Next-generation human iPS cell-derived hepatocytes for metabolic disease modeling and drug discovery

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Abstract

Human pluripotent stem (hPS) cell-derived hepatocytes have the potential to serve as predictive human *in vitro* model systems for drug discovery, drug metabolism research, and hepatotoxicity studies, provided they possess relevant hepatocyte functions. Importantly, some hepatocyte applications, like chronic toxicity testing, demand a 2-week usage window, an order of magnitude outside standard culturing practices.

Here, we show data for a newly developed maintenance medium allowing culturing of the hPS cell-derived functional hepatocytes for 14 days, and thus enables their use for new applications with longer culture times. We have performed multiple analyses, including RT-qPCR, immunostainings, and functional assays, to investigate if our hepatocyte differentiation and maintenance system will 1) generate mature hepatocytes from multiple hPS cell lines, and then 2) to support their functionality during an extended culture time. Importantly, the hPSC-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 novel hPS cell-derived hepatocytes to known hepatotoxins for up to 14 days and found they respond correctly to these toxic compounds with an increasing sensitivity upon longer exposure, 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 we observed that our new maintenance medium substantially extended the lifespan of hPS cell-derived hepatocytes, we tested if it also could extend the lifespan of human primary hepatocytes. Interestingly, we found that cryopreserved human primary hepatocytes cultured in the new maintenance medium were viable and showed stable activities of several key CYP enzymes for several weeks in conventional 2D cultures, sharply contrasting existing commercially available hepatocyte maintenance media. Thus, the novel maintenance medium enables the use of human primary hepatocytes in conventional 2D cultures for applications requiring longer culture times. We hope that the increased assay window of functional hepatocytes in 2D cultures will advance the use of hPS cell-derived hepatocytes in metabolic disease modeling, and empower new areas of liver research and applications.

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

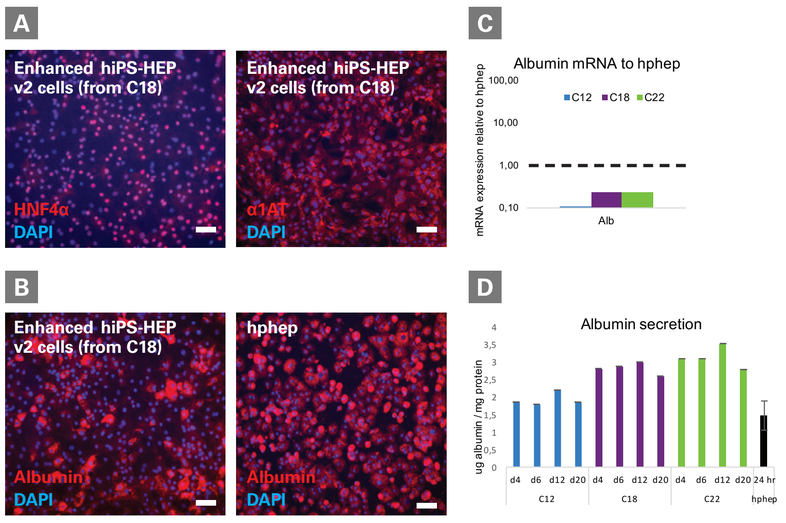

Figure 1. Staining patterns and functional characteristics of Enhanced hiPS-HEP v2 cells in improved maintenance medium.

Panel A. Enhanced hiPS-HEP v2 (on day 12 post-thawing) derived from the hiPSC line ChiPSC18 (C18) 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 gene α1-antitrypsin (α1AT). Scale bar = 50µm.

Panel B. Representative images of Enhanced hiPS-HEP v2 cells (on day 12 post-thawing) derived from C18, and cryopreserved human primary hepatocytes (hphep; 24 hr post-thawing) stained for Albumin and DAPI. Notably, 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 and ChiPSC22 display similar staining patterns (data not shown). Scale bar = 50µm.

Panel C. mRNA expression of albumin (Alb) after 20 days in culture as compared to hphep (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. Enhanced hiPS-HEP v2 display similar or higher Albumin secretion levels as hphep.

2 Enhanced hiPS-HEP v2 cells show functional metabolic characteristics

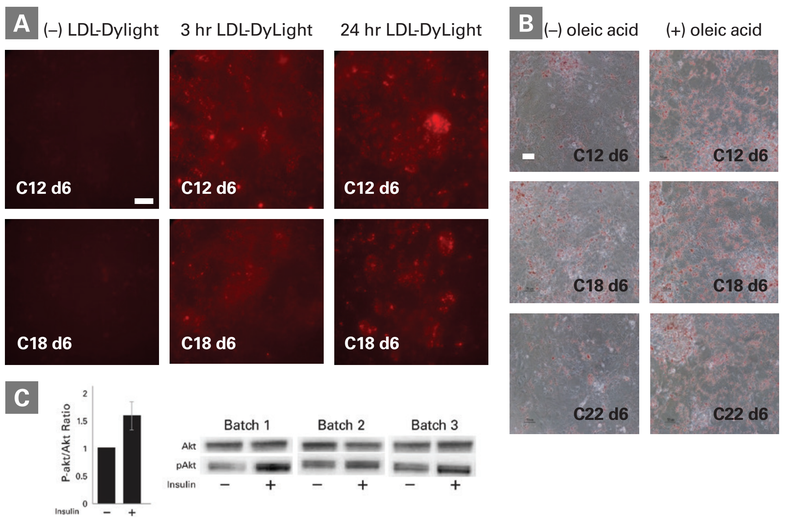

Figure 2. Analysis of metabolic features of Enhanced hiPS-HEP v2 cells. Analyses were performed to assess the uptake of fatty acids and LDL, and response to insulin with phosphorylation of AKT.

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 time points (days 4 and 12 post thaw) 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, then fixed and stained with Oil Red O. Representative images of incubations on Enhanced hiPS-HEP v2 cells derived C12 and C18 on day 6 are shown. Other time points (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-thaw, 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. WB 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 and show a similar profile as human primary hepatocytes

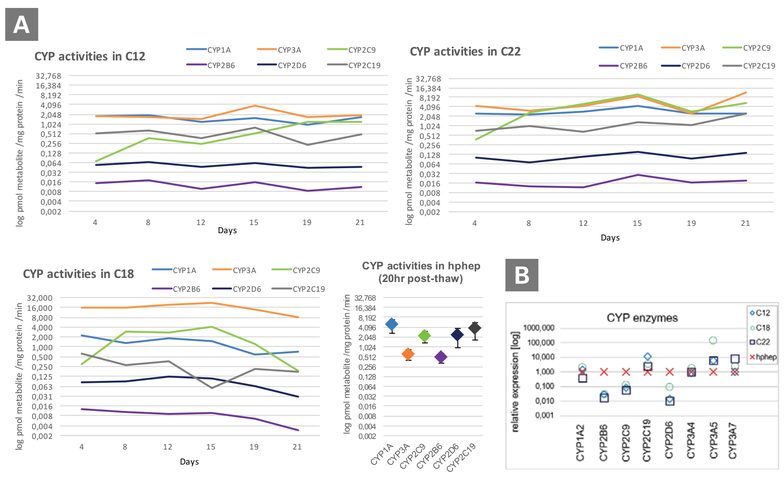

Figure 3. CYP450 activities are stable in Enhanced hiPS-HEP v2 cells over a 21-day time window with an expression profile similar to human primary hepatocytes.

Panel A. CYP activity assays were performed on Enhanced hiPS-HEP v2 cells derived from the hiPS cell lines ChiPSC12, ChiPSC18, and ChiPSC22 (abbreviated as C12, C18, and C22) on days 4, 8, 12, 15, 19, and 21 days 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 2 pooled wells per data point (n=1 batch per hiPSC line). For comparison, CYP activity assay was also performed on hphep (n=4 donors) thawed and cultured for 20hr (including the activity assay). Data is presented as mean ± SEM.

Panel B. mRNA expression of the eight most common drug-metabolizing CYP450 genes in Enhanced hiPS-HEP v2 cells after 20 days of culturing was compared to that of hphep cultured for 24 hr post-thaw. Expression levels of CYP1A2, 2C19, 3A4, 3A5, and 3A7 are similar to that of hphep. Notably, C18-derived Enhanced hiPS-HEP v2 cells show high mRNA expression levels of the polymorphic gene CYP3A5 and also the highest CYP3A activity levels.

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

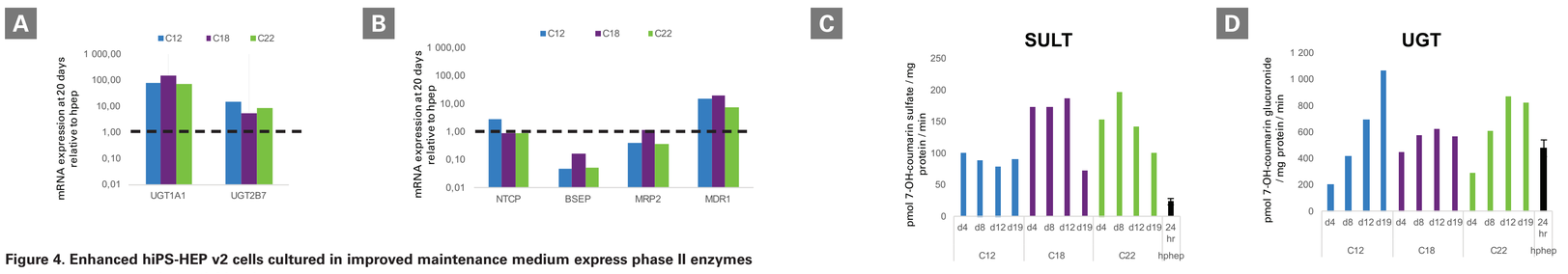

Figure 4. Enhanced hiPS-HEP v2 cells cultured in improved maintenance medium express phase II enzymes and transporters at substantial levels.

Panel A. mRNA expression of phase II enzymes UGT1A1 and 2B7 in in Enhanced hiPS-HEP v2 cells derived from C12, C18, and C22 after 20 days in culture, relative to hphep after 24 hr (dashed line).

Panel B. 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 after 20 days in culture compared to hphep after 24 hr in culture (dashed line).

Panel C and D. In sharp contrast to hphep, 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 compared to hphep 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

	Enhanced hiPS-HEP v2 from C18			Enhanced hiPS-HEP v2 from C22			HepaRG			hphep 3D spheroids		
	48h	7d	14d	48h	7d	14d	48h	7d	14d	48h	7d	14d
Amiodarone	53,8	15,4	10,5	110,0	10,1	5,2	101.0	34.7	18.5	>100	12.3	12.8
Aflatoxin	89,0	0,6	0,1	401,1	6,4	6,9	1.3	0.3	0.1	0.62	0.09	0.02
Troglitazone	221,5	132,5	114,5	262,0	153,4	168,7	n/a	36.5	34.6	32.4	4.5	1.4
Chlorpromazine	213,6	20,0	7,4	51,3	8,5	16,1	67.0	32.4	34.1	15.5	8.1	5.2

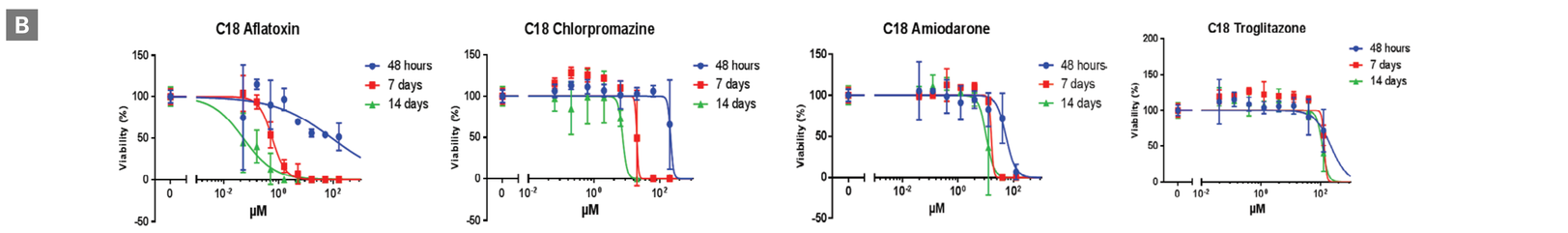

Figure 5. Enhanced hiPS-HEP v2 cells are a high-quality model system for chronic toxicity studies.

Panel A. EC50 (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 hiPSC lines C18 and C22) show increasing sensitivity to compounds after prolonged exposure, similarly to HepaRG and 3D spheroids of hphep.

Panel B. Dose-response curves of Enhanced hiPS-HEP v2 cells. Enhanced hiPS-HEP v2 cells (from C18) were dosed with four known hepatotoxins between days 4 and 18 post-thawing (8 concentrations per compound) and cell viability was assessed after 2, 7, and 14 days of compound exposure using a CellTiterGlo assay. Data is presented as % viability at 8 different compound concentrations.

HepaRG and hphep 3D spheroids 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 and hepatocyte morphology for 4 weeks in conventional 2D cultures

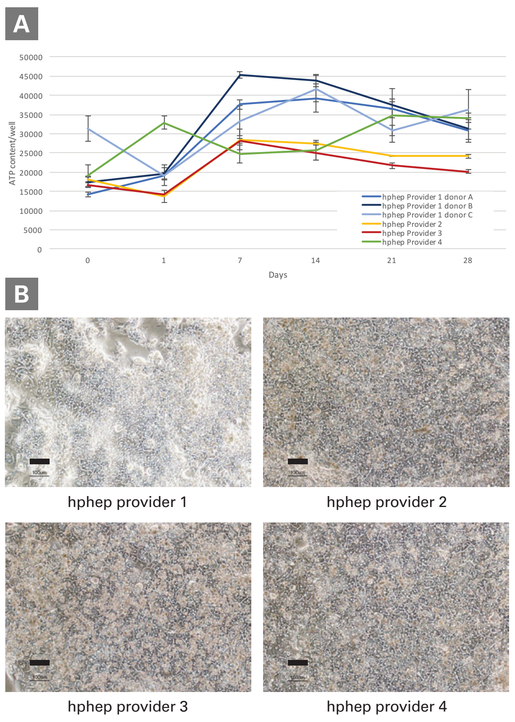

Figure 6. Use of the improved maintenance medium on human primary hepatocytes allows to maintain high cell viability and typical hepatocyte morphology for 4 weeks.

Panel A. Hphep from 4 different providers were thawed and plated according to each supplier's recommendation. Viability of hphep was determined by measuring ATP content using a CellTiterGlo assay after 4 hr and 1, 7, 14, 21, and 28 days post-thawing/plating. Results for 6 different hphep donors from 4 different hphep providers are shown. Data is presented as mean values ± standard deviation (3 wells per time point per donor).

Panel B. Representative phase contrast images showing typical hepatocyte morphology of hphep from 4 different hphep providers cultured for 28 days post-thawing in the improved maintenance medium. Scale bars = 100 µm.

7 Human primary hepatocytes cultured in the improved maintenance medium retain CYP activities for 4 weeks in conventional 2D cultures

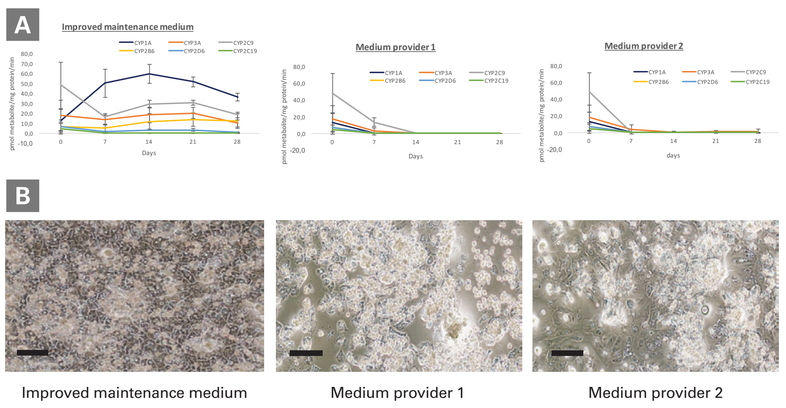

Figure 7. Use of the improved maintenance medium on hphep preserves CYP activities.

Panel A. CYP1A, 3A, 2C9, 2B6, 2D6, and 2C19 activities measured by LC/MS in hphep cultured in improved maintenance medium for 28 days, compared to two conventional media (provider 1 and 2) with claimed prolonged lifespan. Data are presented as mean values ± standard deviation. n=3 donors for improved medium, and n=2 donors for medium provider 1 and 2.

Panel B. Representative phase contrast images showing morphology of hphep cultured for 28 days post-thawing in the improved maintenance medium compared to media from provider 1 and 2. Scale bar = 100 µm.

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

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