



Co-culture with mouse embryonic fibroblasts improves maintenance of metabolic function of human small hepatocyte progenitor cells



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ABSTRACT

Derivation and culture of small hepatocyte progenitor cells (SHPCs) capable of proliferating *in vitro* has been described in rodents and recently in humans. These cells are capable of engrafting in injured livers, however, they display de-differentiated morphology and reduced xenobiotic metabolism activity in culture over passages. Here we report that SHPCs derived from adult primary human hepatocytes (PHHs) and cultured on mouse embryonic fibroblasts (MEFs) not only display differentiated morphology and exhibit gene expression profiles similar to adult PHHs, but importantly, they retain their phenotype over several passages. Further, unlike previous reports, where extensive manipulations of culture conditions are required to convert SHPCs to metabolically functional hepatocytes, SHPCs in our co-culture system maintain expression of xenobiotic metabolism-associated genes. We show that SHPCs in co-culture are able to perform xenobiotic metabolism at rates equal to their parent PHHs as evidenced by the metabolism of acetaminophen to all of its major metabolites. In summary, we present an improved co-culture system that allows generation of SHPCs from adult PHHs that maintain their differentiated phenotype over multiple passages. Our findings would be useful for expansion of limited PHHs for use in studies of drug metabolism and toxicity testing.

Studies in rats have demonstrated that the restoration of tissue mass in injured livers can be achieved through either proliferation of mature hepatocytes (Grisham, 1962) or, under impaired cell division, through the expansion of a population of hepatocytes called small hepatocyte progenitor cells (SHPCs) (Best and Coleman, 2010). SHPCs have also been successfully isolated and grown *ex vivo* from hepatocytes obtained from adult rat livers (Mitaka et al., 1999). In culture, SHPCs present a differentiated morphology reminiscent of mature hepatocytes and, upon transplantation, engraft and heal injured rat livers (Gordon et al., 2002; Ichinohe et al., 2012). These cells are characterized by the expression of CD44 (Kon et al., 2006) and have also been isolated and grown *in vitro* from mouse livers (Katsuda et al., 2017). SHPCs have been shown to originate from mature hepatocytes (Avril et al., 2004) possibly through their transition to a biphenotypic, stem-like state, indicated by the concurrent expression of cholangiocyte

markers. Recently, SHPCs have also been derived from primary human hepatocytes (PHHs) that show engraftment in mouse livers (Zhang et al., 2018) and are capable of supporting human hepatitis B virus infection (Fu et al., 2019), however these cells display diminished metabolic activity over passages. Human SHPCs have been shown to reconvert to a metabolically active state after treatment with specialized conditions, but these cells can only be derived from PHHs obtained from infant livers (Katsuda et al., 2019).

Here we report that culturing adult human donor liver-derived PHHs on mouse embryonic fibroblasts (MEFs) gives rise to CD44⁺ SHPC colonies that not only have high proliferative capacity, but also exhibit a differentiated morphology and xenobiotic metabolic activity. Co-cultured SHPCs display gene expression profiles similar to adult PHHs and, importantly, they retain their phenotype over multiple passages. Further, these cells in our co-culture system are capable of performing xenobiotic metabolism at rates equivalent

Abbreviations: SHPCs, small hepatocyte progenitor cells; PHHs, primary human hepatocytes; MEFs, mouse embryonic fibroblasts; FBS, fetal bovine serum; MACS, magnetic-activated cell sorting; sECs, sinusoidal endothelial cells; AFP, alpha-fetoprotein; SULTs, sulfotransferases; UGTs, glucuronosyl transferases.

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to fully differentiated adult PHHs. Since PHHs are limited, their expansion in culture and, importantly, the preservation of differentiated phenotype over passages will be of great importance. These differentiated SHPCs would be useful in drug discovery, toxicity testing, and modeling liver diseases. They could also potentially be used for cellular transplantation, as a bridge to organ transplant, or for correction of liver diseases (Yovchev et al., 2014).

1. Materials and methods

1.1. Cell culture

Cryopreserved adult liver-derived primary human hepatocytes were purchased from Thermo Fisher (donor data detailed in Supplementary Table 1) and cultured on MEFs. MEFs were obtained from E13.5 embryos of time pregnant CD-1 female mice purchased from Charles River Laboratories. MEFs were cultured to passage 1 (p1) to passage 3 (p3) in growth medium ($1 \times$ DMEM, $10 \times$ heat inactivated fetal bovine serum (FBS), $1 \times$ nonessential amino acids) and irradiated with a dose of 80Gy using a Mark I 137Cs irradiator (from J. L. Shepherd and Associates). Irradiated MEFs were seeded onto 0.1% gelatin coated plates at a concentration of 1.8×10^5 cells/mL to be used for culturing SHPCs. SHPC medium was E6 based (Chen et al., 2011) and contained Dexamethasone ($1 \mu\text{M}$), epidermal growth factor (100 ng/ml), Oncostatin M ($10 \mu\text{g/ml}$), 20% FBS, insulin ($2 \mu\text{l/ml}$), Nicotinamide (100 mM), Y27632 ($10 \mu\text{M}$), A-083 ($0.5 \mu\text{M}$), CHIR99021 ($3 \mu\text{M}$), and N2 and B27 (both at $1 \times$). SHPCs were passaged by dissociation with $10 \times$ Trypsin-EDTA (Sigma-Aldrich) and split 1:2 every 3 days with daily feeding. The SHPCs were not sorted from the MEFs during passaging. After trypsinization for 5 min in a 37°C incubator, trypsin was neutralized with SHPC medium. Next, all the cells (SHPCs, MEFs) were pipetted up and down and transferred to a 50 ml centrifuge tube and allowed to settle for 5 min. The supernatant containing mostly SHPCs were then seeded on new MEF plates leaving majority of the feeder cells at the bottom of the centrifuge tube. Importantly, MEFs were plated 24 h prior to SHPC seeding. Primary human cholangiocytes were obtained from Celprogen Stem Cell Research and Therapeutics and cultured as per manufacturer's guidelines. HepG2 cells (ATCC) were cultured in DMEM-F12 supplemented with 10% FBS on Matrigel coated plates to 40% and 80% confluency prior to RNA isolation (low and high density respectively). Total RNA from human fetal liver tissue was purchased from Clontech. 6-carboxyfluorescein diacetate (6-CFDA, Sigma Aldrich) metabolism assay to visualize bile canaliculi was performed following Ardalani et al. (2019) with modifications. SHPCs were incubated with 6-CFDA and DAPI (4',6-diamidino-2-phenylindole) (Thermo Fisher), washed with PBS and imaged immediately to minimize loss of the excreted fluorogenic carboxy fluorescein (6-CF) through diffusion into the surrounding medium. To reduce this diffusion as well as background fluorescence, the PBS was aspirated off prior to imaging.

1.2. Gene expression analyses

Total RNA from cells were isolated with RNeasy Plus kit (QIAGEN), and sequencing libraries were prepared following the LM-seq protocol (Hou et al., 2015). Bioinformatic analyses of gene expression levels were performed using the following packages: normalization was performed with EB-Seq (Leng et al., 2015); clustering (K-Means) with R v3.6.1; and PCA and elbowing with factextra v1.0.5 in R package (<https://rdocumentation.org>). Gene enrichment analysis was performed with ConsensusPathDB (Kamburov et al., 2011; Kamburov et al., 2009). We added one to the expected counts and then Log2 transformed them. We then used this normalized data to generate line graphs. For correlation plots, we kept only the first 2 significant digits of each expression value before making the plots. We used Spearman's rank correlation coefficient to find the correlation between the samples. All RNA_Seq data have been submitted to the GEO database (accession number GSE157791).

1.3. Acetaminophen metabolism study

SHPCs were separated from MEFs by magnetic-activated cell sorting (MACS) using anti-CD44 conjugated magnetic beads (Miltenyi Biotec). PHHs, HepG2 cells, and sorted SHPCs were resuspended in E6 medium containing 10 mM acetaminophen (Sigma-Aldrich) and incubated at 37°C for an hour. Post incubation, cellular media was stored at -80°C and later thawed, protein precipitated, and metabolites generated were quantified by LC/MS/MS using the standard curves of spike metabolites. Details of mass spectrometry methodology are described in the Supplementary data section. All standards used in this study were provided by Dr. Chris Bradfield, McArdle Laboratories, University of Wisconsin-Madison and validated by the m/z ratio.

2. Results

2.1. SHPCs on MEFs display differentiated phenotype

1×10^6 cryopreserved PHHs were seeded per 9.6 cm^2 in plates coated with Matrigel (Corning Life Sciences) from Donor 1 (donor data detailed in Supplementary Table 1). To induce generation of SHPCs, we tried combinations of small molecules and growth factors that have been reported for SHPC generation from mouse (Katsuda et al., 2017) and rat (Mitaka et al., 1999) in E6 medium (Chen et al., 2011) with PHHs obtained from Donor 1. The mouse or rat media components alone or in combination failed to generate SHPC colonies. The introduction of N2 and B27, supplements that support adult stem cell culture, was successful in producing SHPC colonies between day 7 to 10 post plating. N2 has 5 (Nagy et al., 2006) and B27 has 20 components (Brewer et al., 1993) and both have traditionally been used for neuronal cultures (Brewer and Cotman, 1989; Brewer et al., 1993) and neural lineage differentiation from pluripotent stem cells (Brewer et al., 1993; Dhara et al., 2008; Li et al., 1998). However, our rationale in using these supplements in our medium stems from their successful use in hepatoblast generation (Zhang et al., 2015) as hepatoblasts and hepatic progenitor cells are hypothesized to be similar, sharing expression of CD44 (Yovchev et al., 2007). SHPCs morphologically resembled PHHs in monolayer culture, except they were smaller than primary cells (between 8 and $20 \mu\text{m}$ compared to $\sim 30 \mu\text{m}$ of PHHs in monolayer culture). Though the cells in the middle of the colony displayed typical differentiated hepatocyte morphology with bile canalicular structures, these colonies did not display a tight boundary as seen in rat SHPC colonies, and cells at the periphery of our colonies displayed a dedifferentiated morphology (Supplementary Fig. 1a). Further, upon passaging, the dedifferentiated cells took over and the colonies lost the bile canalicular network (Supplementary Fig. 1b, donor 1, p1). Differentiated hepatocyte morphology is associated with hepatocyte-specific functions in rats (Arterburn et al., 1995), and hence we explored ways of generating SHPCs that would display tight colonies with differentiated morphology.

Mouse SHPCs have been reported to proliferate rapidly while maintaining their differentiated morphology and having well-defined boundaries when co-cultured in direct contact with MEFs (Zhou et al., 2019). We next explored derivation of human SHPCs by seeding cryopreserved PHHs on MEF plates using the previous medium that gave rise to SHPCs on Matrigel. Between 7 and 10 days post plating, SHPC colonies were observed to emerge and no new colonies were produced after 14 days post-plating. At this time 56 ± 2 colonies were observed/ 10^6 PHHs plated and contained 50 ± 10 cells/colony (donor 1). These colonies exhibited the tight, well-differentiated clusters of smaller sized hepatocytes reminiscent of rat SHPC colonies (Fig. 1a). Adult PHH-derived SHPCs on MEFs displayed typical polarized hepatocyte morphology with prominent bile canaliculi. To ascertain formation of functional bile canaliculi, we performed 6-carboxyfluorescein diacetate (6-CFDA) metabolism assay. 6-CFDA is taken up by hepatocytes, where intracellular esterase metabolizes it into 6-CF (carboxy fluorescein, green fluorescent) and is excreted through bile canaliculi (Lin et al., 2016b). Fig. 1b shows an SHPC colony (donor 1, p1) in phase contrast (top) and its excretion of 6-CF in the canalicular space (bottom), marked by arrows. The network-like pattern seen is typically observed in CFDA staining of polarized hepatocytes in

sandwich cultures. Primary rat hepatocytes have been shown to retain their differentiated morphology for extended periods of time and form extensive canalicular networks in collagen (LeCluyse et al., 1994) and heparin-PEG hydrogel (Elena Foster et al., 2015) sandwich cultures. However, in our culture system without any overlay, the fluorogenic 6-CF excreted by the SHPCs rapidly diffused into the surrounding medium leaving scattered pockets of fluorescence prompting us to image the cells immediately after washing. We postulate that encapsulation of the bile canaliculi in sandwich cultures hinders diffusion of 6-CF into the surrounding medium resulting in a better and more complete visualization of the canalicular network. The feeder MEFs around the SHPCs display fluorescence as they do not excrete the dye. Further, expression of hepatocyte, hepatic progenitor, and xenobiotic metabolism-associated genes were higher in SHPCs cultured on MEFs compared to those cultured on Matrigel (both from donor 1, p1, Fig. 1c).

The SHPC colonies were passaged on day 14 post PHH plating. They doubled every 3 days thereafter and were passaged at 3-day intervals at 1:2 ratio by dissociating in $10\times$ Trypsin-EDTA and reseeding on fresh MEF plates. They could be frozen in SHPC medium supplemented with serum and DMSO and successfully cultured after being thawed. We froze SHPCs after magnetically sorting them with microbeads conjugated to CD44 antibody. After thawing, cells were $60 \pm 3\%$ viable and when plated gave rise to differentiated looking colonies (donor 1, Fig. 1d).

We next characterized SHPCs along with their parental PHHs (from Donors 2 and 3) in MEF co-culture *via* whole transcriptome sequencing (Supplementary Table 2) and bioinformatic analyses. Elbow method (Thorndike, 1953) identified 5 clusters to ideally represent the whole RNA-Seq dataset (elbow plot in Supplementary Fig. 2). Clustering with K

= 5 grouped the SHPCs derived from different donors (as well as different passages) together, close to their parental adult PHHs, whereas control HepG2 (a hepatoma-derived undifferentiated cell line), human fetal liver tissue and cholangiocytes grouped separately (Fig. 1e). We included cholangiocytes in our analysis since SHPCs have been postulated to represent a biphenotypic nature as they express cholangiocyte markers CK-7 and CK-19. We also included HepG2s grown at different densities as expression of xenobiotic metabolism-associated genes in cultured hepatocytes are a function of their confluency (Hamilton et al., 2001). Further, whole genome PCA analysis also grouped the SHPCs together, close to adult PHHs (Fig. 1f), away from control HepG2, fetal liver, and cholangiocytes. Clustering of SHPCs derived from different donors together suggests they share a unique phenotype that is closer to, but not entirely similar to, differentiated adult cells.

2.2. SHPCs on MEFs maintain differentiated phenotype over multiple passages

To investigate whether SHPCs on MEFs can retain their differentiated phenotype over prolonged passaging, we performed RNA-Seq on SHPCs through multiple passages as well as the parental uncultured PHH (Donor 1) from which they were derived (Supplementary Table 3). SHPCs showed a doubling time of 3 days and were cultured for 7 passages (2^7 doublings), and they continued to grow exponentially even after p7. The SHPC colonies on MEFs displayed differentiated and polarized morphology at p7 that were cultured for over a month akin to cells at p0 (Fig. 2a and b). Spearman's rank-order correlation analysis of whole transcriptome RNA-Seq data of these cells showed a correlation of 0.56 between the parental PHH and

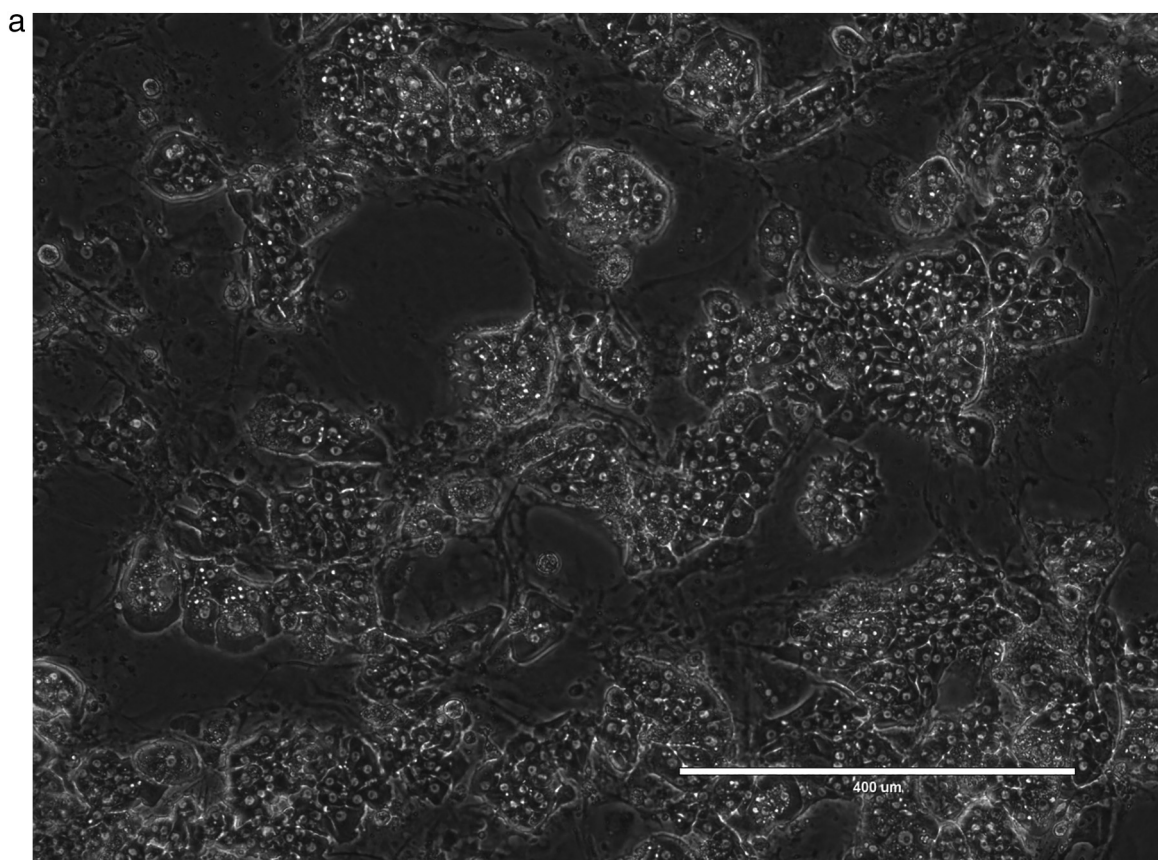


Fig. 1. (a) SHPCs cultured on MEFs (p1, donor 1) retain defined boundaries and polarized hepatic morphology upon passaging. (b) SHPC colony on MEF (p1, donor 1) displays functional bile canaliculi. Top panel shows phase contrast image of a colony that was incubated with 6-CFDA. Bottom panel shows 6-CF to be excreted into the canalicular network (green fluorescent, nuclei counterstained with DAPI) by the SHPCs while the surrounding MEF cells do not excrete it and are green. (c) Expression of hepatocyte, hepatic progenitor, and xenobiotic metabolism-associated genes are higher in SHPCs cultured on MEFs compared to those cultured on Matrigel (donor 1, p1). (d) SHPCs plated after thawing frozen cells gives rise to colonies with differentiated morphology. (e) Whole transcriptome analysis shows SHPCs to cluster together, next to adult uncultured parental PHHs by K-means clustering and by (f) PCA analysis.

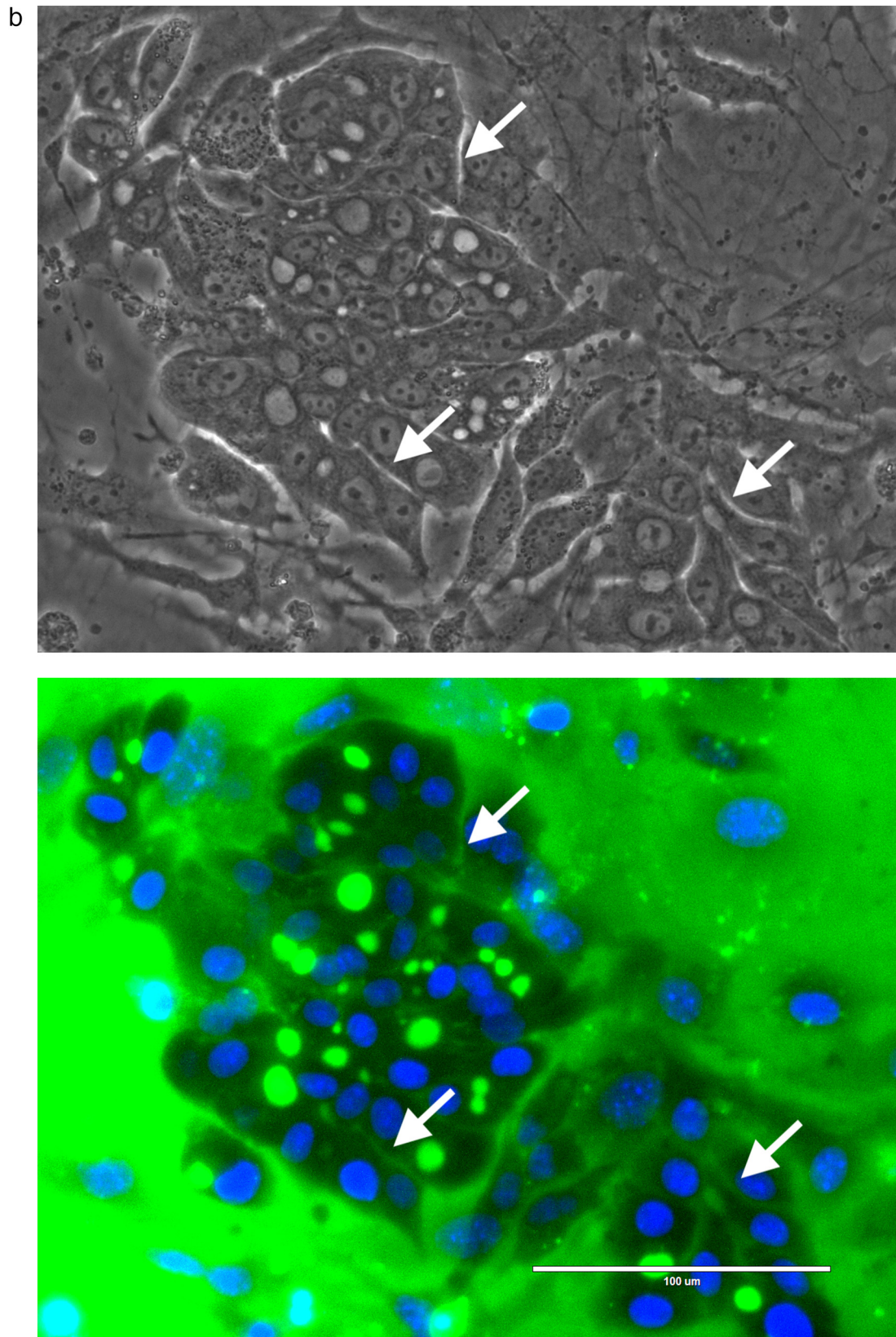
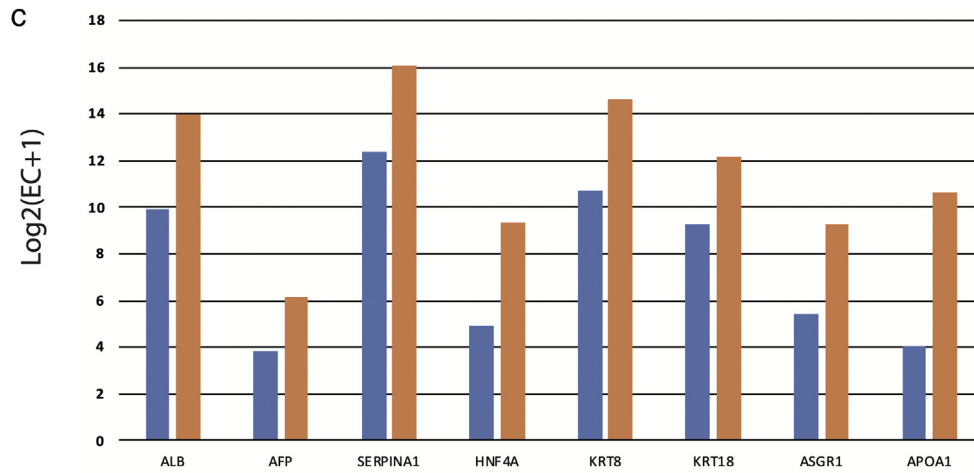


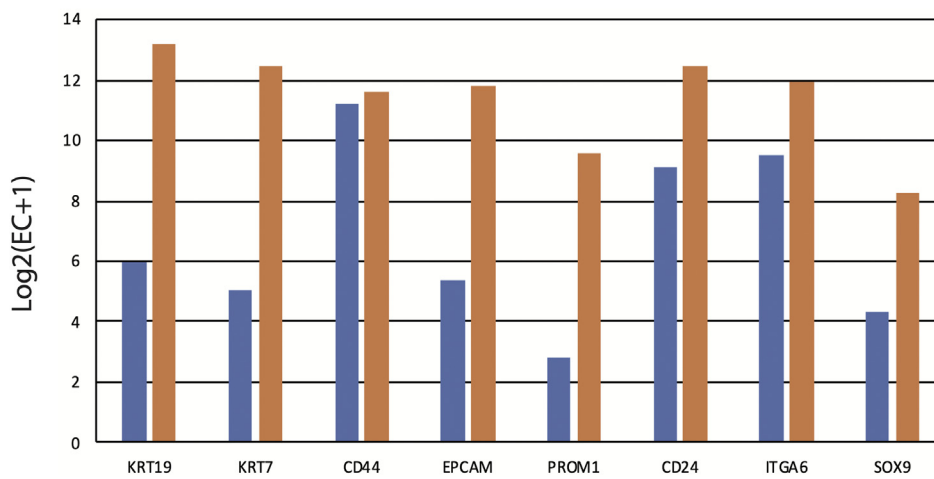
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p0, suggesting SHPCs have a different gene expression signature than parental PHHs, however, p0 had a high correlation to all later passages (average Rho^2 of 0.89 up to p7) indicating they strongly retained their phenotype through multiple passages on MEFs (Fig. 2c). Hepatic markers

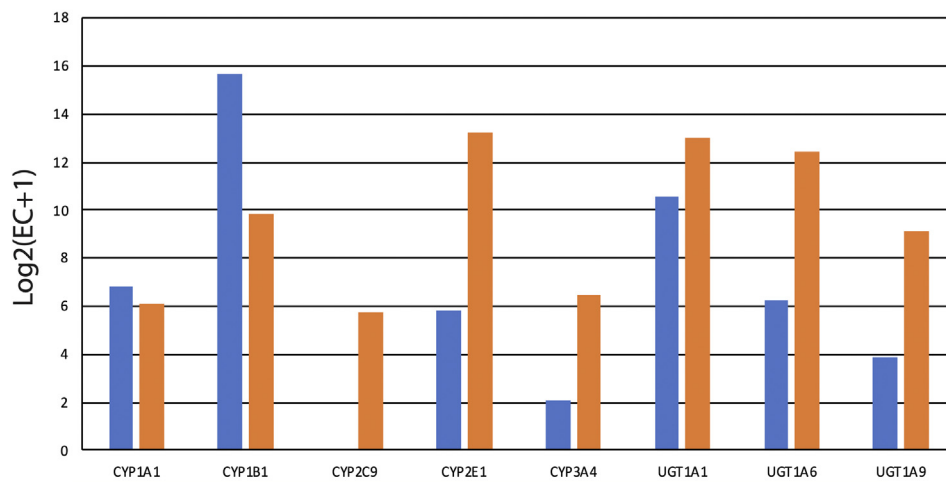
(HNF4A, APOA1, SERPINA1, ASGR1, KRT 8 and 18) retained similar expression levels through passages, with/without changes from parental PHH (Fig. 2d). They retained high expression of the mature hepatic gene albumin concomitant with low expression of the fetal marker alpha-



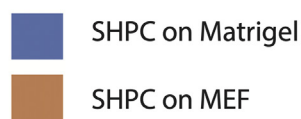
Hepatocyte marker genes



Progenitor marker genes



Metabolic marker genes



d

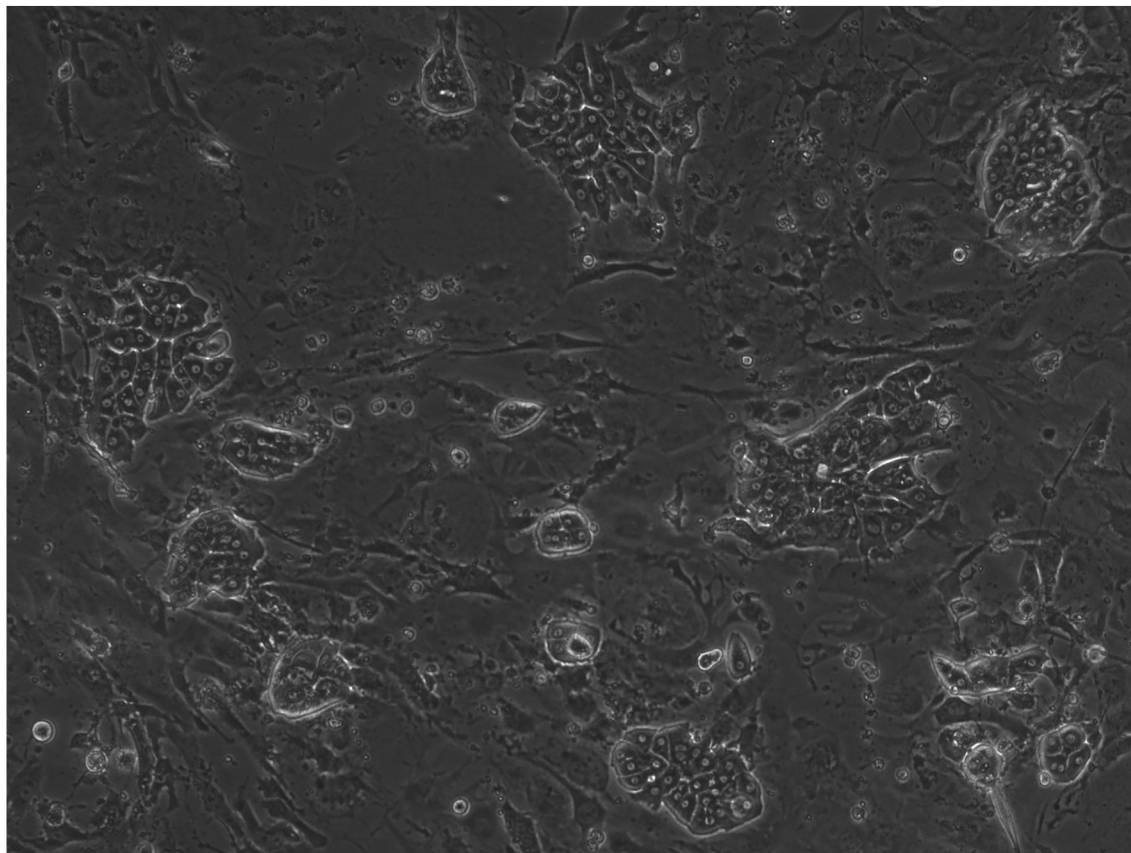


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fetoprotein (AFP) indicating they conserve features of mature differentiated cells. This was also validated by their consistent expression of important phase I and II drug metabolizing genes over passages, whether they increased/decreased from parental PHH (CYP1A1, CYP1B1, CYP2C9, CYP2E1, CYP3A4, UGT1A1, UGT1A6, UGT1A9) (Fig. 2d). To note, two genes whose expression decreased from parental PHHs were CYP3A4 and CYP2C9, both being important drug metabolizers. At the same time, SHPCs at all passages displayed high expression of progenitor markers, absent in PHHs (CD44, EPCAM, PROM1, CD24, ITGA6, SOX9, KRT 7 and 19), placing them in a unique group, apart from mature differentiated adult cells (Fig. 2d).

2.3. SHPCs maintain xenobiotic metabolism activity similar to PHHs

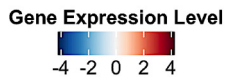
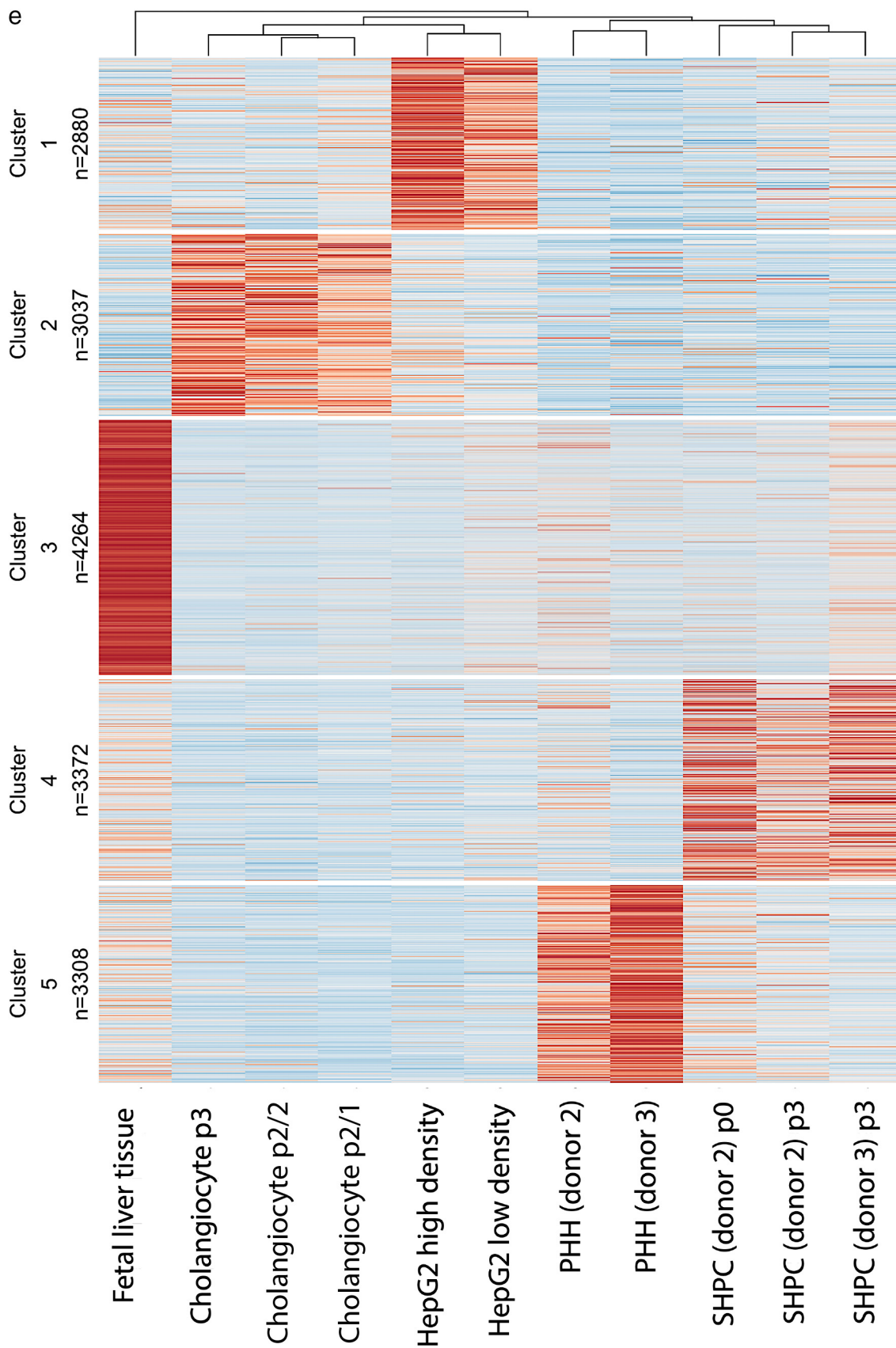
We next tested metabolic function of the SHPCs and their parental PHHs (from Donors 1 and 2) along with control HepG2 cells, a hepatoma-derived undifferentiated cell line with poor metabolic activity. The SHPCs (both at p3) were magnetically separated from feeder MEFs with anti-CD44 conjugated antibody; PHHs were thawed and washed and were not put into culture prior to testing. We chose to use magnetic sorting to isolate SHPCs over fluorescent sorting as the latter not only results in poor cell viability (Sutermaster and Darling, 2019), but in our experience, FAC sorting also results in lowered metabolic activity of hepatocytes. All the cell types were incubated for an hour in E6 medium containing 10 mM acetaminophen (APAP), a common hepatotoxicant used in toxicity studies. The media was collected post incubation, and the amount of metabolites secreted was measured by LC/MS/MS. APAP is mainly cleared *in vivo* by the formation of APAP-sulfate (APAP-sulf) and APAP-glucuronide (APAP-gluc) (Chen et al., 2008); while at high doses when these pathways are saturated, APAP forms a minor oxidative hepatotoxic metabolite, NAPQI, which has a very short half-life (Mannery et al., 2010) and is reduced back to APAP or conjugated to glutathione, forming the APAP-glutathione (APAP-gsh) conjugate. Fig. 3a illustrates SHPCs from both donors generated all three metabolites

at rates equal to or better than parental PHHs, whereas HepG2 cells, except for APAP-sulfate, were ineffective in APAP metabolism. Both Donors 1 and 2 derived SHPCs were superior to their parental PHHs in formation of APAP-gluc, APAP-sulf, and APAP-gsh conjugate. However, there were significant differences in generation of metabolites by the SHPCs obtained from the two donors, in particular in generation of APAP-gsh (Fig. 3a). Formation of glutathione conjugates by hepatocytes have been noted previously to vary significantly between donors (Prabhu et al., 2002) and might be the reason for the observed difference in APAP-gsh generated by the SHPCs derived from hepatocytes obtained from the two different donors. However, further studies are required to address the inter-donor variability in glutathione conjugation in SHPCs.

A major goal of our work was to develop culture conditions that will maintain metabolic function of SHPCs through passages. To investigate this, we compared expression of major genes associated with APAP metabolism (Mazaleuskaya et al., 2015) (Krasniak et al., 2014) between p3 (when APAP metabolism was tested) and at late passage (p7) (donor 1). We found all genes involved in generation of the major metabolites and their secretion to be comparable between p3 and p7, indicating that our co-culture system is able to preserve metabolic capabilities of SHPCs over multiple passages (Fig. 3b, Supplementary Table 3).

3. Discussion

Fibroblast co-culture has mostly been used with cancer cells to generate clinically relevant tumor models as it recreates the tumor microenvironment faithfully (Jeong et al., 2016; Lee et al., 2018; Majety et al., 2015; Miki et al., 2012). Bhatia and colleagues (Bhatia et al., 1998) and later other workers have described fibroblast co-culture with normal primary hepatocytes, (Cho et al., 2010; Khetani and Bhatia, 2008; Lin and Khetani, 2017; Lin et al., 2016a). These works demonstrated fibroblasts to prolong maturity and drug metabolizing capabilities of primary human and rat hepatocytes *in vitro*. The first report of progenitor cell co-culture with



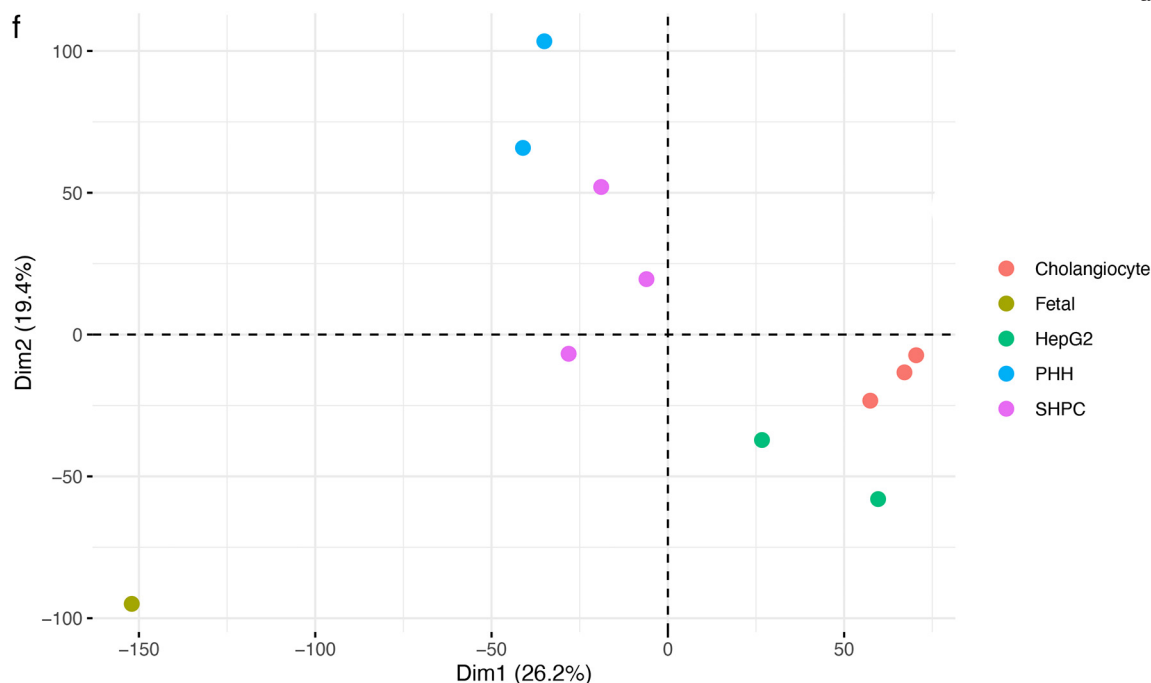


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fibroblasts was by Zhou et al. (2019). They demonstrated mouse fetal liver-derived progenitor cells to differentiate to hepatocytes or cholangiocytes depending on direct or indirect contact with the feeder fibroblasts. Therefore, fibroblast co-culture models are not novel; however, we believe that we are the first to report derivation and successful propagation of progenitor cells from primary human hepatocytes in a fibroblast co-culture system.

Here we report derivation of CD44⁺ SHPCs from adult primary human hepatocytes (PHHs) in co-culture with MEFs. We also show that our co-culture system enables the SHPCs to retain their differentiated phenotype throughout multiple passages. They also display gene expression profiles similar to differentiated mature PHHs, as well as competency in xenobiotic metabolism equivalent to PHHs. However, they concurrently express hepatic progenitor markers, setting them apart as a unique cell type. Both Matrigel and MEF conditioned media (data not shown) failed to maintain differentiated phenotype of SHPCs, indicating that SHPCs require direct cell-cell contact with MEFs for maintenance of their phenotype. We are in the process of identifying cell signaling mechanism(s) responsible for this, as well as identifying the minimal essential components for our medium.

Recently proliferative hepatocytes have been reported to have been derived from PHHs (Fu et al., 2019; Hu et al., 2018; Katsuda et al., 2019; Kim et al., 2019; Zhang et al., 2018). Hu and colleagues (Hu et al., 2018) showed mouse and human primary hepatocytes to proliferate when cultured in 3-dimensional (3D) organoids. 3D culture has been shown to improve metabolic functions of hepatocytes (Ardalani et al., 2019), however, successful aggregation of PHHs vary from lot to lot (Baze et al., 2018). Kim et al., described a chemical cocktail to induce ‘reprogramming’ of PHHs into CD44⁺ bipotent progenitor cells; however, it required further steps for differentiation into hepatocytes (Kim et al., 2019). Zhang and colleagues showed PHH-derived CD44 expressing SHPCs to engraft in livers of immunodeficient mice, but they displayed de-differentiated morphology in culture, and the authors did not report any xenobiotic metabolism of these cells (Zhang et al., 2018). While it has been possible to derive CD44⁺ SHPCs from livers of infants that displayed differentiated morphology (Katsuda et al., 2019), these conditions did not allow generation of SHPCs from adult PHHs, and shortage of infant liver donors makes this work challenging to translate into practice for drug and toxicity testing. Further, manipulation of culture conditions to reconvert these cells to a metabolically

active state was required, and only two phase II enzyme activity of these cells were tested in this work: sulfotransferases (SULTs) and glucuronosyl transferases (UGTs), both of which were much lower than PHHs.

A recent work (Fu et al., 2019) also showed generation of proliferative hepatocytes from PHHs, termed liver progenitor-like cells (HepLPCs) but the authors did not report them to express CD44. HepLPCs showed great reduction in hepatic marker genes (ALB, G6PC, HNF4A) in a very short period of time in culture (10 days). The HepLPCs could be converted back to functional cells, but showed considerably lower xenobiotic metabolic activity (at p5) compared to PHHs.

The three seminal works in PHH proliferation (Fu et al., 2019; Katsuda et al., 2019; Zhang et al., 2018) showed reduction/absence of xenobiotic metabolism activity in the proliferating cells. These works also did not perform detailed correlation analysis of gene expression through passages. We, for the first time, not only demonstrate maintenance of functional metabolism-associated genes over passages in adult PHH-derived SHPCs, but also demonstrate the SHPCs perform at equal or even better levels in xenobiotic metabolism in both phase I and II metabolic pathways. However, it must be noted that we found a significant drop in expression of CYP3A4 and CYP2C9 in SHPCs over parental PHHs. CYP2C9 and CYP3A4 are two important drug metabolizing enzymes, with the latter responsible for metabolizing over 50% of all clinically used drugs (Zanger and Schwab, 2013). Therefore, it is imperative to identify and remedy the cause of downregulation of CYP3A4 in SHPCs before this system could be used in drug development and toxicity testing. The reduction of CYP3A4 in SHPCs could be due to a myriad of reasons: inhibitors may be present in the currently undefined SHPC medium; CYP3A4 expression is dependent on confluency (Sivertsson et al., 2010); and SHPCs might need higher cell density to express this enzyme at higher levels. CYP3A4 levels as well as inducibility varies from donor to donor (Bulutoglu et al., 2020), and it is conceivable that CYP3A4 expression in SHPCs derived from different donors would also vary. We are investigating ways to overcome this shortcoming to make our system a practical and useful tool in drug discovery and toxicity testing.

We studied acetaminophen metabolism to assess metabolic capabilities of proliferating SHPCs compared to their parental PHHs. Acetaminophen metabolism is well-studied and undergoes both phase I oxidative metabolism primarily through Cytochrome P450 2E1 and phase II conjugation to

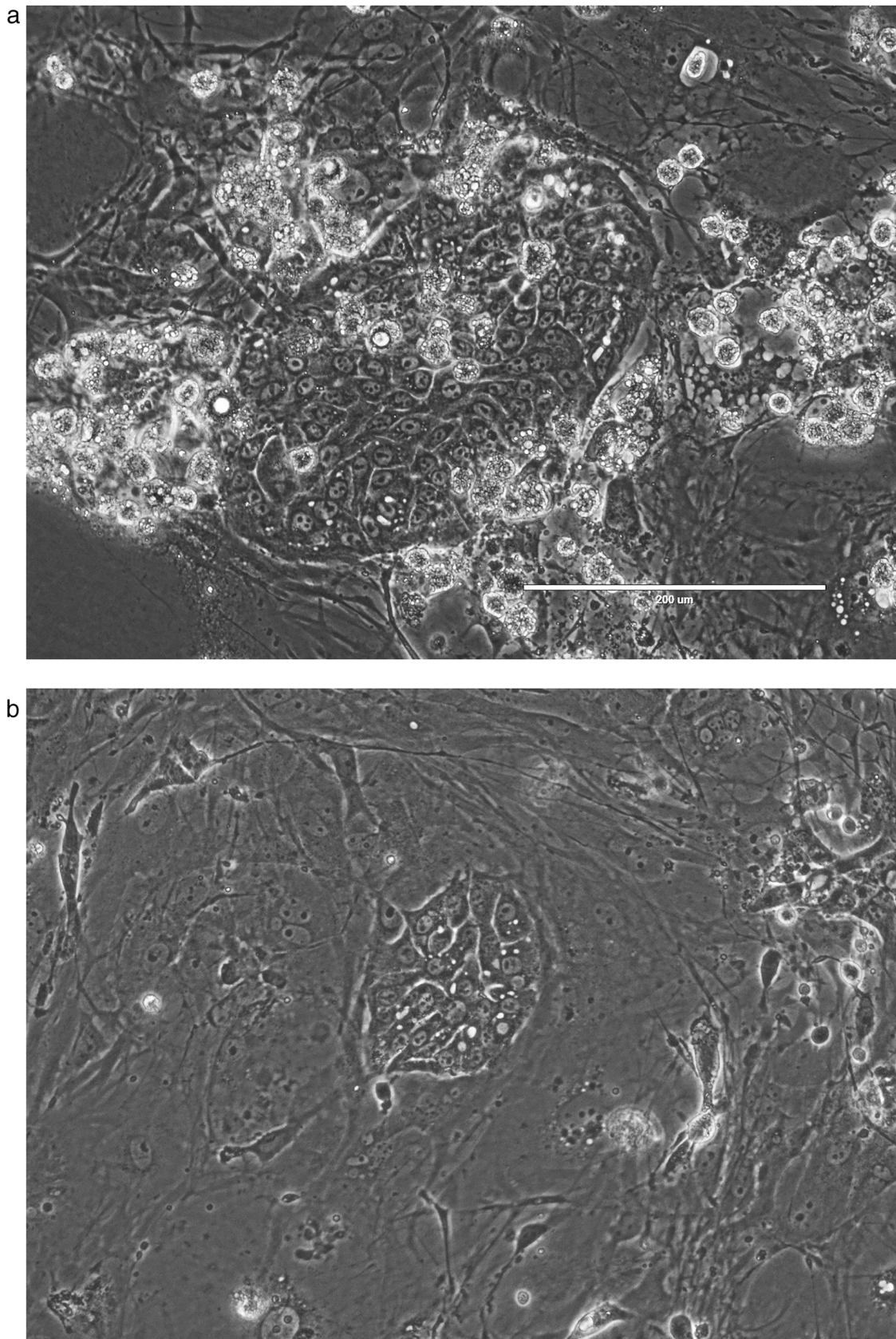
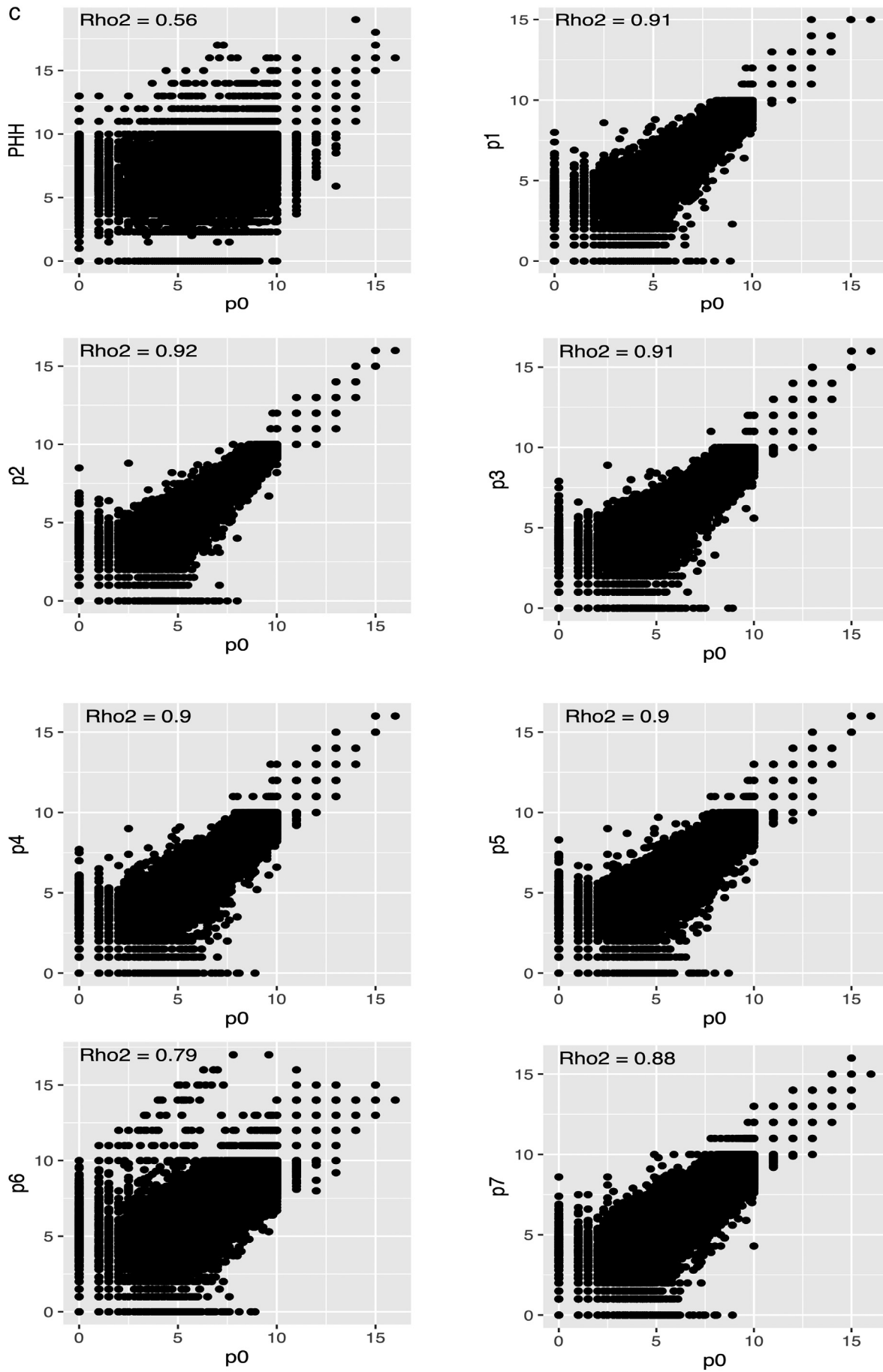


Fig. 2. SHPCs maintain differentiated morphology and gene expression through late passages. (a) SHPC colonies at p0 display tight boundaries and polarized morphology. (b) Same morphology is maintained by the SHPCs at late passage (p7). (c) SHPCs maintain gene expression profiles that show high correlation between p0 and later passages. Cells through passages retain average correlation of 0.89 Rho^2 with p0 calculated from whole genome RNA-sequencing. (d) SHPCs also continue expressing hepatocyte and xenobiotic metabolism-associated genes that is otherwise lost in culture. They also express progenitor marker genes at similar levels throughout passages.



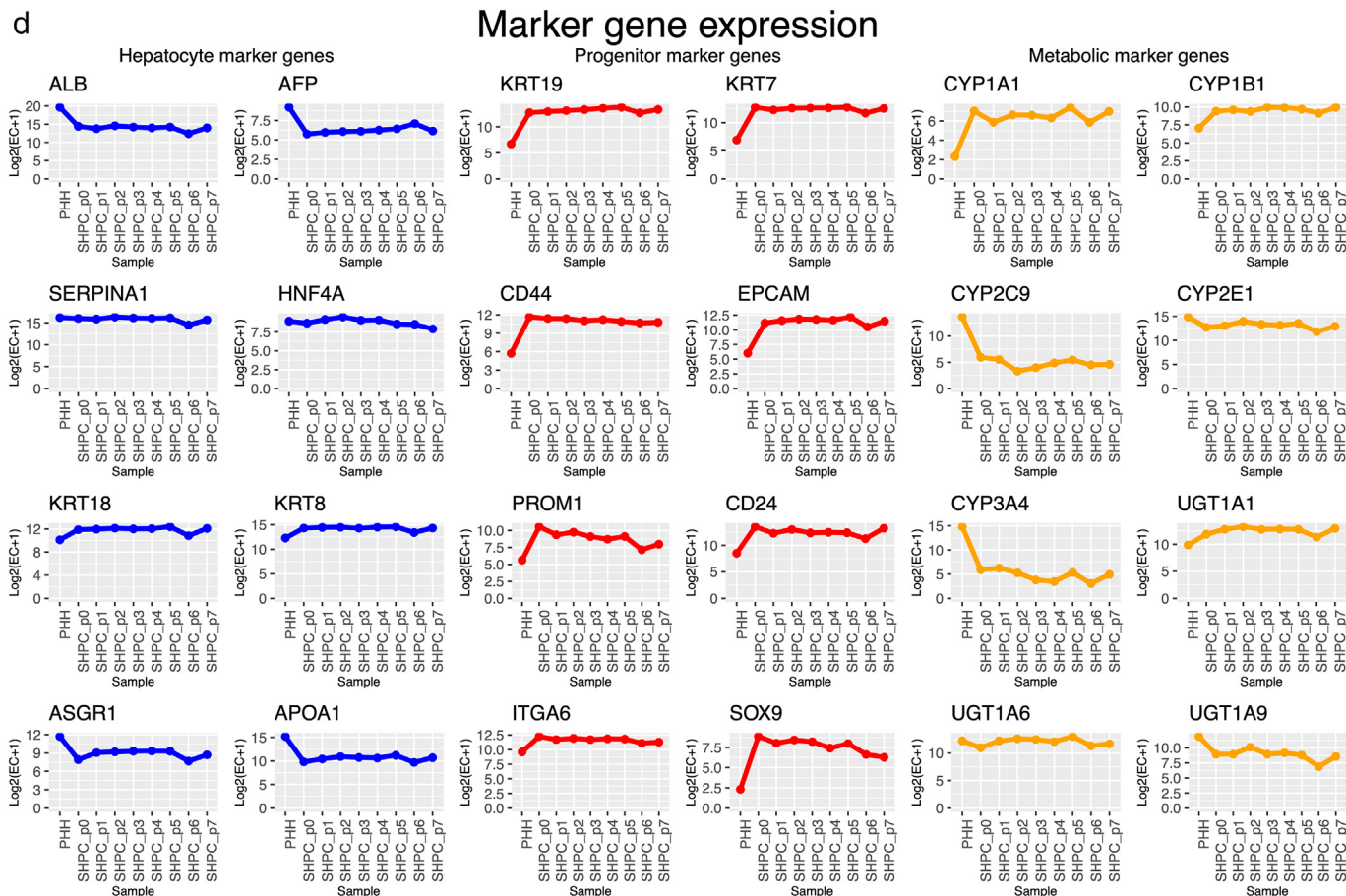


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sulfate or glucuronide *via* SULTs and UGTs respectively. We showed that SHPCs even at p3 are equivalent or better than PHHs in xenobiotic metabolism through phase I and II pathways. PHHs are considered the gold standard for *in vitro* cell based metabolism studies, but as they are limited and lose their metabolic capacity, including those involved in xenobiotic metabolism (Cassim et al., 2017; Holme, 1985) rapidly in culture. Thus, SHPCs could be useful for drug metabolism testing; however, future studies are necessary to determine if these cells have broad metabolic activity in other important phase I and phase II enzymes. Further, drug induction and baseline metabolism studies also need to be performed to confirm the functionality of SHPCs in MEF co-culture system.

We used acetaminophen metabolism as an initial indication of their metabolic capacity because of our previous work in this area (Sengupta et al., 2014). Together with CYP2E1, oxidative metabolism for most drugs (>90%) occurs through cytochromes P450 CYP1A2, CYP2C9, 2C19, CYP2D6 and CYP3A4 (Bibi, 2008). Expression of the above genes in SHPCs varied from donor to donor, except CYP 2D6, which was poor in SHPCs derived from all three donors we tested. Thus, pooled SHPCs from a large number of donors may be required for accurate drug and toxicity testing. Further, we performed colony formation and cell viability assays with hepatocytes obtained from donor 1. These parameters may vary from donor to donor.

A recent work (Xiang et al., 2019) reported long-term maintenance of PHHs in culture, but these cells did not proliferate. Xiang et al., and Fu et al., also demonstrated infection of the cultured cells by hepatitis B virus (Fu et al., 2019; Xiang et al., 2019). Another recent work also showed generation of proliferative hepatocytes from primary cells supportive of hepatitis B as well as hepatitis delta virus replication (Unzu et al., 2019). We did not explore virus replication capabilities of our SHPCs as there are

multiple hepatic cell types including immortalized lines and pluripotent stem cell derived hepatocytes that allow productive infection by HBV (Gripon et al., 2002; Shlomai et al., 2014).

A major use of passagable differentiated SHPCs will be in the field of drug development and toxicity testing, as PHHs, that are currently employed for such studies are limited and have a very short useful life in culture due to rapid de-differentiation. Proliferation of SHPCs would also allow their genetic manipulation in culture for various research purposes. Patient-derived SHPCs can also be used for the development of drugs against inherited liver diseases. SHPCs also hold the promise of clinical transplantation for regeneration of diseased livers, acute failures, correction of metabolic diseases, and as a bridge to organ transplantation.

4. Conclusion

Here we report that a) small hepatocyte progenitor cells (SHPCs) derived from adult human liver-derived primary hepatocytes and cultured on MEFs retain their differentiated phenotype and mature gene expression over multiple passages and b) SHPCs in MEF co-culture system are as competent as adult primary human hepatocytes in their xenobiotic metabolic activity. We conclude that MEF co-cultured SHPCs may be useful for drug development and toxicity testing.

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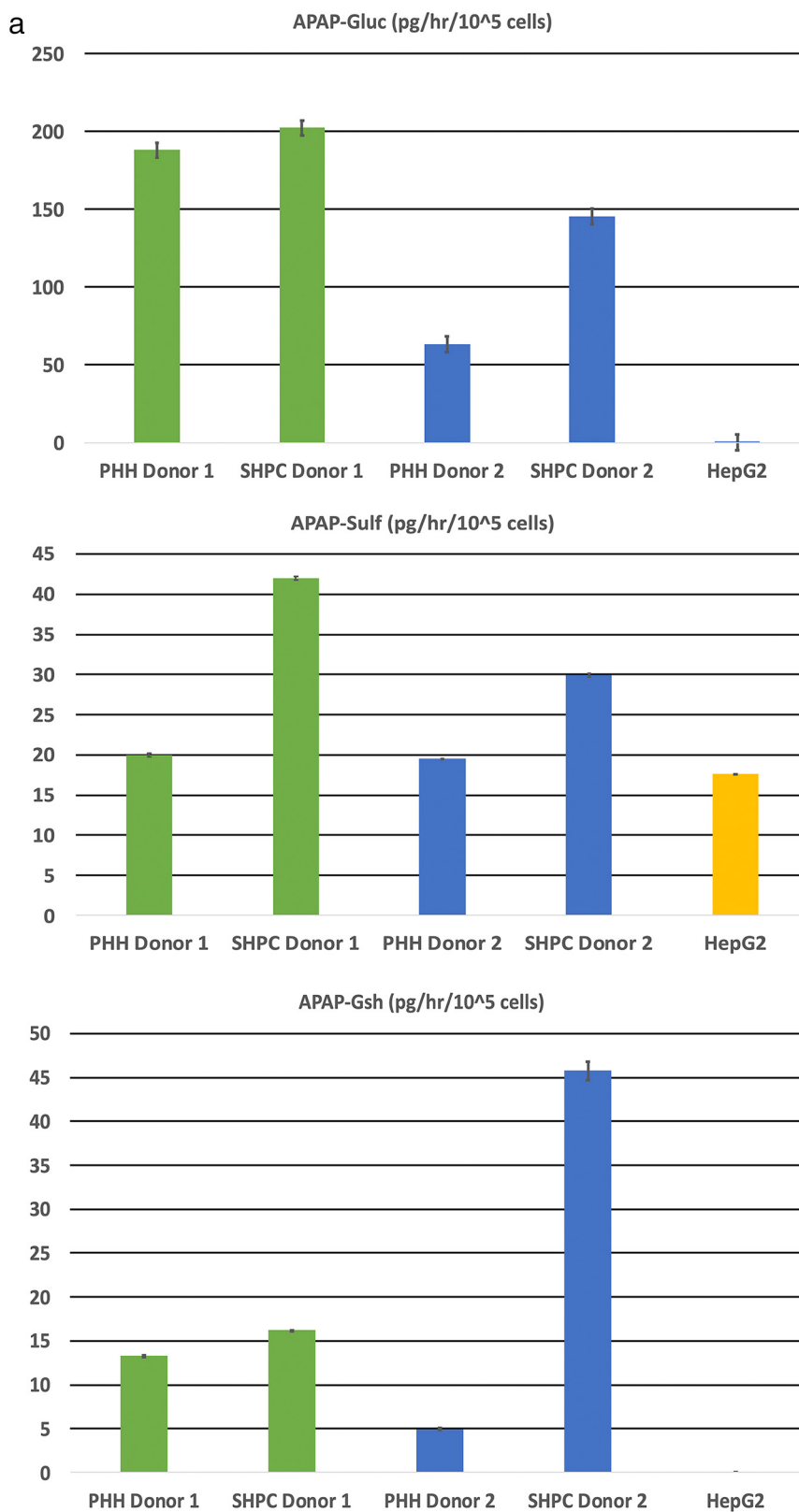


Fig. 3. (a). SHPCs (p3) metabolize acetaminophen (APAP) to all its major metabolites at rates equal to or greater than uncultured adult parental PHHs. (b) Genes involved in APAP metabolism shows similar levels between p3 (when APAP metabolism was tested) and at late passage (p7). Gene expression values are in ECs (expected counts).

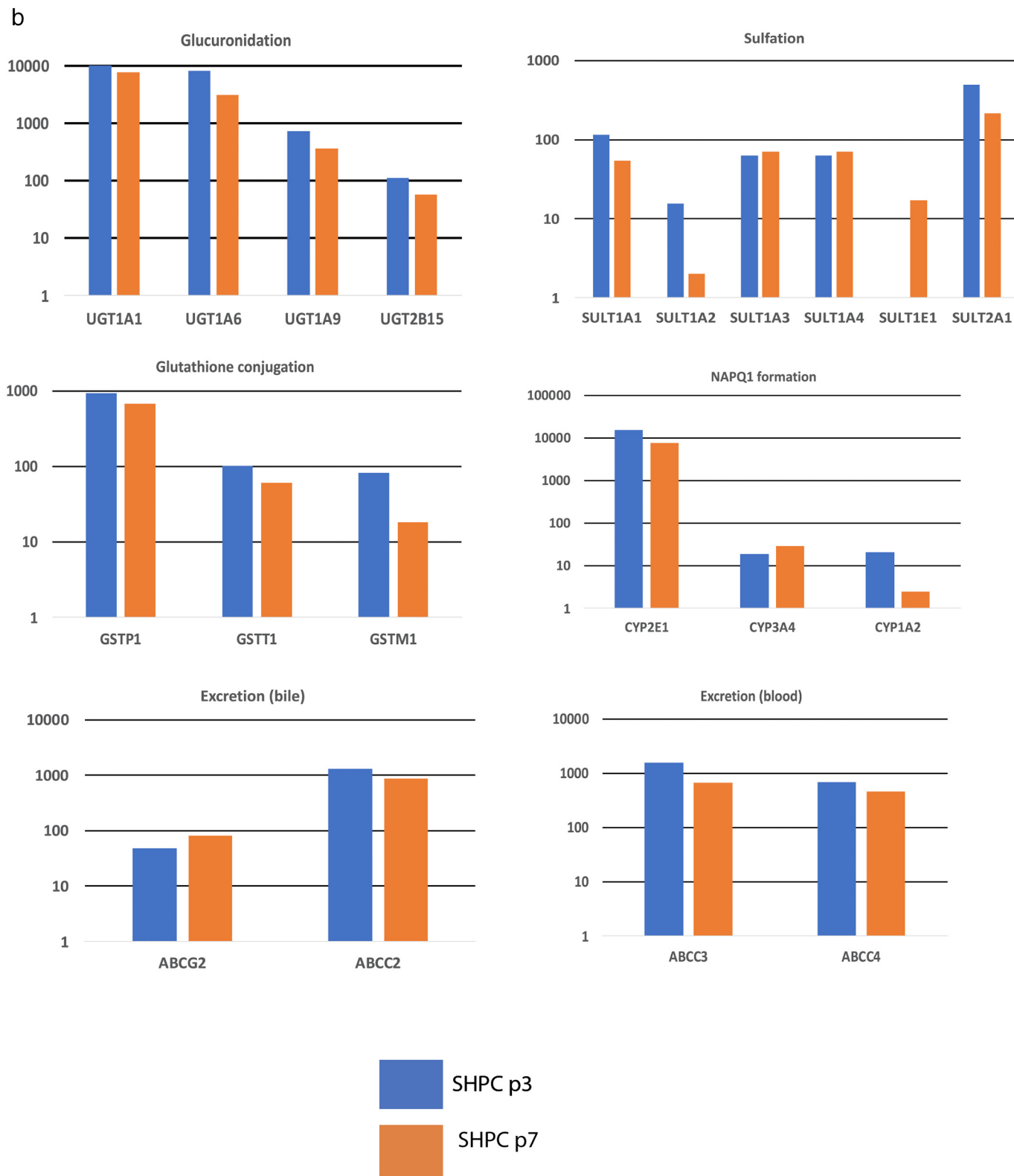


Fig. 3 (continued).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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