDL. Enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 (not shown) were incubated for 3 or 24 hr with LDL-DyLight. Representative images of LDL uptake on Day 6 post-thawing are shown. Other timepoints (Days 4 and 12 post-thawing) showed similar LDL uptake. Scale bar = 50 µm. **Panel B.** Enhanced hiPS-HEP v2 cells exposed to oleic acid show accumulation of lipid droplets as detected by Oil Red O staining. Enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 were incubated for 24 hr with 600 µM oleic acid or BSA vehicle control, and then fixed and stained with Oil Red O. Representative images of incubations on enhanced hiPS-HEP v2 cells on Day 6 post-thawing are shown. Other timepoints (Days 4 and 12 post-thawing) showed similar levels of Oil Red O staining. Scale bar = 25 µm. **Panel C.** Enhanced hiPS-HEP v2 cells respond to insulin with phosphorylation of AKT. Enhanced hiPS-HEP v2 cells (from C18, Day 12 post-thawing, n = 3) were incubated in insulin-free medium for 3 hr, and then treated for 10 min with 0 nM (–) and 100 nM insulin (+), respectively. Phosphorylated AKT and total AKT in insulin-treated cells (+) and untreated controls (–) were quantified using Western Blot. Western Blot data were kindly provided by Dr. Ann Hammarstedt, Department of Molecular and Clinical Medicine, Gothenburg University, Sweden.

3 Enhanced hiPS-HEP v2 cells have stable CYP enzyme activities for 20 days in culture

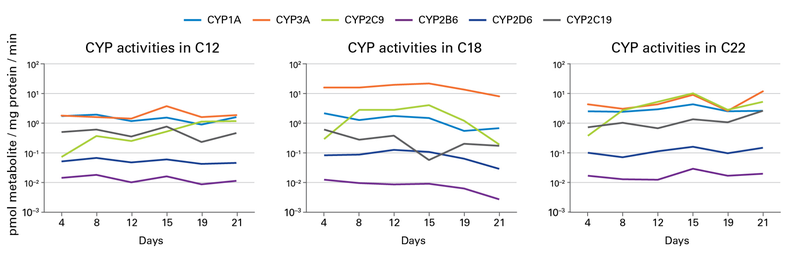

Figure 3. CYP450 activities are stable in enhanced hiPS-HEP v2 cells over a 21-day time window. CYP activity assays were performed on enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 on Days 4, 8, 12, 15, 19, and 21 post-thawing. Metabolite formation was analyzed using LC/MS. Importantly, CYP activities in enhanced hiPS-HEP v2 cells are stable over an extended culture time. Data shows two pooled wells per data point (n = 1 batch per hiPS cell line). Data are presented as mean ± SEM.

4 Enhanced hiPS-HEP v2 cells demonstrate effective activity and expression of phase II enzymes and transporters over an extended culture time

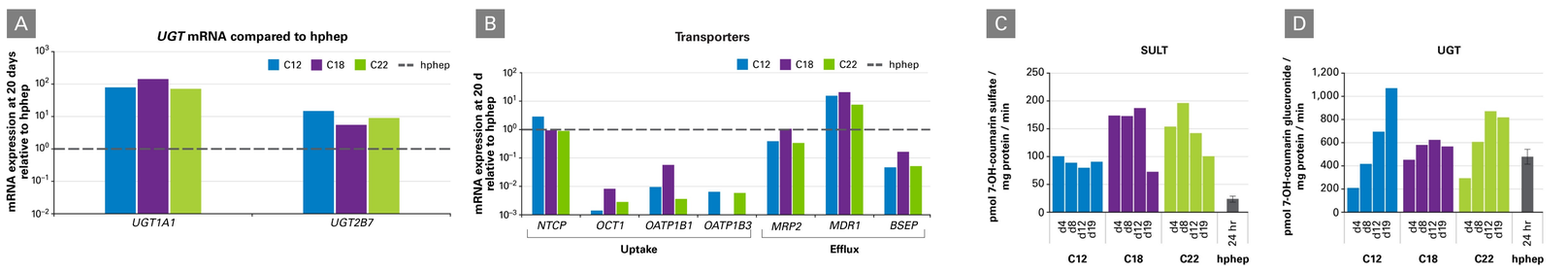

Figure 4. Enhanced hiPS-HEP v2 cells cultured in improved maintenance medium express phase II enzymes and transporters at substantial levels. Panel A. mRNA expression of phase II enzymes *UGT1A1* and *UGT2B7* in enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 on Day 20 post-thawing, relative to hphep cells at 24 hr post-thawing (dashed line). **Panel B**. mRNA expression of uptake transporter *NTCP* and efflux transporters *BSEP*, *MRP2*, and *MDR1* (P-gp) in enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 on Day 20 post-thawing, as compared to hphep cells after 24 hr post-thawing (dashed line).

Panels C and D. In sharp contrast to hphep cells, enhanced hiPS-HEP v2 cells have high SULT and UGT activities over time, as evidenced by the accumulation of the SULT metabolite 7-OH-coumarin sulfate (**Panel C**) as well as the UGT metabolite 7-OH-coumarin glucuronide (**Panel D**). These samples both represent data pooled from two wells as compared to hphep cells from n = 3 donors, and levels were measured by LC/MS.

5 Enhanced hiPS-HEP v2 cells show increasing sensitivity to hepatotoxic compounds upon chronic exposure

A

Sensitivity to hepatotoxic compounds (EC_{50} values) over time per cell type

Compound	Enhanced hiPS-HEP v2 from C18			Enhanced hiPS-HEP v2 from C22			HepaRG			hphep 3D spheroids		
	2 d	7 d	14 d	2 d	7 d	14 d	2 d	7 d	14 d	2 d	7 d	14 d
Amiodarone	54	15	11	110	10	5	101	35	19	100	12	13
Aflatoxin	89	0.6	0.1	401	6	7	1	0.3	0.1	0.6	0.1	<0.1
Troglitazone	221	133	115	262	153	169	ND	37	35	32	5	1
Chlorpromazine	214	20	7	51	9	16	67	32	34	16	8	5

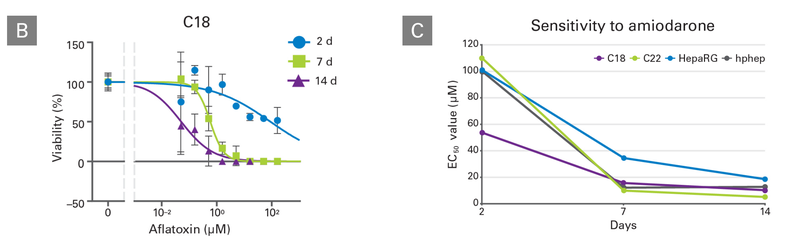

Figure 8. Enhanced hiPS-HEP v2 cells are a high-quality model system for chronic toxicity studies. Panel A. EC_{50} (concentration with 50% loss of viability) of four different known hepatotoxic compounds metabolized by a variety of CYP enzymes. Enhanced hiPS-HEP v2 cells (derived from C18 and C22) show increasing sensitivity to compounds after prolonged exposure, similar to HepaRG cells and 3D spheroids of hphep cells. **Panels B and C.** Dose-response curves of enhanced hiPS-HEP v2 cells. Enhanced hiPS-HEP v2 cells (from C18) were dosed with two known hepatotoxins (eight concentrations per compound) between Days 4 and 18 post-thawing, and cell viability was assessed after 2, 7, and 14 days of compound exposure using a CellTiterGlo assay. Panel B shows percent viability at eight different concentrations of aflatoxin. Panel C shows EC_{50} concentrations of amiodarone and demonstrates increasing sensitivity after prolonged exposure in all cell models tested. HepaRG and hphep cell 3D spheroid experiments were performed at AstraZeneca (Mölndal, Sweden) and Karolinska Institute (Stockholm, Sweden), respectively, within the Scr&Tox EU project.

6 Human primary hepatocytes maintained in improved maintenance medium retain viability, CYP enzyme activity, and hepatocyte morphology for 4 weeks in conventional 2D cultures

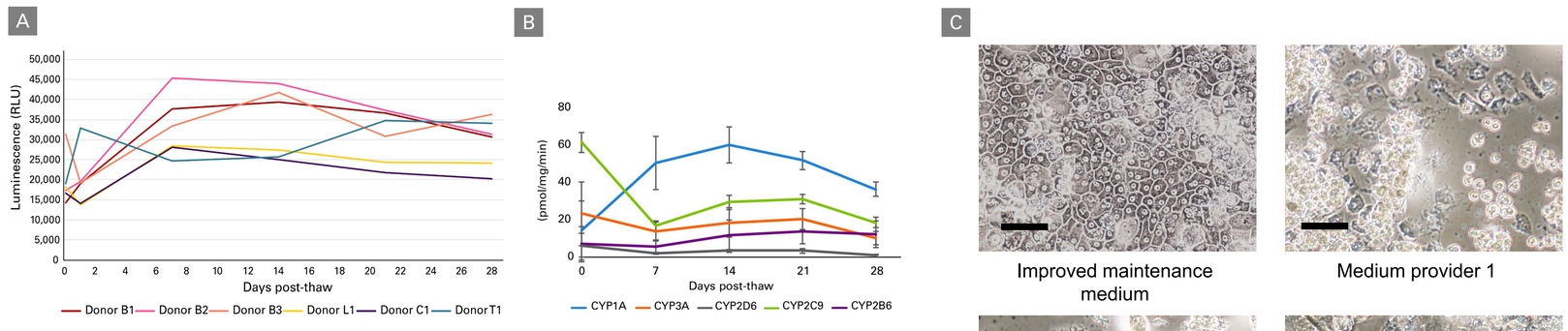

Figure 6. Use of the improved maintenance medium on human primary hepatocytes maintains high cell viability, stable CYP enzyme activity, and typical hepatocyte morphology for 4 weeks. Panel A. Hphep cells from four different providers were thawed and plated according to each supplier's recommendation. Viability of hphep cells was determined by measuring ATP content using a CellTiterGlo assay after 4 hr and 1, 7, 14, 21, and 28 days post-thawing. Results for six different hphep cell donors from four different hphep cell providers are shown. Data are presented as mean values for each donor (three technical triplicates). **Panel B.** CYP1A, 3A, 2D6, 2C9, and 2B6 activities were measured by LC/MS in hphep cells cultured in improved maintenance medium. Data are presented as mean values ± standard deviation (n = 2 donors). **Panel C.** Representative phase contract images showing morphology of hphep cells from one donor cultured for 28 days post-thawing in the improved maintenance medium compared to media from providers 1, 2, and 3. Scale bars = 100 µm.

7 Human primary hepatocytes are healthy and functional for 10 days in improved maintenance medium without medium change

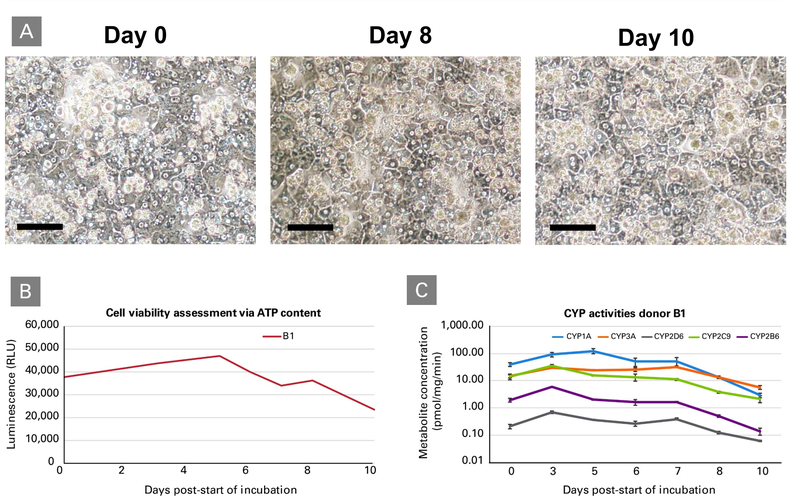

Figure 7. Human primary hepatocytes cultured for 10 days in improved maintenance medium without medium changes. Panel A. Hphep cells were thawed and plated according to each supplier's recommendation. At 4 hours post-thawing, the medium was changed to the improved maintenance medium. On Day 7 post-thawing, a 10-day incubation without medium change was initiated by adding fresh medium to each well. Representative phase contrast images of hphep cells at the start of incubation (Day 0), and after 8 and 10 days of incubation are shown here. Scale bars = 100 µm. **Panel B.** Viability of hphep cells was determined by measuring ATP content using a CellTiterGlo assay at the start of incubation (Day 0), and again 3, 5, 6, 7, 8, and 10 days after the start of incubation. Data are presented as mean values (two technical replicates). **Panel C.** CYP activity assays were performed at the same time points as above. The formation of specific metabolites was measured by LC/MS in hphep cells cultured in improved maintenance medium. Data are presented as mean values +/– standard deviation for each CYP enzyme tested (duplicates per donor).

8 Human primary hepatocytes in improved maintenance medium can be used to accurately predict *in vivo* intrinsic clearance

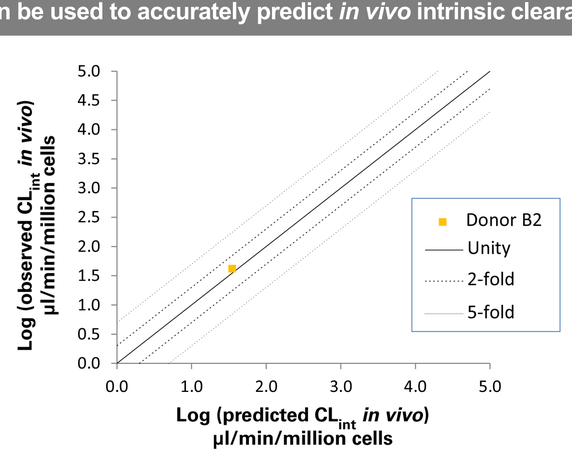

Figure 8. Prediction of *in vivo* CL_{int} of quinidine using primary hepatocytes cultured in improved maintenance medium. Hphep cells from a single donor were thawed and plated according to the manufacturer's instructions. On Day 7 post-thawing, 230 µl of fresh medium supplemented with 1 µM quinidine was added to each well of a 96-well plate. The incubation was continued for 10 days without medium change. Concentration of quinidine at the start of incubation (0 hr), 1 hr, 3 hr, 5 hr, and 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days after the start of incubation was monitored using LC/MS. Disappearance of quinidine was determined using Multiple Reaction Monitoring (MRM) and integration of chromatographic peaks. The *in vitro* CL_{int} was calculated and scaled to the liver to give a predicted *in vivo* CL_{int} that is in agreement with the observed *in vivo* CL_{int}. (Data kindly provided by AstraZeneca.)

Conclusions

- Enhanced hiPS-HEP v2 cells cultured with the improved maintenance medium:
  - Contain many mature hepatic features, including expression of mature hepatic markers and albumin secretion
  - Show functional metabolic features including insulin signaling response and uptake of fatty acids and LDL
  - Show activity and expression of *CYP450* genes as well as phase II enzymes and transporters
  - Retain all of these hepatic characteristics over a 2-week culture window
- Cryopreserved human primary hepatocytes grown in conventional 2D cultures with the improved maintenance medium:
  - Retain viability and typical hepatocyte morphology for 4 weeks
  - Maintain key CYP450 enzyme activity for 4 weeks, significantly longer than with conventional maintenance media
  - Are healthy and maintain CYP450 enzyme activity for 10 days without medium change
  - Can be used to accurately predict *in vivo* intrinsic clearance of slowly metabolized compounds

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