TECH NOTE

Cellartis Power Primary HEP Medium, a novel maintenance medium, enables long-term culture of human primary hepatocytes

Maintain human primary hepatocyte viability and typical morphology >>

Support stable albumin secretion for four weeks in culture >>

Sustain CYP activities of human primary hepatocytes long term >>

Induce CYP expression in human primary hepatocytes long term >>

Introduction

The liver is a vital organ, consisting of mostly hepatocytes, which performs over 500 functions including protein synthesis and detoxification of various metabolites. Human primary hepatocytes (hphep cells) are the gold standard for *in vitro* evaluation of drug metabolism, drug-drug interactions, safety assessment of drug candidates, and disease modeling. However, a significant limitation of hphep cells is their rapid loss of function when cultured *in vitro* (Richert et al. 2006). This fundamental limitation restricts the applications for which hphep cells can be used. To address this problem, 3D sandwich cultures with matrix overlays (Liu et al. 1999), bioreactors (Hoffman et al. 2012), and 3D spheroid cultures (Proctor et al. 2017) have been developed. Although these approaches can maintain some hepatocyte functions for several weeks *in vitro*, they do not entirely overcome the limitations of hphep cells because these culture systems restrict the types of assays that can be performed. Further, they require advanced and expensive lab equipment, are not easy to use, or are not generally applicable to hphep cells from different donors.

To enable long-term cultures of primary hepatocytes in user-friendly 2D-culture formats, we developed Cellartis Power Primary HEP Medium (Power HEP medium), a new medium that maintains healthy, functional human primary hepatocytes for up to four weeks in conventional 2D cultures—without the need of overlays or sandwich cultures—overcoming a key limitation of hphep cells. Furthermore, the recommended culture schedule is weekend free, requiring media changes only on Mondays, Wednesdays, and Fridays.

Results

Culture with Cellartis Power Primary HEP Medium results in viable hphep cells that display a typical hepatocyte morphology for four weeks

The first basic requirement for successful long-term culture is maintained cell viability. Therefore, we measured the ATP content of the cultures at multiple timepoints post-thawing as an indicator of cell viability and overall health of the hepatocytes. To this end, we thawed and plated primary hepatocytes from six donors (purchased from four commercial manufacturers) according to the recommendations from each manufacturer and then changed the medium to Power HEP medium 4 hr post-thawing. We then measured the ATP content four hours after the media change (Day 0) and again on days 1, 7, 14, 21, and 28. We found that ATP levels were stable, showing little variation between Days 7 and 28 post-thawing, indicating that the hepatocytes stayed viable for four weeks post-thawing (Figure 1). Interestingly, during the first week of culture, we observed an increase in ATP content between 4 hr and 7 days post-thawing, with some variations between donors, likely due to a recovery phase that varies between donors.

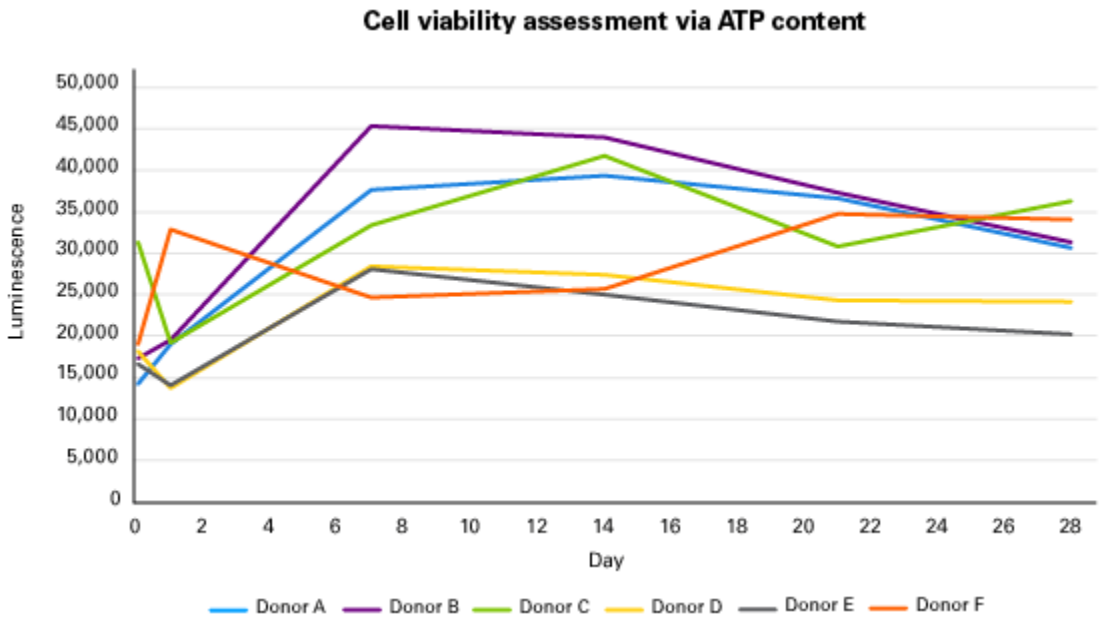

Figure 1. ATP content in primary hepatocytes cultured in Cellartis Power Primary HEP Medium. Human primary hepatocytes (six donors from four vendors) were thawed and plated according to each manufacturer's instructions. 4 hr post-thawing, medium was changed to Power HEP medium. Cell viability was assessed 4 hours after the media change (Day 0) and again at 1, 7, 14, 21, and 28 days in culture by measuring ATP content using the CellTiterGlo assay. Data are presented as mean values for each donor (three technical triplicates).

Next, we monitored cell morphology, which is a strong indicator of the differentiation status of hphep cells. Hphep cells were cultured for four weeks in Power HEP medium or the maintenance media from three different vendors, and the morphology of the cells was monitored. The phase contrast images show that the hphep cells cultured in Power HEP medium displayed typical hepatocyte morphology after 28 days in culture: the cells were polygonal; had distinct, white cell-to-cell borders; and were occasionally bi-nucleated (Figure 2, Panel A). In contrast, hphep cells cultured in the competitors' media rapidly lost hepatocyte morphology or viability (Figure 2, Panels B, C, and D).

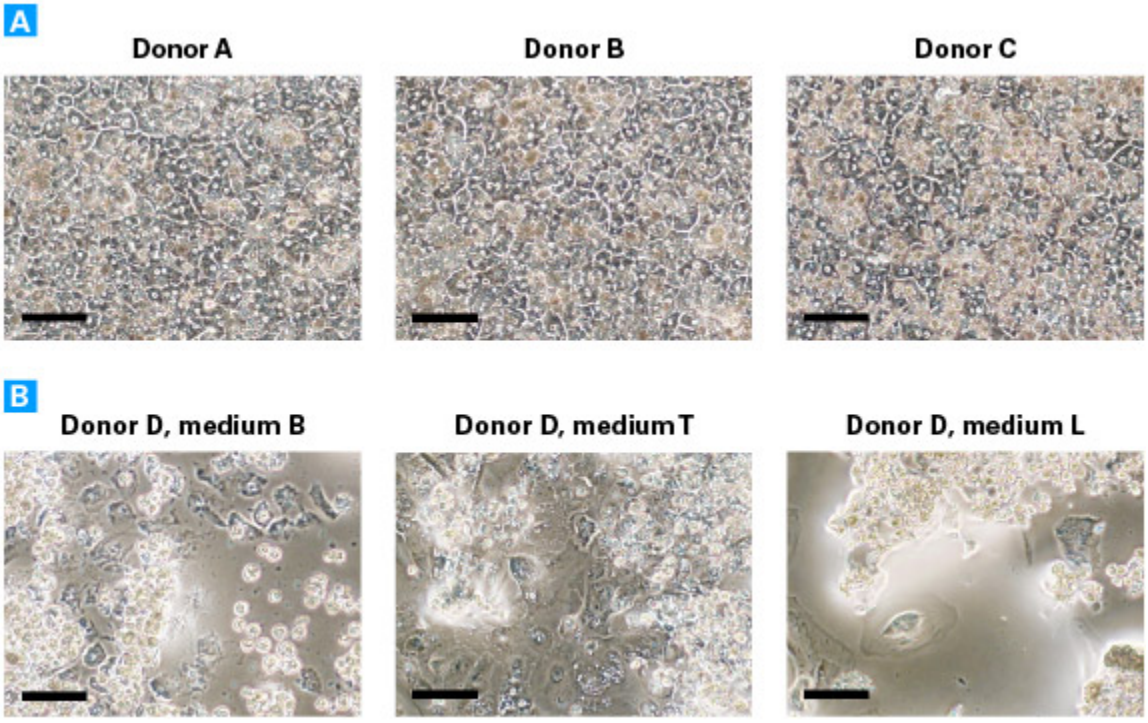

Figure 2. Morphology of primary hepatocytes cultured in Cellartis Power Primary HEP Medium and three commercially available hepatocyte maintenance media at Day 28 post-thawing. Panel A. Human primary hepatocytes were thawed and plated according to the manufacturer's instructions. 4 hr post-thawing, the medium was changed to Power HEP medium (shown for three donors). **Panel B.** Cells from a fourth donor (Donor D) were cultured in medium from Vendor B, Vendor T, or Vendor L. For all panels, the morphology of the hepatocytes was monitored during a 28-day culture period. Representative phase contrast images of hphep cells cultured after 28 days in the various media are shown. For all images, the scale bar is 100 µm.

Cellartis Power Primary HEP Medium supports stable albumin secretion from hphep cells for four weeks

The model of metabolic zonation proposes a functional specialization of hepatocytes depending on the cell's location in the liver lobe (Jungermann and Kietzmann 2000). According to this model, albumin secretion is a predominant function of hepatocytes in the periportal zone, while hepatocytes performing drug metabolism via cytochrome P450 (CYP) enzymes are in the perivenous zone.

Albumin is the main protein of human blood plasma, and albumin secretion is a commonly used characteristic to evaluate the quality of *in vitro* hepatocyte models, particularly for periportal hepatocytes. Overall, we show that not only was albumin already secreted after one day in culture, but secretion was also stably maintained throughout the 28-day culture period (Figure 3). Interestingly, we also observed that while total albumin secretion stayed stable for all 28 days (Figure 3, Panel A), total protein content increased from Day 1 to Day 7; therefore, once albumin is normalized to protein content, we saw an initial drop in the normalized albumin content before it stabilized to a constant level that was maintained out to 28 days. We hypothesize that the initial rise of protein content correlates with a recovery period after thawing and plating.

Taken together, these data demonstrate that when cultured in Power HEP medium, hphep cells secrete albumin—an important indicator of high-quality, functional hepatocytes—for up to 28 days.

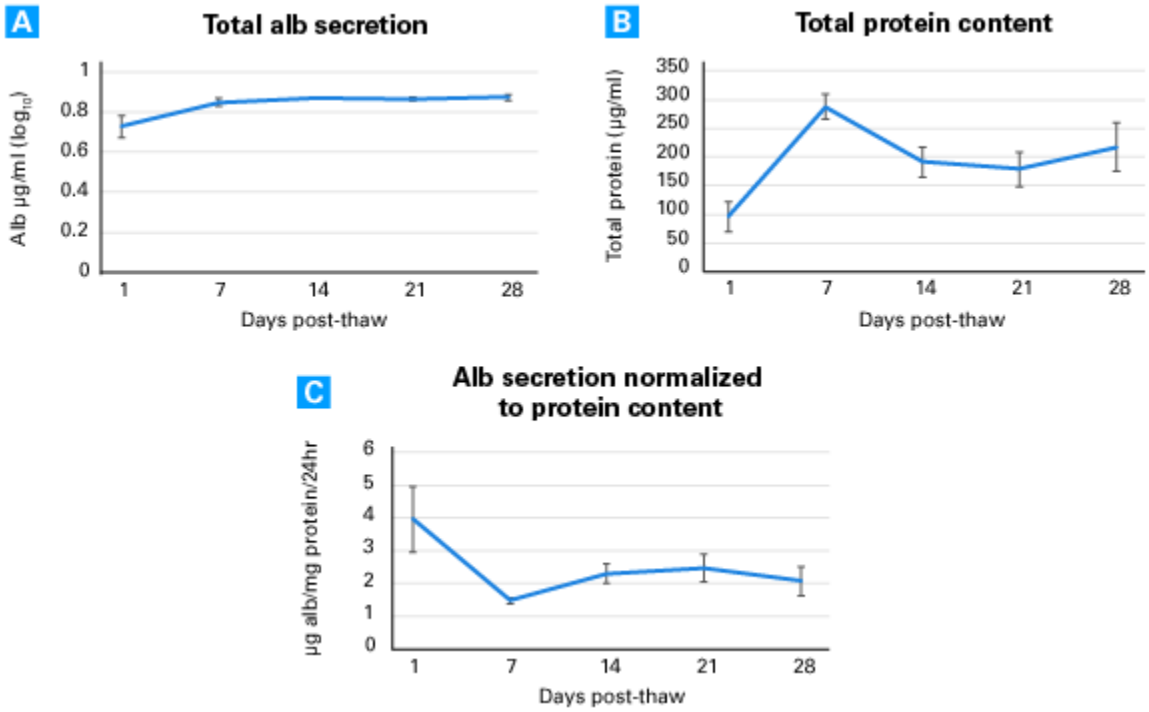

Figure 3. Albumin (alb) secretion in primary hepatocytes cultured in Cellartis Power Primary HEP Medium. Human primary hepatocytes (six donors from four vendors) were thawed and plated according to each manufacturer's instructions. 4 hr post-plating, the media was changed to Power HEP medium. **Panel A.** Albumin secreted into the medium during a 24-hr period was measured by ELISA on Days 0–1, 6–7, 13–14, and 27–28 post-thawing. **Panel B.** Protein content per well was quantified using the Pierce BCA Protein Assay kit. **Panel C.** Normalization of albumin secretion to total protein content. For all graphs, data are presented as mean values +/– standard deviation for n = 6 donors.

Cellartis Power Primary HEP Medium results in hphep cells with stable CYP activity for four weeks

Another major function of hepatocytes is the expression and activity of drug metabolizing CYP enzymes, which are important for the detoxification of drug compounds. This function occurs in hepatocytes from the perivenous zone and is often used to evaluate the functionality of *in vitro* hepatocyte models. We investigated the activities of five key CYP enzymes in primary hepatocytes from six donors (from four commercial vendors) cultured in Power HEP medium during a 4-week period. CYP activities were measured at 4 hr (Day 0) and 1, 7, 14, 21, and 28 days post-thawing, and levels were normalized to total protein content. CYP activities were sustained in all six hphep donors for the entire 4-week culture period, and levels were remarkably stable between Days 7 and 28 post-thawing (Figure 4). CYP activities were more variable during the first seven days of culturing, likely due to the recovery period after thawing. We observed the expected interindividual variation found in the general population, as demonstrated by variation in activity levels of different CYPs depending on the donor. This variation was also maintained over the 28-day culture period.

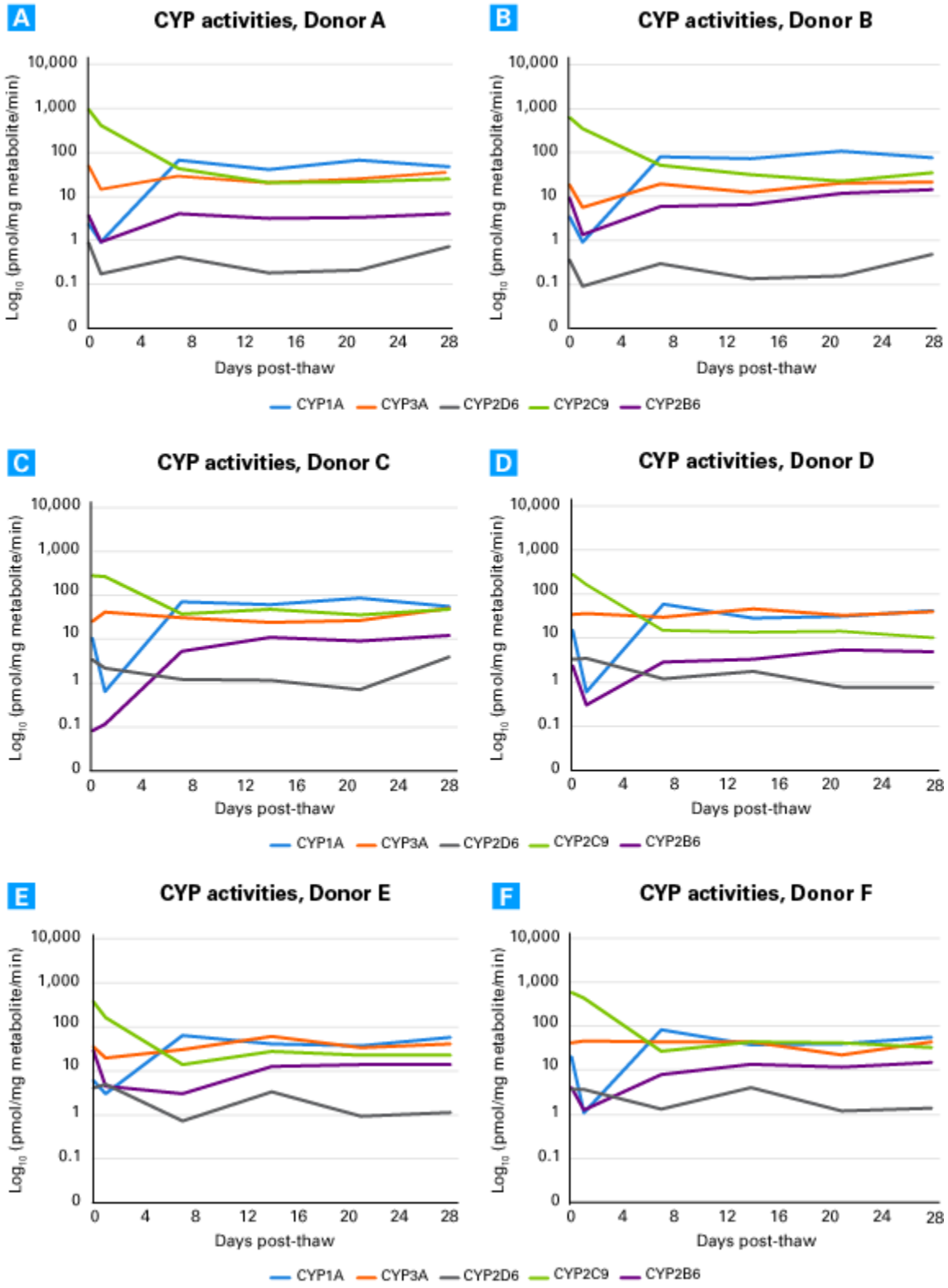

Figure 4. CYP activities in primary hepatocytes cultured in Cellartis Power Primary HEP Medium for 28 days. Panels A–F. Human primary hepatocytes (six donors from four vendors) were thawed and plated according to each manufacturer's instructions. CYP activity assays were performed 4 hr (Day 0) and 1, 7, 14, 21, and 28 days post-thawing. The formation of

specific metabolites (CYP1A = Paracetamol, CYP3A = OH-Midazolam, CYP2C9 = OH-Diclofenac, CYP2B6 = OH-Bupropion, and CYP2D6 = OH-Bufuralol) was measured by liquid chromatography/mass spectrometry (LC/MS). Metabolite concentrations were normalized to protein content and activity assay duration. Data are presented as mean values for each CYP enzyme tested (three technical triplicates).

To investigate how the performance of hphep cells cultured in Power HEP medium compares to other commercially available hepatocyte maintenance media, we cultured primary hepatocytes from two different donors either in Power HEP medium or in hepatocyte media from three vendors. Again, hphep cells showed sustained CYP activities for 28 days when cultured in Power HEP medium (Figure 5, Panel A), which is in sharp contrast to hphep cells cultured in other commercially available media where the activities rapidly dropped off (Figure 5, Panels B–D). This clearly demonstrates that in comparison to other media, Power HEP medium preserves CYP activity levels in plated hphep cells, as it is the only medium that stably maintains CYP enzyme function.

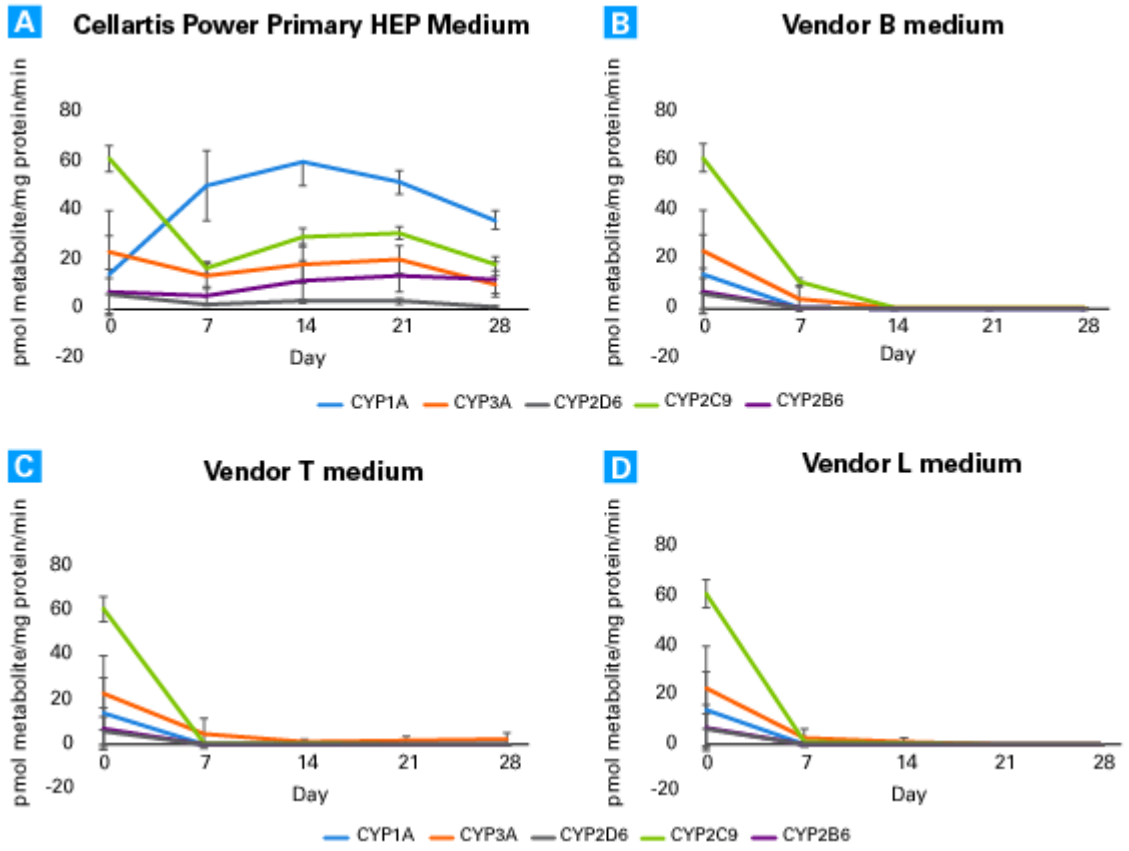

Figure 5. CYP activities in primary hepatocytes cultured in Cellartis Power Primary HEP Medium and three commercially available hepatocyte maintenance media. Human primary hepatocytes from two donors were thawed and plated according to the manufacturer's instructions. The hphep cells were cultured in Power HEP medium (**Panel A**) or medium from Vendor B (**Panel B**), Vendor T (**Panel C**), and Vendor L (**Panel D**). CYP activities were measured 4 hr (Day 0) and 7, 14, 21, and 28 days post-plating by performing a CYP activity assay for the formation of specific metabolites (CYP1A = Paracetamol, CYP3A = OH-Midazolam, CYP2C9 = OH-Diclofenac, CYP2B6 = OH-Bupropion, CYP2D6 = OH-Bufuralol) and measuring by LC/MS. Metabolite concentrations were normalized to protein content and activity assay duration. Data is presented as mean values +/– standard deviation for each donor (n = 2).

Cellartis Power Primary HEP Medium results in hphep cells that show inducible CYP expression after four weeks

Drugs can increase CYP enzyme levels by inducing their mRNA expression, which can cause a change in the effects of coadministered drugs, leading to serious problems for patients taking multiple medications. Consequently, the

assessment of potential drug-drug interactions is important in drug development, and hphep cells are the gold standard for assessing potential CYP inductions by a drug candidate.

To evaluate whether this important hepatocyte feature is preserved in hphep cells cultured with Power HEP medium, we exposed hphep cells to typical inducers (Table I) for 48 hr after they had reached 26 days in culture. We then analyzed *CYP1A2*, *2B6*, *2C9*, and *3A4* mRNA expression on Day 28 and compared them to levels in DMSO-treated control cells.

CYP	Induced by
1A2	Omeprazole (Ome)
2B6	Phenobarbital (PB), Rifampicin (Rif)
2C9	Phenobarbital (PB), Rifampicin (Rif)
3A4	Phenobarbital (PB), Rifampicin (Rif)

Table I. Overview of CYP enzymes induced by three typical inducers.

Surprisingly, we found that even after an extended time in culture prior to induction, all four CYPs tested were induced at high levels (Figure 6) comparable to the fold induction shown for hphep cells induced 2–4 days post-thawing (Yajima et al. 2014). The only exception to the pattern of high levels of CYP inducibility across donors is that *CYP2C9* induction by Rif was observed in only 2 out of 3 donors (Figure 6, Panel D); however, this is consistent with reports that *CYP2C9* is not inducible in some donors (Yajima et al. 2014).

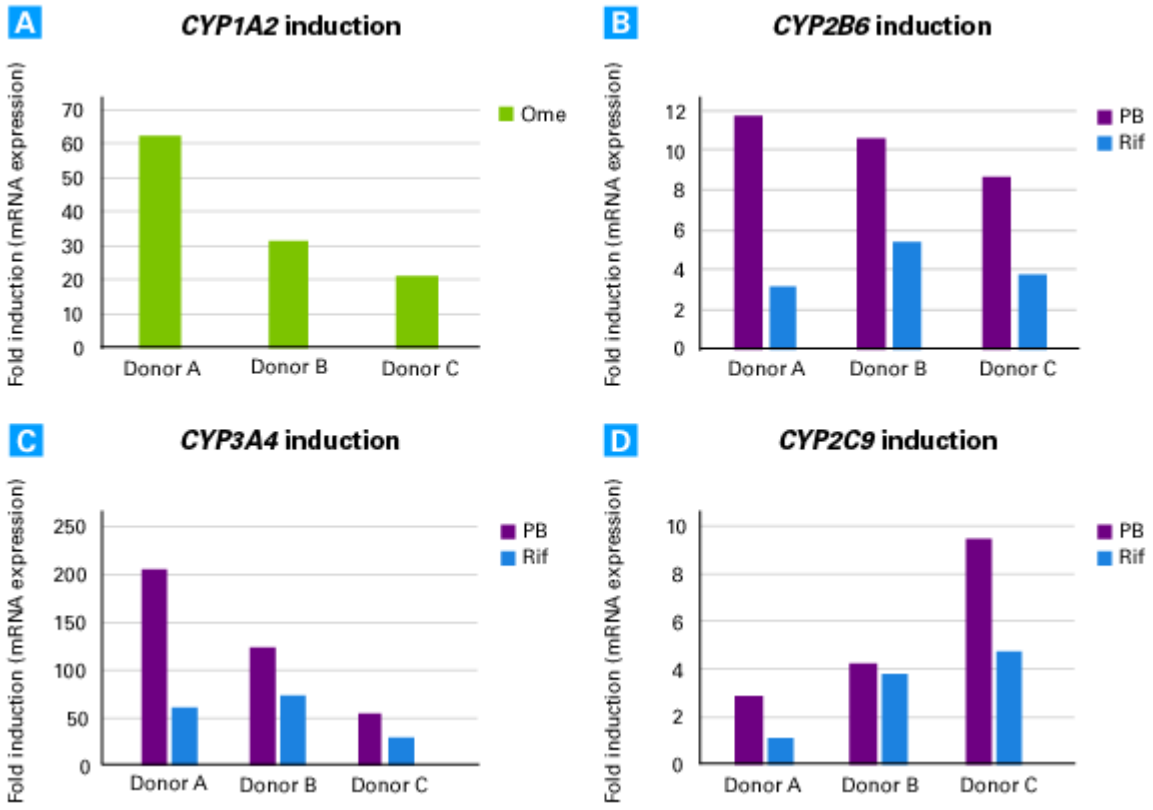

Figure 6. Induction of CYP mRNA expression in primary hepatocytes cultured in Cellartis Power Primary HEP Medium. Three donors of human primary hepatocytes were cultured in Power HEP medium for 26 days and subsequently treated

for 48 hr with 25 µM omeprazole (Ome), 1 mM phenobarbital (PB), 50 µM rifampicin (Rif), and/or 0.2% DMSO (vehicle control). On Day 28, cells were harvested and *CYP1A2* expression in response to Ome (**Panel A**), *CYP2B6* to PB and Rif (**Panel B**), *CYP3A4* to PB and Rif (**Panel C**), and *CYP2C9* to PB and Rif (**Panel D**) were measured by qRT-PCR and compared to the DMSO control. CYP levels are presented as fold induction over DMSO control (n = 1 for each donor).

Conclusions

Primary hepatocytes are the gold standard for liver research but, to date, their utility has been strongly limited by their short functional lifespan in user-friendly 2D cultures. This short lifespan prevents hphep cells from being used for assays requiring longer time in culture such as chronic toxicity or drug clearance experiments. Our novel Cellartis Power Primary HEP Medium enables maintenance of viable and functional hepatocytes for four weeks in conventional 2D cultures. Importantly, key hepatocyte functions such as albumin secretion, CYP activities, and CYP inducibility are maintained for the four-week culture period. Notably, both periportal and perivenous features, typical for the different hepatocyte phenotypes present in the different zones of the liver lobe, are preserved.

The extended lifespan of primary hepatocytes cultured with Power HEP medium in user-friendly 2D cultures and with a weekend-free feeding schedule significantly enhances the utility of primary hepatocytes in multiple applications, such as drug discovery and safety toxicology studies.

Methods

Primary hepatocyte culture

Cryoplateable human primary hepatocytes were purchased from four vendors (BioreclamationIVT, Lonza, Corning, and Thermo Fisher Scientific), thawed, and plated according to each vendor's instructions. 4 hr post-thawing, plating medium was carefully removed and replaced with prewarmed Cellartis Power Primary HEP Medium or media from other vendors. Cells were maintained for up to 28 days post-thawing. Cells cultured in Power HEP medium were cultured according to the Cellartis Power Primary HEP Medium User Manual, with media changes every second or third day. Cells cultured in medium from other vendors were cultured according to each manufacturer's instructions.

Protein quantification

Cells were washed once with D-PBS (1X Dulbecco's Phosphate-buffered saline with Calcium and Magnesium), lysed in 0.02 mM NaOH overnight at 4°C, and stored at –20°C until analysis. Protein amount was quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

ATP quantification

The viability of the hphep cells was determined at multiple timepoints after thawing using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Luminescence was measured and the values were blank corrected.

Albumin secretion

Albumin secretion from primary hepatocytes was analyzed at multiple timepoints after thawing. The culture medium was collected after 24 hr of conditioning, and albumin content was analyzed using the Albuwell kit (Exocell) according to the manufacturer's instructions, then normalized to the total protein amount per well.

CYP induction

CYP induction was assessed between Days 26 and 28 post-thawing. Hphep cells were incubated with the inducers omeprazole (25 µM), phenobarbital (1 mM), and rifampicin (50 µM) in William medium E containing 0.1% Penicillin-Streptomycin (PEST) and HCM SingleQuots (w/o GA1000 and hydrocortisone). DMSO (0.2%) was used as a vehicle control. Medium with inducers was refreshed after 24 hr of induction, and after 48 hr cells were harvested in RNA protect reagent (Thermo Fisher Scientific). Total RNA was extracted using the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized, and qRT-PCR amplification reactions were performed using an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific). Gene expression was analyzed using TaqMan® Gene Expression Assays (Thermo Fisher Scientific) according to the manufacturer's recommendations. Each sample was analyzed in duplicate. The following assays (Thermo Fisher Scientific) were used:

CEBPα (Assay ID Hs00269972_s1), CYP1A2 (Assay ID Hs 01070374_m1), CYP2B6 (Assay ID Hs04183483_g1), CYP3A4 (Assay ID Hs00604506_m1), and CYP2C9 (Assay ID Hs004260376_m1). Expression levels were calculated using the ΔΔCt method and normalized to a calibrator mix consisting of cDNA from human pluripotent stem cells (hPSC), hPSC-derived embryoid bodies, hPSC-derived definitive endoderm cells, hPSC-derived cardiomyocytes, hphep cells, HepG2 cells, and HEK293 cells. Expression was normalized to *CEBPα* expression and presented as relative quantification. ΔΔCt was transformed into fold change by the formula: fold change = 2–ΔΔCt.

CYP activity assay

The CYP activities of primary hepatocytes were analyzed at multiple timepoints after thawing. LC/MS was used to measure the formation of specific metabolites: paracetamol/acetaminophen (CYP1A), OH-Bupropion (CYP2B6), 4-OH-Diclofenac (CYP2C9), OH-Bufuralol (CYP2D6), and 1-OH-Midazolam (CYP3A). LC/MS analysis was performed at Pharmacelsus GmbH. The cells were carefully washed twice with Phenol-red-free prewarmed William medium E containing 0.1% PEST. Then, the activity assay was started by adding 110 µl/cm^2 culture area of prewarmed Phenol-red-free William medium E containing 0.1% PEST, 25 mM HEPES, 2 mM L-Glutamine, and the probe substrate cocktail (see Table II, below). After a 2-hr incubation at 37°C, 100 µl of the supernatant was collected and kept at –80°C until LC/MS analysis. The metabolite concentrations measured by LC/MS were normalized to the amount of protein per well (determined using the Pierce BCA Protein Assay Kit) and the assay duration (120 min).

CYP	Drug substrate	Metabolite
3A4/5	Midazolam	α-OH-midazolam
2D6	Bufuralol	1'-OH-bufuralol
2B6	Bupropion	(2S,3R)-OH bupropion
2C9	Diclofenac	4'-OH-diclofenac
1A	Phenacetin	Paracetamol

Table II. Metabolites produced from the drug substrates for the tested CYP enzymes

References

Hoffmann, S. A. *et al*. Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies. *Biotechnol. Bioeng.* **109,** 3172–81 (2012).

Jungermann, K. & Kietzmann, T. Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology* **31,** 255–60 (2000).

Lim, Y.-P. & Huang, J. Interplay of pregnane X receptor with other nuclear receptors on gene regulation. *Drug Metab. Pharmacokinet.* **23,** 14–21 (2008).

Liu, X., Chism, J. P., LeCluyse, E. L., Brouwer, K. R. & Brouwer, K. L. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab. Dispos.* **27,** 637–44 (1999).

Proctor, W. R. *et al*. Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. *Arch. Toxicol.* **91,** 2849–2863 (2017).

Richert, L. *et al*. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. *Drug Metab. Dispos.* **34,** 870–9 (2006).

Yajima, K. *et al*. Evaluation of 23 lots of commercially available cryopreserved hepatocytes for induction assays of human cytochromes P450. *Drug Metab. Dispos.* **42,** 867–71 (2014).