

A novel maintenance medium extends the lifespan and enables long-term applications for both human primary hepatocytes and human pluripotent stem cell-derived hepatocytes in conventional 2D cultures



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Abstract

Human primary hepatocytes are considered the gold standard for *in vitro* model systems of liver function for drug development, toxicity assessment, and metabolic disease research; however, their rapid loss of cell viability in conventional 2D culture limits their utility in these applications. Human induced pluripotent stem (hiPS) cell-derived hepatocytes have potential as a better *in vitro* model if they possess a relevant usage window and functionality—but this is challenging to accomplish.

Addressing these problems, our newly developed hepatocyte maintenance medium enables the culture of cryopreserved human primary hepatocytes or hiPS cell-derived hepatocytes for four or two weeks, respectively, with maintained viability and stable activities of several key cytochrome P450 enzymes (CYPs). Multiple analyses on cryopreserved hiPS cell-derived hepatocytes, including RT-qPCR, immunostainings, functional assays such as albumin secretion, and CYP activity assays demonstrate mature features and high functionality. Importantly, the hiPS cell-derived hepatocytes show expression of the essential genes of the drug-metabolizing machinery, such as CYPs, phase II enzymes, and transporters.

An extended *in vitro* culture time for hepatocytes enables chronic toxicity testing. We show that hiPS cell-derived hepatocytes can be exposed to known hepatotoxins for up to 14 days. Cells respond as expected to these toxic compounds, demonstrating their utility for chronic toxicity studies. The hiPS cell-derived hepatocytes also respond to insulin, and they can take up and store low-density lipoproteins and fatty acids.

The novel maintenance medium presented here maintains the viability and functionality of cryopreserved human primary hepatocytes and hiPS cell-derived hepatocytes from multiple lines for a much longer time than existing commercially available hepatocyte maintenance media. We hope that the increased assay window of functional hepatocytes in 2D cultures will empower new areas of liver research and applications.

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

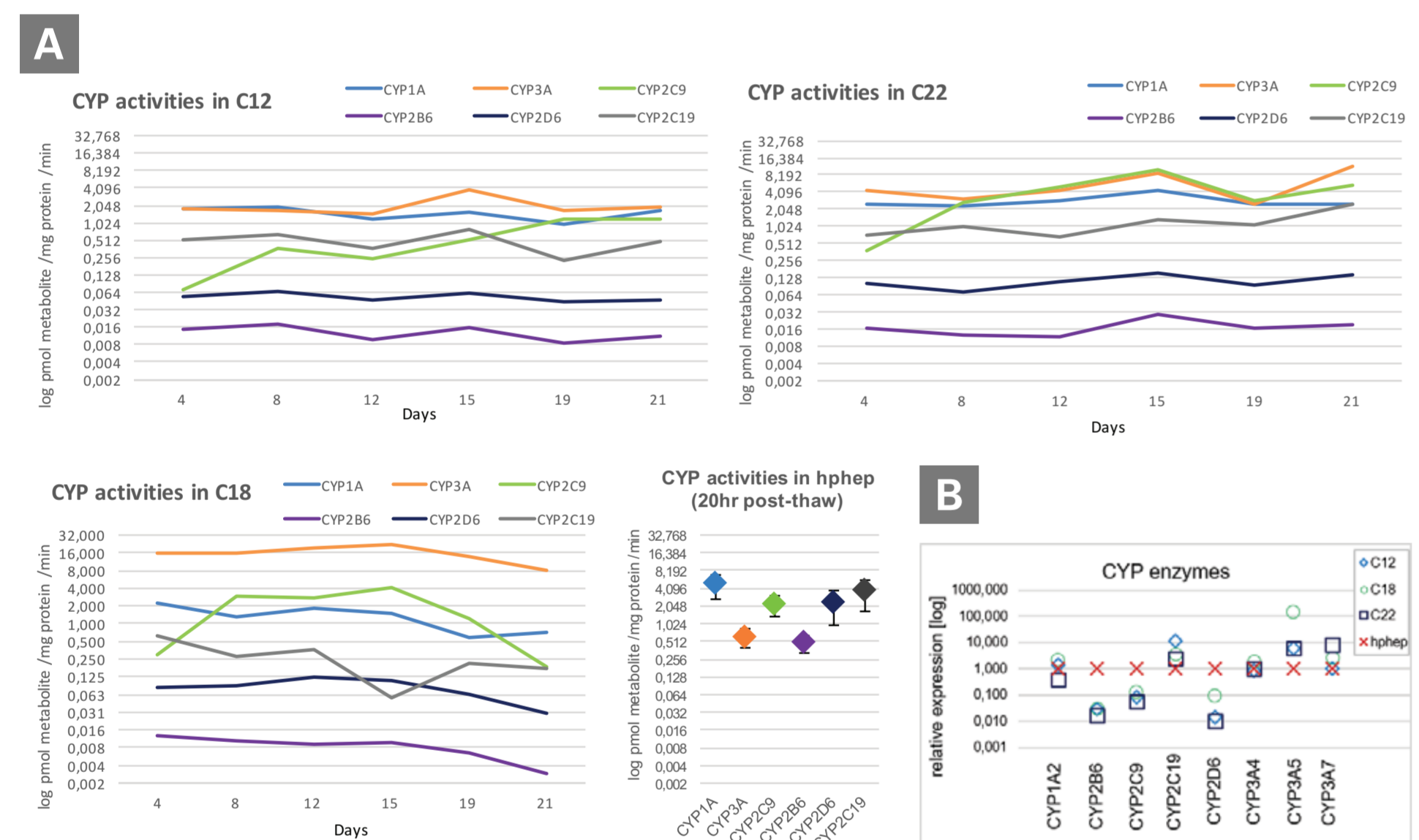


Figure 3. CYP450 activities are stable in Enhanced hiPS-HEP v2 cells over a 21-day time window with an expression profile similar to human primary hepatocytes.

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 hhphep (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 hhphep cultured for 24 hr post-thaw. Expression levels of CYP1A2, 2C19, 3A4, 3A5, and 3A7 are similar to that of hhphep. 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.

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

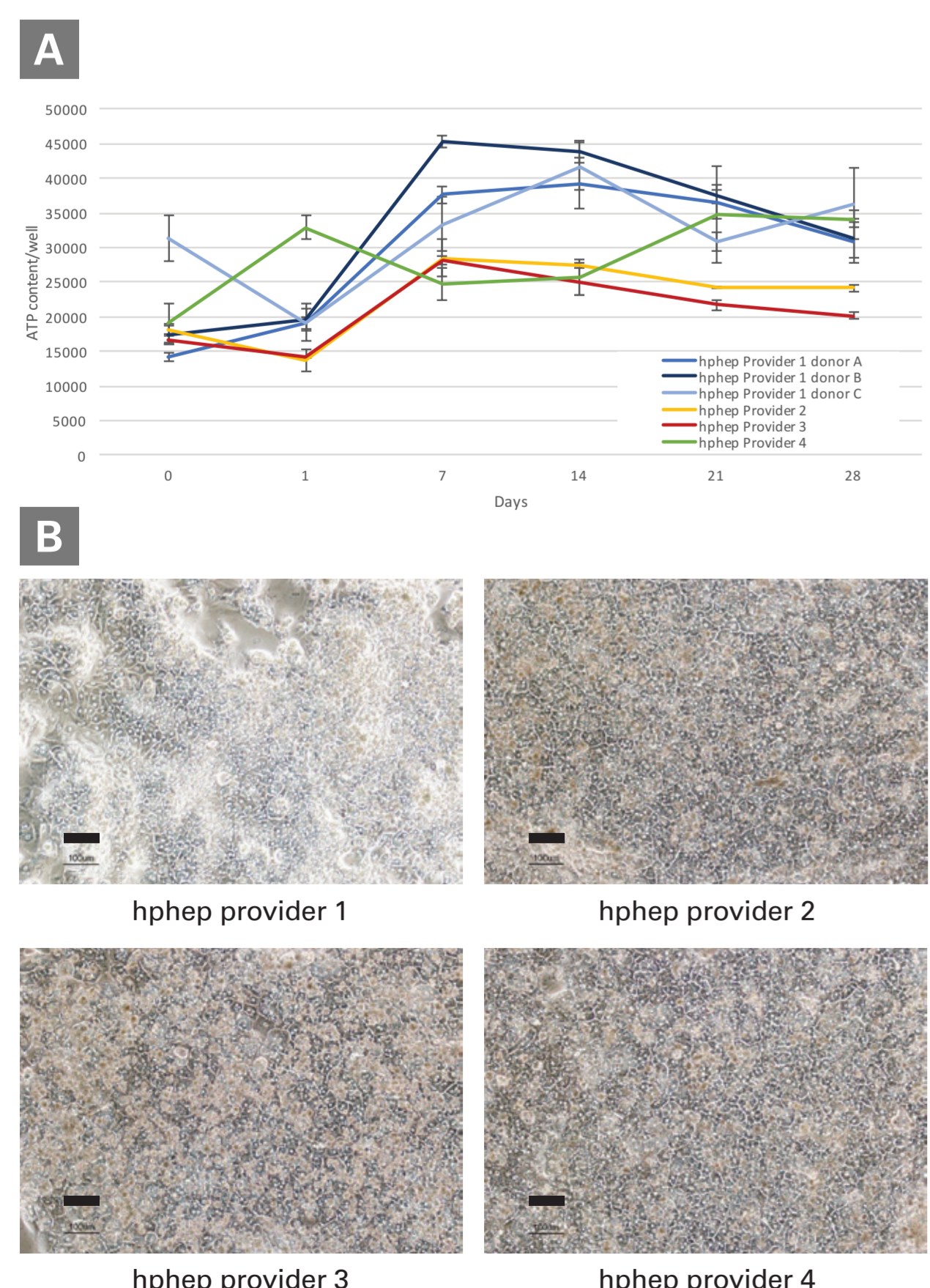


Figure 6. Use of the improved maintenance medium on human primary hepatocytes allows to maintain high cell viability and typical hepatocyte morphology for 4 weeks.

Panel A. Hhphep from 4 different providers were thawed and plated according to each supplier's recommendation. Viability of hhphep 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 hhphep donors from 4 different hhphep 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 hhphep from 4 different hhphep providers cultured for 28 days post-thawing in the improved maintenance medium. Scale bars = 100 µm.

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

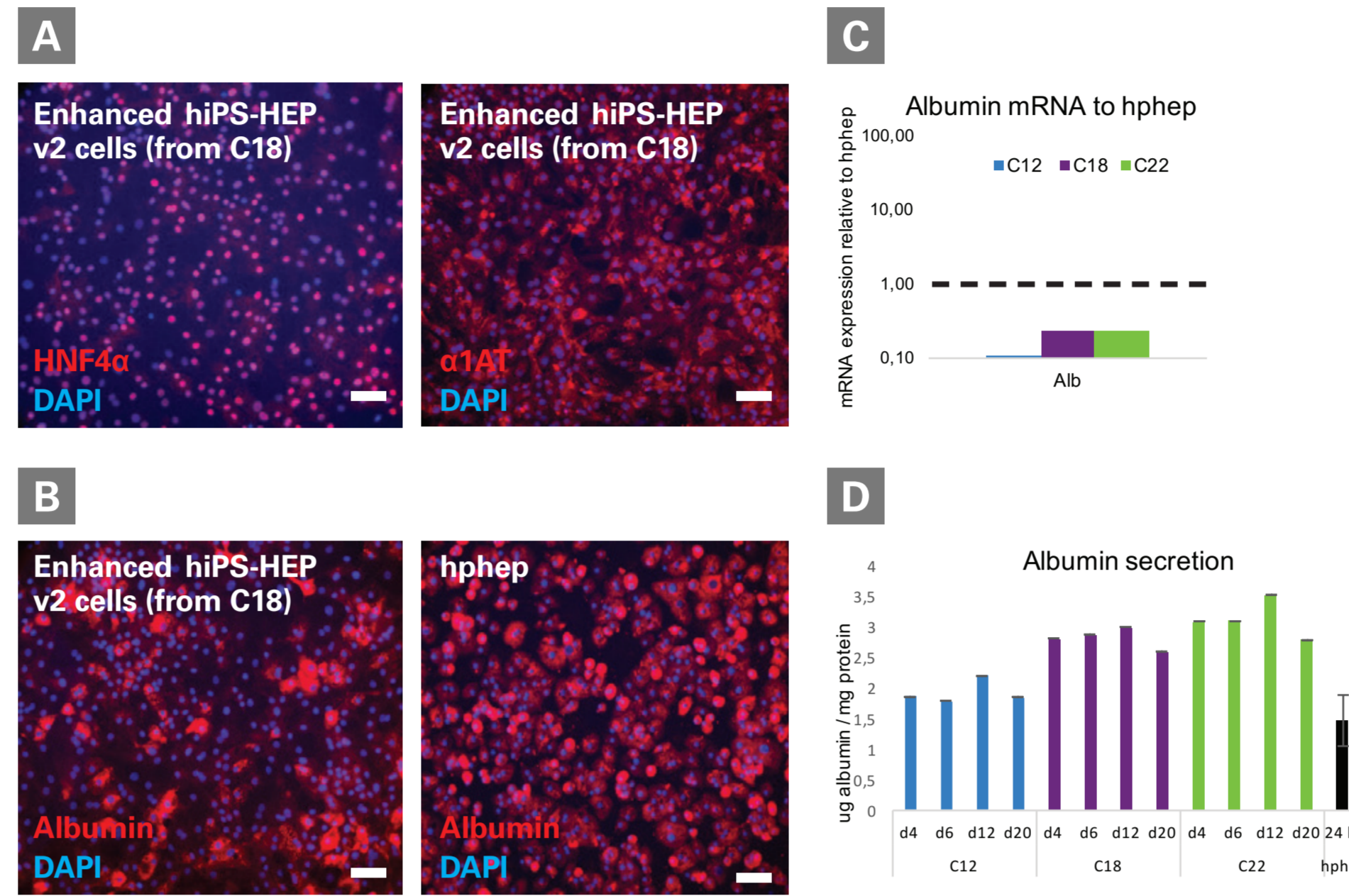


Figure 1. Staining patterns and functional characteristics of Enhanced hiPS-HEP v2 cells in improved maintenance medium.

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 = 50 µm.

Panel B. Representative images of Enhanced hiPS-HEP v2 cells (on day 12 post-thawing) derived from C18, and cryopreserved human primary hepatocytes (hhphep; 24 hr post-thawing) stained for Albumin and DAPI. Notably, in both cultures only a subset of hepatocytes is strongly immunopositive 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 = 50 µm.

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

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

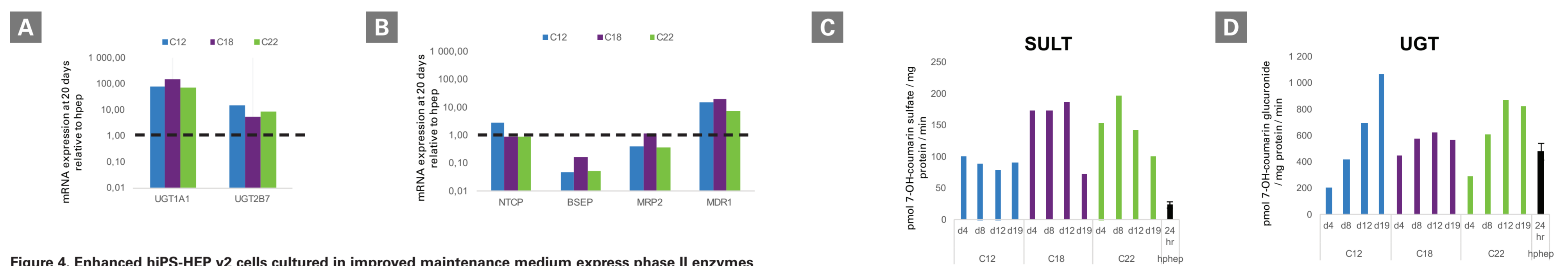


Figure 4. Enhanced hiPS-HEP v2 cells cultured in improved maintenance medium express phase II enzymes and transporters at substantial levels.

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

2. Enhanced hiPS-HEP v2 cells show functional metabolic characteristics

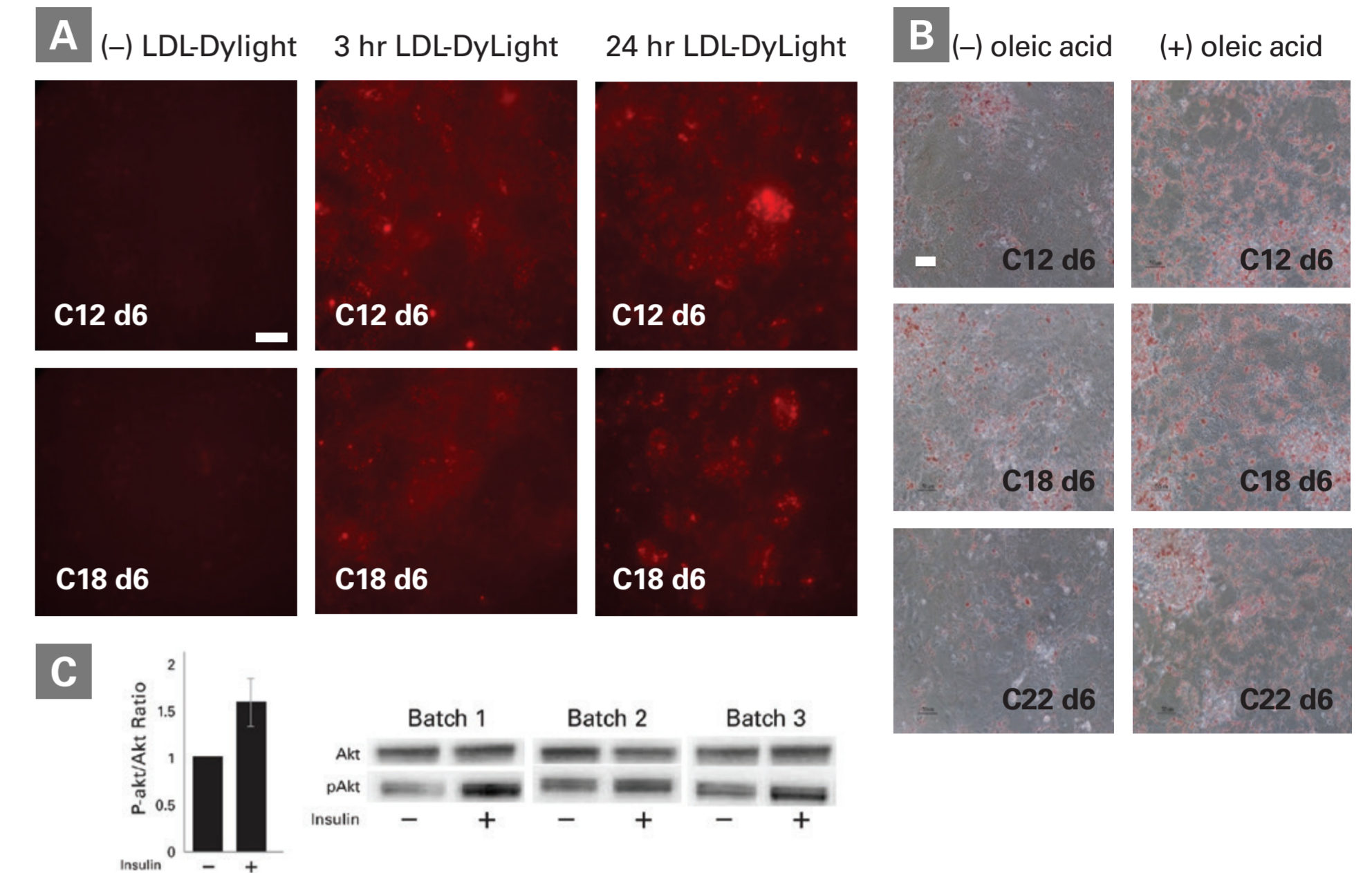


Figure 2. Analysis of metabolic features of Enhanced hiPS-HEP v2 cells. Analyses were performed to assess the uptake of fatty acids and LDL, and response to insulin with phosphorylation of AKT.

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.

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

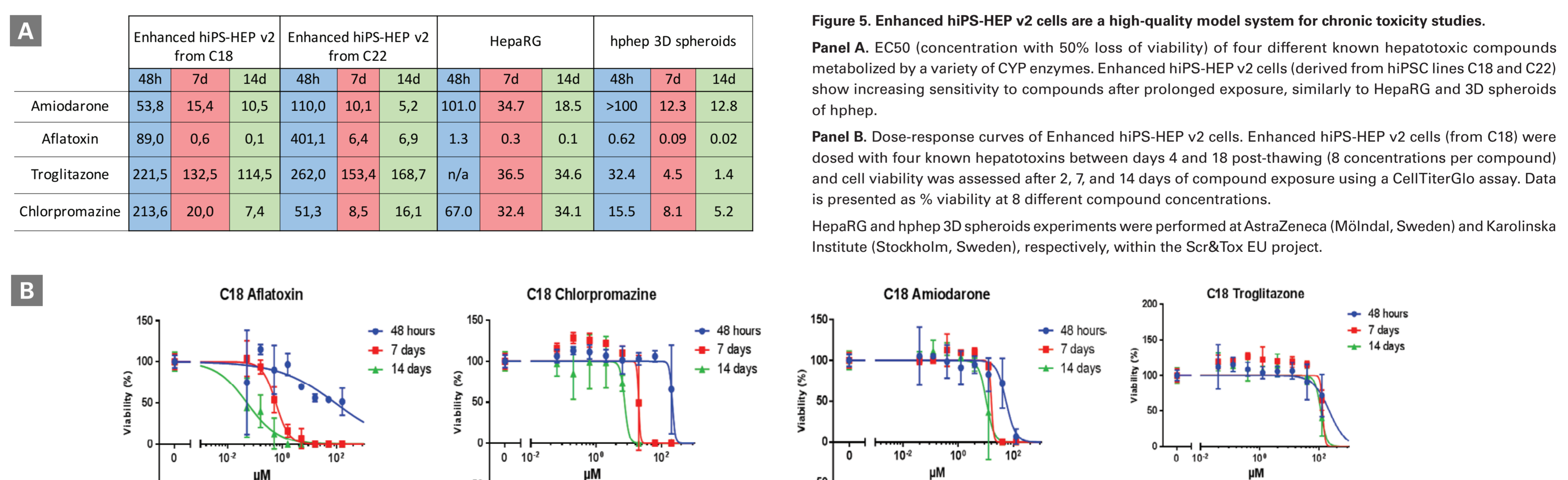


Figure 5. Enhanced hiPS-HEP v2 cells are a high-quality model system for chronic toxicity studies.

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 hhphep.

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 hhphep 3D spheroids experiments were performed at AstraZeneca (Mölnådal, Sweden) and Karolinska Institute (Stockholm, Sweden), respectively, within the Scr&Tox EU project.

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

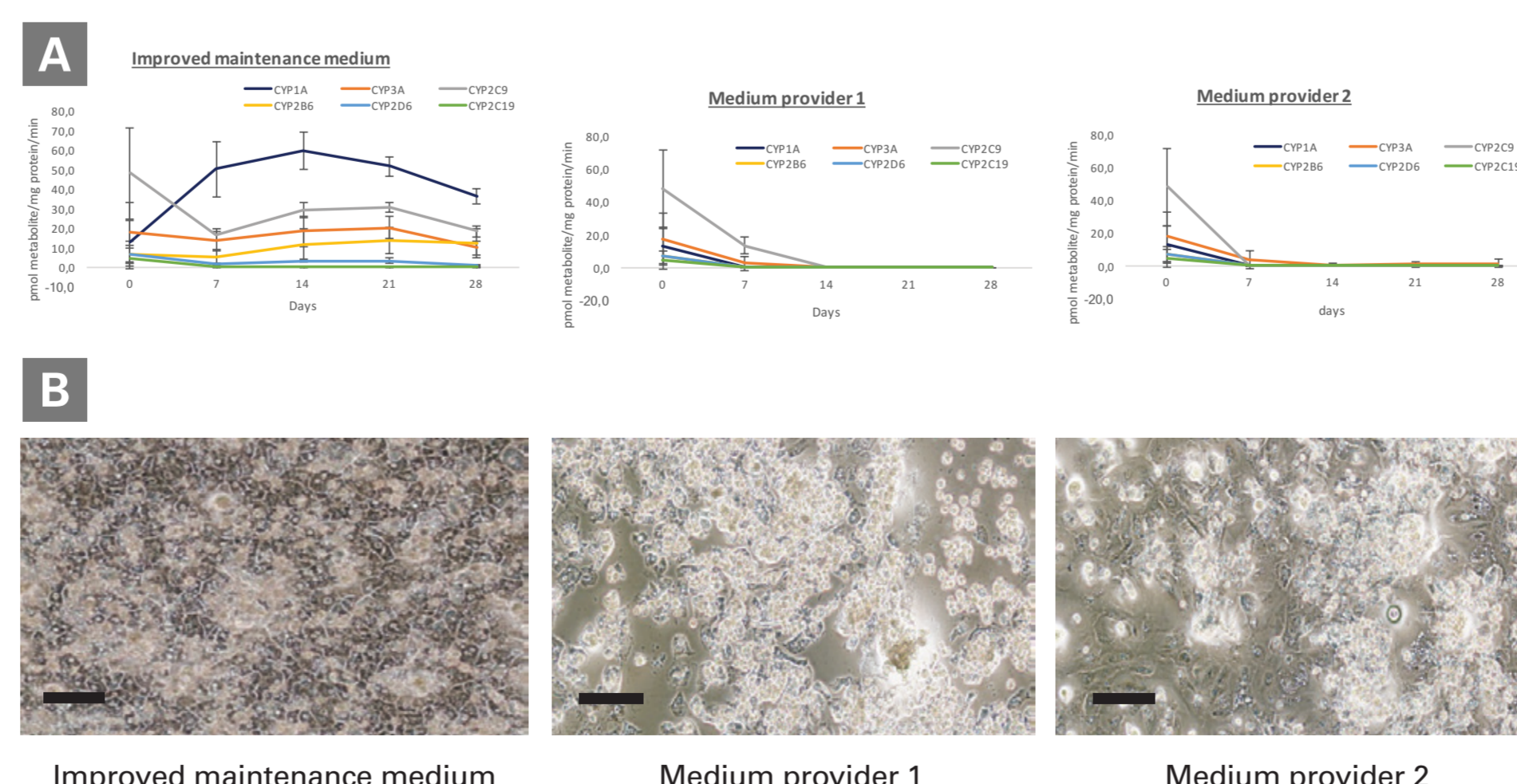


Figure 8. Use of the improved maintenance medium on hhphep preserves CYP activities.

Panel A. CYP1A, 3A, 2C9, 2B6, 2D6, and 2C19 activities measured by LC/MS in hhphep 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 hhphep 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