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Title

Functional proliferating human hepatocytes: *in vitro* hepatocyte model for drug metabolism, excretion and toxicity

Authors

Shida Qiao[a,b,1], Sisi Feng[c,1], Zhitao Wu[a,b,d], Ting He[e], Chen Ma[a,b], Zhaoliang Peng[a,b], E Tian[c], Guoyu Pan[a,b,*]

[a] Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

[b] University of Chinese Academy of Sciences, Beijing 100049, China

[c] Shanghai Hexaell Biotech Co., Ltd, 201807, Shanghai, China

[d] Nanjing University of Chinese Medicine, Nanjing 210029, China

[e] Nanjing Tech University, Nanjing, 211816, China

[1] These authors contributed equally to this work.

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Running Title：Functional ProliHH derived from PHH

* Corresponding Author:

Guoyu Pan, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Haike Road 501, Shanghai 201203, (China), Tel. +86-21-20231000-1715, E-Mail gypan@simm.ac.cn

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List of Abbreviations:

3D, three dimensional; BEI, bile excretion index; BSEP, bile salt export pump; CDFDA, 5-(and-6)-carboxy-2', 7'-dichlorofluorescein diacetate; CLF, Cholyl-Lys-Fluorescein; CL_{int}, intrinsic clearance; CYP450, cytochrome P450; d8-TCA, deuterium-labelled sodium taurocholate acid; DMEs, drug metabolic enzymes; MRP2, multidrug resistance-associated protein 2; NTCP, sodium-taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; PHH, primary human hepatocytes; ProliHH, proliferating human hepatocytes; ProliHH-P, proliferative status of ProliHH; ProliHH-M, maturation status of ProliHH; SC, sandwich culture;

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Abstract

To develop a functional alternative hepatocyte model for primary human hepatocytes (PHH) with proliferative property, essential drug metabolic and transporter functions, proliferating human hepatocytes (ProliHH) expanded from PHH were fully characterized *in vitro*. Herein, ProliHH generated from multiple PHH donors could be expanded more than 200 folds within four passages, and maintained their metabolic or transporter capacities partially. Further, ProliHH was able to regain of the mature hepatic property after three-dimensional (3D) culture. Particularly, the downregulated mRNA expression and function of three major CYP450 enzymes (CYP1A2, CYP2B6, and CYP3A4) in the proliferating process (ProliHH-P) could be recovered by 3D culture. The metabolic variabilities across different PHH donors could be inherited to their matured ProliHH (ProliHH-M). The intrinsic clearances (CL_{int}) of seven major CYP450 enzymes in ProliHH-M were correlated well (r = 0.87) with that in PHH. Also, bile canaliculi structures could be observed in sandwich cultured ProliHH (SC-ProliHH), and the biliary excretion index (BEI) of four probe compounds (CLF, CDF, d8-TCA and rosuvastatin) in SC-ProliHH (>10%) were close to SC-PHH. More importantly, both ProliHH-P and ProliHH-M could be used to evaluate hepatotoxicity. Therefore, these findings demonstrated that the 3D and sandwich culture system could be utilized to recover the metabolic and transporter functions in ProliHH for clearance prediction and cholestasis risk assessment, respectively. Together, ProliHH could be a promising substitute for PHH in drug metabolism, transport and hepatotoxicity screening.

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Significance Statement

We firstly report the study of drug metabolic capacities and efflux transporter functions, as well as toxicity assessments of ProliHH. The metabolic variability in different primary human hepatocytes (PHH) donors could be inherited by their matured ProliHH derivatives. Also, ProliHH could form canalicular networks in sandwich culture and display the biliary excretion capacities. More importantly, both ProliHH-P and ProliHH-M could be used to evaluate hepatotoxicity. Together, these results make it feasible for ProliHH to support drug candidate screening in hepatic metabolism, disposition and toxicity.

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Introduction

Assessments of drug metabolism, transport and hepatotoxicity are essential parts for early drug discovery (Godoy et al., 2013; Zhang et al., 2016). Primary human hepatocytes (PHH) have been widely recognized as the gold standard for predict drug clearance and hepatotoxicity (Gomez-Lechon et al., 2003; Gomez-Lechon et al., 2004; Hewitt et al., 2007). However, the loss of characters, including proliferative properties, metabolic and transported functions of PHH after *in vitro* culture, limits their applications (Gomez-Lechon et al., 2003; O'brien et al., 2006). Moreover, it is time-consuming and costly to determine the variabilities of drug metabolizing enzymes (DMEs) and transporters from different PHH batches (Teresa et al., 2013). Therefore, the establishment of a functional substitutable hepatocyte model is an important issue.

Significant efforts have been made in the past decade. In addition to PHH, the embryonic stem cell (ESC)-derived, induced pluripotent stem cell (iPSC)-derived or epigenetic reprogramming induced hepatocytes technology were developed to solve the lacking of donor, However, a better differentiation protocol was still missing to generate physiological relevant hepatocytes (Brolén et al., 2010; Si-Tayeb et al., 2010; Ogawa et al., 2013; Huang et al., 2014). Also, there are several investigations about reprogramming PHH into hepatic progenitor cells to obtain cell proliferation features and keep physiologically relevant, but these proliferative cells lose mature hepatic features quickly and have not been widely used in drug toxicity screening (Kim et al., 2018; Fu et al., 2019). Moreover, multiple reports on applications of HepaRG and Upcyte® human hepatocytes (UHHs) in metabolic and toxicological evaluation were published (Andersson et al., 2012; Levy et al., 2015; Ramachandran et al., 2015). However, to the best of our knowledge, different clones, passages and differentiation protocols in HepaRG may lead to poor repeatability (Petrov et al., 2018), the transcriptional levels of major CYP450 enzymes in UHHs still to be improved at donor-specific levels, and the efflux transporter functions for

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biliary excretion were not reported (Levy et al., 2015; Schaefer et al., 2016). Furthermore, genetic manipulation by overexpressing HPV genes (E6 and E7) or other immortal gene is not the best way to obtain expanded hepatocytes with genomic instability (Liang and Zhang, 2013; Ma H, 2014).

Recently, the development of de-differentition strategy, a breakthrough in developing *in vitro* surrogate hepatocyte model for PHH, made it feasible for PHH to expand as proliferating human hepatocytes (ProliHH) and ProliHH could be matured in a 3D culture environment (Zhang et al., 2018). Unlike other dedifferentiation strategies for PHH, ProliHH are bi-phenotypic, partially maintaining mature hepatocyte features and gaining of expression of progenitor-associated genes. Compared to hPSC-derived hepatocytes and hiHeps, ProliHH show promising potential for *in vitro* drug safety assessment because of expression of transporter and activity of CYP2B6 (Zhang et al., 2018). However, it is still unclear whether ProliHH is feasible to predict drug hepatic clearance, biliary excretion and hepatotoxicity *in vitro*.

In this study, we generated ProliHH from different PHH donors. Three major DMEs expression and function in ProliHH were systematically characterized, and the relevance of seven major DMEs was then investigated between ProliHH-M and PHH. After sandwich culture, cell polarization and efflux transporter function were identified in ProliHH. Also, selected compounds induced cytotoxicity were tested in ProliHH. Together, these results suggested ProliHH can be an alternative hepatocyte model for PHH in drug metabolism, transport and hepatotoxicity screening.

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Materials and Methods

Chemicals and Reagents

Phenacetin, bupropion, testosterone, chlorzoxazone, diclofenac acid, s-mephenytoin, dextromethorphan, dexamethasone, n-acetyl-cysteine, human [leu15]-gastrin I, penicillin-streptomycin (100×), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), chir99021, ibuprofen, lithocholic acid (LCA), chenodeoxycholic acid (CDCA), chlorpromazine, cyclosporin A, rifampin, troglitazone, tamoxifen, amiodarone, imipramine, isoniazid, valproic acid, rosuvastatin, glibenciamide, ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Advanced DMEM/F-12 (Dulbecco's Modified Eagle Medium/Ham's F-12), DMEM (Dulbecco's Modified Eagle Medium), Williams' Medium E (WME), Hanks' balanced salt solution (HBSS), Fetal bovine serum (FBS), ITS^{TM+} Premix (insulin, transferrin, selenium) and 0.25% trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human epidermal growth factor (rhEGF), recombinant human hepatocyte growth factor (rhHGF), oncostatin M (OSM) and recombinant human fibroblast growth factor 10 (rhFGF10) were purchased from PeproTech (Rocky Hill, NJ, USA). Cholyl-Lys-Fluorescein (CLF) and 5-(and-6)-carboxy-2', 7'-dichlorofluorescein diacetate (CDFDA) were purchased from AAT bioquest (Sunnyvale, CA, USA). Deuterium-labeled sodium taurocholate (d8-TCA) was purchased from GIBCO (Carlsbad, CA, USA).

The culture of PHH

Cryopreserved PHH were purchased from BioreclamationIVT (Baltimore, MD, USA) and XenoTech (Lenexa, KS, USA). The human hepatocytes donor information were presented in Supplemental Table 1. Briefly, the cryopreserved PHH were resuscitated and suspended following vendor's

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recommendations. PHH cells were diluted to 3.5×10^5 cells/well and plated onto 24 well plates, coated by collagen-I in plating medium (PM, WME supplemented with 1 μM dexamethasone, 5% FBS and 1% PS). Attached PHH were maintained in feeding medium (FM, WME supplemented with 1× ITS^{TM+} Premix, 0.1 μM dexamethasone, 1% FBS and 1% PS) and changed FM medium every day (Pan et al., 2012).

The derivation and proliferation of ProliHH from PHH

Cryopreserved PHH were firstly seeded at required density (5×10^5 cells/well) in a 6 well plate which coated by collagen I and cultured under hypoxia conditions (5% O_2, 5% CO_2, 37℃) (Zhang et al., 2018). To obtain proliferative status of ProliHH (ProliHH-P), plating medium was replaced with HM medium (HM, advanced DMEM/F12 supplemented with10 μM Y-27632, 5 μM A83-01, 50 ng/mL Wnt3a protein or 5 μM chir99021, 2 ng/mL rhFGF10, 50 ng/mL rhEGF, 25 ng/mL rhHGF, 10 nM Human [Leu15]-gastrin I, 1× N2 supplement 100×, 1 mM N-acetyl-cystein, 1× B27 supplement 50× minus vitamin A, 1% FBS, 10 mM nicotinamide and 1% PS) in 10 - 24 hours after PHH seeding and the HM medium was changed every two days. For monolayer expansion, ProliHH were seeded at 6 well plate (1×10^5 cells/well) under hypoxia condition and trypsinized at day 7 for passaging. The HM medium was changed every two days.

The maturation of ProliHH in 3D culture system

For rapid hepatic maturation, ProliHH were seeded at 2.5×10^5 cells/well and aggregated in an Ultra-Low Attachment (ULA) 24-well plate (Kuraray, Corning, Tewksbury, MA, USA) and formed spheres in hepatic maturation medium (HIM, HM supplemented with 5 μM forskolin, 1 μM dexamethasone, 20

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ng/mL OSM) under normoxia condition (5% CO_2 incubator). A 50% HIM medium was then exchanged daily to allow further maturation about 7-10 days. These ProliHH-M were further used in CYP metabolic assay or sandwich culture.

The sandwich culture of ProliHH

For sandwich culture, trypsinized ProliHH (3.5×10^5 cells/well) from proliferative or maturation status were seeded onto collagen I coated 24-well plates. ProliHH were incubated overnight, and HIM medium was renewed. Attached ProliHH were overlaid with 0.25 mg/mL Matrigel® in HIM and incubated under normoxia condition (5% CO_2 incubator) to form sandwich configuration. Sandwich cultured ProliHH were further used in CDFDA staining and biliary excretion measurement at day 7.

Phase microscopy imaging

Hepatocyte morphology was assessed using phase contrast microscopy to ensure healthy optimal culture for experiments. ProliHH-P and PHH were monitored in a daily basis. The images of ProliHH-P were recorded on day 1, 7, 14 and 28. ProliHH-M were recorded on day 1, day 3 and day 7 to recheck the sphere stage. SC-PHH were recorded and checked on day 1 and day 5 to ensure bile canaliculi formation. SC-ProliHH were recorded and checked at day 1 and day 7.

RNA isolation and RT-qPCR

Total RNA extracted from ProliHH and PHH was isolated from TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was transcribed by 1000 ng total RNA with a Primescript RT Reagent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR was performed with a real-time qPCR kit (Yeasen, Shanghai,

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China) and SYBR Green Master Mix (Yeasen, Shanghai, China) on Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA). The Primers were listed in Supplemental Table 2.

Measurement of Human Albumin levels

For ProliHH-P and PHH, supernatants were collected from 6-well plate when cells were attached to the plated at day 1. For ProliHH-M, supernatants were collected from 24-well plate at day 0 (trypsinized from ProliHH-P), 2 and 8. Human albumin ELISA Quantitation Set was purchased from Bethyl Laboratory (Montgomery, TX, USA) and used to measure human albumin secretion level.

CYP metabolic capacity determination

Clearance of probe compounds was determined at designated time points (day 7, passage 4) in ProliHH-P and day 7 in ProliHH-M. For measurement of activities of three major DMEs (CYP1A2, CYP2B6 and CYP3A4), ProliHH-P (5×10^5 cells/well) and PHH (1×10^5 cells/well) were seeded in a 24-well plate. For the measurement of seven metabolic activities of CYP450 enzymes, ProliHH-M were seeded at 2.5×10^5 cells/well and cultured in a 24-well ULA plate in HIM for 7 days. ProliHH and PHH were incubated in 1 mL medium with probe substrates listed in Supplemental Table 3. The supernatants (100 μL) were collected from PHH, ProliHH-P, and ProliHH-M at 0, 1, 2, and 3 h after administration of substrate. The metabolic assay was stopped by adding 300 μL ice-cold acetonitrile. The probe compounds were analysed by LCMS-8030 (Shimadzu, Kyoto, Japan). Drug clearance was determined through parent compound disappearance rate as follows:

Elimination rate constant (k) = (− gradient)

$$\text{Half life } (t_{1/2})(\text{min}) = \frac{0.693}{k}$$

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$$V\ (\mu L/\text{million cells}) = \frac{\text{Incubation volume } (\mu L)}{\text{Number of cells in incubation } (\times 10^6)}$$

$$\text{Intrinsic Clearance } (CL_{int})\ (\mu L/\text{min/million cells}) = \frac{0.693 \times V}{t_{1/2}}$$

Hepatocyte polarization and biliary excretion capacity measurement assay

ProliHH-P, ProliHH-M and PHH were seeded at required density (3.5×10^5 cells/well) in 24-well plates. The formation of bile canaliculi networks in polarized hepatocytes were visualized by CDFDA (Levy et al., 2015). Generally, dilute 5 mM stock CDFDA solution 1:500 in culture medium and incubate at 37℃ for 30 min in the dark to detect the hepatocyte polarization. In order to measure the efflux transporter activities of ProliHH and PHH, different transporter substrates were selected: CLF (bile salt efflux pump, BSEP), CDF (multidrug resistance associated protein-2, MRP2), d8-TCA (BSEP) and rosuvastatin (MRP2). Biliary excretion index (BEI) values were determined over a 15 min interval after pre-incubated with warm HBSS with or without Ca^{2+} (Liu et al., 1999). For PHH, the BEI study was performed on day 5. The fluorescence intensity of CDF and CLF in sandwich cultured ProliHH or PHH were measured at excitation/emission wavelengths of 495/529 nm and 492/536 nm, respectively. According to our previous report protocol, the concentration of d8-TCA and rosuvastatin in sandwich cultured ProliHH or PHH was analysed by LCMS-8030 (Shimadzu, Kyoto, Japan). (Pan et al., 2012; Guo et al., 2014).

The BEI value was calculated as follows:

$$BEI = \left(1 - \frac{A_{minus\_Ca++}}{A_{plus\_Ca++}}\right) \times 100\%$$

A was the concentration or amount of test compound accumulated in the absence (A_{minus_Ca++}) or presence (A_{plus_Ca++}) of Ca^{2+}, which was achieved through adding warm Ca^{2+}-free or standard HBSS buffer (Wu et al., 2016).

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***In vitro* cytotoxicity assay**

ProliHH-P and PHH were seeded at required density (3×10^4 cells/well) in collagen I coated 96-well plates. The medium containing test compounds were replaced to each well after cell attached and then incubated 24 hours. For ProliHH-M spheroids in toxicity testing, 1.5% agarose coated 96-well plates which stored at 4°C were prepared prior to ProliHH seeding. ProliHH were then seeded at required density (3×10^4 cells/well) at agarose coated 96-well plates to form spheres and change 50% HIM medium every other day. At the fifth day, tested compounds were replaced and incubated for 24 hours in ProliHH-M. Cell viability was measured by Cell counting kit 8 (CCK-8, Yeasen, Shanghai, China) or MTT assay.

Statistical analysis

All data were expressed as the mean ± SD unless other stated using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Unpaired t-test and one-way analysis of variance (ANOVA) was used to analyse the two or more than two group comparisons, respectively. Correlations were calculated using Pearson correlation coefficient. In all analyses, a difference was considered as significant: p-value < 0.05, p-value < 0.01 and p-value < 0.001. The CL_{int} and TC_{50} value in ProliHH was compared with the observed value in PHHs to determine the predictability using absolute average fold error (AAFE) and AAFE value was calculated in equation as follows (Kimoto et al., 2017):

$$AAFE = 10^{\frac{1}{N}\Sigma\left|log_{10}\frac{ProliHH}{PHH}\right|}$$

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Results

Generation, maturation and cultured characteristics of ProliHH

The generation and maturation strategies were illustrated in Fig.1A. ProliHH in proliferative or maturation status (2D or 3D cultured) were abbreviated by ProliHH-P or ProliHH-M, respectively. In this study, with the help of HM medium and hypoxia culture conditions, PHH started to proliferate and gradually transformed from hepatocytes to progenitor-like cells: the proliferative status of ProliHH (ProliHH-P) (Fig.1B and S1). After 4 passages, the cell number of ProliHH could be expanded 200 folds, especially more than 1000 folds in donor MRW (Fig. S2).

When the proliferated culture conditions (HM medium/hypoxia) were removed and ProliHH were transferred to 3D culture plates with HIM medium, ProliHH were able to form spheroids and display a well-defined shape (Fig. 1B, right), which suggested the maturation of ProliHH. Meanwhile, the progenitor genes, such as *SOX17* and *CK7*, were significantly upregulated in ProliHH-P, and returned to the levels comparable to PHH after 3D culture (Fig. 1C). In contrast, the hepatic genes, such as *HNF4A*, *CEBPA*, *ALB* and *AAT*, were significantly downregulated in ProliHH-P and went back to the levels comparable to PHH in ProliHH-M (Fig. 1D and E).

To our knowledge, albumin secretion was always used as a functional biomarker of hepatocytes. Being consistent with the gene expression levels, albumin excretion was gradually reduced with serial passages in ProliHH-P (Fig. 1F), and significantly increase to 3.8 ± 0.1 μg/d/million cells in ProliHH-M after 8 days 3D culture comparable to, even better than their original PHH (Fig. 1F). Together, these results indicated that ProliHH were able to generate from PHH and mature after 3D culture.

The expressions and metabolic activities of three major CYP450 enzymes in ProliHH

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The metabolic capacities of ProliHH were assessed by gene expressions and probe substrate clearances of selected major DMEs. We first analysed the expression levels of *CYP1A2*, *CYP2B6*, and *CYP3A4* in ProliHH-P and found that the expression levels of these genes were downregulated dramatically with serial passage compared with PHH (Fig. 2A and S3). Similarly, a decrease of metabolic capacity was also observed in ProliHH-P, but weak metabolic activities were still maintained at passage 4 (Fig. 2B). While, after the 3D maturation (ProliHH-M), the expressions and functions of these indicated enzymes recovered significantly. The expression levels of *CYP1A2*, *2B6* and *3A4* in ProliHH-M were significantly higher than ProliHH-P but still less than PHH (Fig. 2A). The intrinsic clearances of selected CYP substrates also increased significantly (Fig. 2B). The results suggested that ProliHH-M could rank the CYP metabolic capacities of their donor PHH to some extent, especially for CYP1A2 and 3A4 (Fig. 2C). Together, these findings indicated that ProliHH were able to improve the gene expression and metabolic activities of three major DMEs after 3D culture.

The metabolic activities of CYP450 enzymes in ProliHH-M and PHH

Further, a comparison of metabolic capacities of seven DMEs between ProliHH-M and their derived PHH were performed with three independent donors (Fig. 3). Except for CYP2C19, the intrinsic clearance of CYP1A2, 2D6, 3A4 and 2E1 in ProliHH-M were approximately within 3-fold of PHH. CYP2B6 and 2C9 enzyme activities were within a 5-fold range compared between ProliHH-M and PHH. The ProliHH-M CL_{int} values of CYP2C19 and 2D6 were above the proportion of PHH whereas CYP2B6, 2C9 and 3A4 were below the proportion of PHH. Together, these data suggested that the ProliHH-M system may be utilized to estimate the clearance of major DMEs in their PHH counterparts to some extent.

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The formation of bile canalicular networks in sandwich-cultured ProliHH

According to the preliminary study, ProliHH can only revert back to mature status in 3D culture environment (e.g., ProliHH-derived 3D organoids) (Zhang et al., 2018). However, 3D organoids are not suitable for quantitative transporter studies. Therefore, sandwich-culture model was employed to evaluate if ProliHH could form canalicular networks after sandwich culture.

The sandwich-culture strategy was illustrated in Fig. 4A. Typically, ProliHH-P or ProliHH-M were trypsinized and then sandwich-cultured in 24-well plates. Sandwich-cultured PHH (SC-PHH) for 5 days was used as a positive control. After sandwich culture for 7 days, cell morphology, bile canaliculi structure and cell polarization of ProliHH were investigated. SC-ProliHH-M exhibited well hepatocyte-like morphology, including cubical shape, tight junctions and bile canalicular-like structures, while the hepatocyte-like cell appearance in SC-ProliHH-P was not as obvious as SC-ProliHH-M (Fig 4B). Both SC-ProliHH-P and SC-ProliHH-M were able to form bile canalicular networks, evidenced by fluorescence dye (Fig. 4C).

Further, the expression levels of essential uptake/efflux transporters were investigated for that cells derived from both ProliHH systems. First, RT-qPCR results showed the expression levels of these transporters were downregulated significantly in ProliHH-P, while significantly upregulated in ProliHH-M (Fig. 5A). To examine efflux transporter functions, such as BSEP and MRP2, sandwich cultured cells were incubated with their substrates (CLF and d8-TCA for BSEP, CDF and rosuvastatin for MRP2). Although the expression levels of these genes in ProliHH-P were rarely detected than PHH, all the tested compounds showed biliary excretion trends in ProliHH-P (BEI values >10%). For SC-ProliHH-M, the BEI values of tested compounds were increased or even comparable to PHH (Fig. 5B). Together, these

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findings suggested that ProliHH in sandwich-culture could maintain polarity, form bile canaliculi networks and display biliary excretion capacity.

Hepatotoxicity assessment in ProliHH

To further investigate whether ProliHH can be used to evaluate drug induced hepatotoxicity, twelve compounds were selected to perform 24 h acute toxicity assessment. The TC_{50} values of three compounds (rifampicin, isoniazid and valproic acid) in PHH were from literature reported (Supplemental Table 4). Both ProliHH-P and ProliHH-M responded to these drugs in 24 hours and displayed well correlation with PHH (Fig. 6A and B). Compared with PHH, the 2D culture system gave much higher TC_{50} estimation for imipramine and tamoxifen approximately above 3-fold (Fig. 6A). While the TC_{50} value of troglitazone and imipramine in ProliHH-M was much higher than PHH (Fig. 6B). When TC_{50} values were compared to PHH, the AAFE was 1.27 and 2.13 for ProliHH-P and ProliHH-M, respectively. Together, these results demonstrated that both ProliHH-M and ProliHH-P could be an alternative hepatocyte model for PHH in drug cytotoxicity evaluation. Considering the cost and convenience, ProliHH-P should be a better choice for early discovery drug screening phase.

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Discussions:

A variety of hepatocyte models, such as ESC or iPSC-derived hepatocytes-like cells (HLCs), hiHeps, HepaRG and UHHs, have been developed to study drug metabolism, transport and drug induced liver injury (DILI) (Mcgill et al., 2011; Levy et al., 2015; Ni et al., 2016; Bell et al., 2017). However, these hepatocyte models for application are limited by the lack of physiological relevance or donor variability (Brolén et al., 2010; Katsuda et al., 2012; Huang et al., 2014). For example, the transcripts of major DMEs in UHHs still need to be improved at donor-specific levels (Levy et al., 2015; Schaefer et al., 2016). It is vital to develop new promising cell models that address these issues. Recently, with the help of de-differentiation strategy, PHH could be expanded, displayed bi-phenotypic and stable genetic characteristics, and also could be converted back to mature "hepatocytes" after 3D culture (Zhang et al., 2018). Compared with PHH, the advantages of ProliHH include low cost, easy to obtain, diversified donor background, etc. However, it is not clear if it is feasible to evaluate drug metabolism, biliary excretion and toxicity, especially from the transport aspect, using this novel cell model. Therefore, in this paper, for the first time, the drug metabolic capacities and efflux transporter functions of ProliHH, as well as their potential in toxicity assessments, were fully characterized.

According to the previous study, ProliHH could be expanded up to 10,000 folds at P8 from young PHH donor cells (Zhang et al., 2018). In this study, the total cell number of ProliHH was enlarged more than 200 folds at P4, which is enough for the following studies and no further proliferation test was performed (Fig. S2). Being consistent with previous reporting that PHH will lose their hepatic phenotype and metabolic capacity rapidly during *in vitro* culture (Gómez-Lechón et al., 2014) and the 3D scaffold culture system could preserve the CYP450 gene expression and hepatocyte-specific functions for PHH (Schaefer et al., 2016; Heslop et al., 2017; Bell et al., 2018). These data indicated ProliHH from different

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donors gradually lost hepatic functions with serial passages and regained primary hepatocyte appearances and functions after 3D culture.

The loss of the phenotype of hepatocytes and the increase of the phenotype of liver progenitors were observed during the proliferating process of ProliHH (Fig. 1C-E and S3). The expression of major DMEs and transporters also rapidly declined in ProliHH-P (Fig. 2A and 5A), which were common for the de-differentiation process (Kim et al., 2018; Zhang et al., 2018; Fu et al., 2019). The phenotypes of PHH could be regained in ProliHH-M after 3D culture. For instance, the improvement of metabolic capacity given the fact that ProliHH-M was more flexible to predict intrinsic clearances of DMEs (Fig. 2B, 2C and 3). The intrinsic clearances of selected CYP substrates in ProliHH-M were able to predict the CL_{int} ranking of their PHH donors to some extent (Fig. 2C). These results indicated that the metabolic variabilities in different donors (inter-individual variability) could be inherited by their derived ProliHH, respectively, and the donor specific could be rebuilt in a short term.

It is worthwhile to note, that although mRNA expression of CYP enzymes in ProliHH-M is much less than that in PHH, metabolic activity mediated by these enzymes in ProliHH-M was close to that in PHH. For example, CYP2B6 mRNA level in ProliHH-M was about 1/300 as much as that in PHH, while its Cl_{int} was about 1/3 of PHH (Fig. 2A and 2B). In order to clarify this disconnection, the protein expression level of CYP2B6 in ProliHH-M and PHH was determined by Western Blotting. It was found that although the mRNA level of CYP2B6 in ProliHH was about 1/300 in PHHs, its protein level was comparable to PHHs (~1/3 of PHHs) (Fig. S4). This disconnection could partially be attributed to the post-transcriptional process of these related genes, and the different half-life of mRNA and protein (Berger et al., 2016).

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In addition, ProliHH-M was more efficient as an alternative hepatocyte model for drug metabolic investigation with donor specific character compared with ProliHH-P (Fig. 2C). Based on it, the functional comparison of seven major DMEs between ProliHH-M and PHH indicated ProliHH-M could predict intrinsic clearances of these DMEs in PHH successfully. A positive correlation ($r = 0.87$) was found and four DMEs of the CL_{int} values were within 3-fold (AAFE=0.92) accuracy (Fig. 3). Especially, a good prediction of CYP2E1 was displayed in ProliHH-M, which may be attributed to their higher mRNA expression level as previously reported (Zhang et al., 2018). The higher CYP2C19 activity (above 3-fold accuracy range) in ProliHH-M may be attributed to dexamethasone (CYP2C19 inducer) added in HIM medium (Raucy and J., 2002). However, being different from HepaRG and UHHs, the expression and function of CYP2B6 in ProliHH-M was lower than PHH, which might be associated with suboptimal 3D culture conditions (without dimethylsulfoxide or FGF19 in HIM medium) (Kanebratt and Andersson, 2008; Schaefer et al., 2016). Therefore, further manipulation of the culture system will be required to generate more functional ProliHH-M that are capable of efficiently replacing the PHH for drug metabolic studies.

Sinusoidal and canalicular transport properties are critical for primary hepatocytes. The lack of uptake and efflux transporter functions limits various alternative hepatocyte models in the preclinical evaluation of DILI and cholestasis (Teresa et al., 2013; Bell et al., 2016; Zhang et al., 2016). Previous studies reported, CDFDA has been commonly used to study the function of efflux transporters, e.g., MRP2 (Anthérieu et al., 2010; Levy et al., 2015). In this study, we found that ProliHH could be polarized in sandwich culture, which was essential for the proper efflux of bile acids or toxic drugs, and both cells from proliferative and maturation status of ProliHH could form canalicular networks in sandwich culture (Fig.4B and 4C). Further, compounds which may lead to cholestasis were investigated for their potential

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DILI risk using SC-ProliHH. To evaluate the BSEP efflux function, d8-TCA was selected to assess the efflux transporter functions in SC-ProliHH (Fig.5B). Three selected cholestatic drugs (rifampin, glibenclamide and ketoconazole) were reported as the inhibitors of BSEP (Ni et al., 2016; Wu et al., 2016). In this study, all of them significantly inhibited d8-TCA biliary excretion in SC-ProliHH-P (Fig. S5). Of course, the sandwich cultured cells derived from ProliHH-M displayed higher BEI values than ProliHH-P, which were close to the BEI values of PHH (Fig.5B). However, considering the timing and cost of 3D culture, SC-ProliHH-P was a better choice for the early drug discovery stage. More comprehensive studies, including OATPs/NTCP uptake and MDR1 (P-gp) efflux function, are required to fully characterized in SC-ProliHH in the future.

The evaluation of drug hepatotoxicity is essential for preclinical drug safety study (Teresa et al., 2013; Sebastian et al., 2014; Zhang et al., 2016). In this study, to investigate whether ProliHH can be used to evaluate drug-induced cytotoxicity, twelve compounds were selected to perform acute toxicity assessment. All tested compounds covered a broad toxicity dose range (TC_{50} ranged from 1 to 5000 μM) with varied liver toxic mechanisms, for example, metabolic activation (CDCA, ibuprofen and isoniazid), cholestasis (LCA, chlorpromazine and imipramine), reactive oxygen species (cyclosporin A, rifampin, amiodarone and valproic acid and multiple mechanisms (troglitazone and tamoxifen) (Wang et al., 2002; Kemp and Brouwer, 2004; Ni et al., 2016; Xie et al., 2019). In most cases, the TC_{50} values of the selected compounds in both ProliHH-M and ProliHH-P were within 3-fold accuracy with those in PHH (Fig. 6A, B). The higher TC_{50} values of tamoxifen in ProliHH-P may be attributed to the low expression of uptake transporter OATP1B1 during the proliferating process (Fig. 5A) (Gao et al., 2017). While, the higher TC_{50} value of troglitazone in ProliHH-M may be attributed to the increasing function of DMEs (CYP2C19, CYP2D6 and CYP3A4) and efflux transporters (BSEP and MRP2) in ProliHHs-M (Fig. 3

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and 5B) (Mueller et al., 2013). In addition, both ProliHH-P and ProliHH-M were insensitive to imipramine, for which liver toxicity was related to the production of metabolic intermediates (Lemoine et al., 1993; Su et al., 1993). We attributed these to the lower functions of related metabolic enzymes compared to their PHH counterparts. Together, these results showed that both of 3D cultured ProliHH-M spheroids and ProliHH-P were able to predict transporter mediated cytotoxicity, while ProliHH-P could be used to assess drug cytotoxicity in a quick, convenient and efficient way.

In conclusion, the proliferative and maturation protocol of ProliHH were fully characterized from perspectives of metabolic enzyme and transporter. The metabolic capacities of 3D cultured ProliHH-M are comparable to PHH. The sandwich cultured ProliHH recovered and maintained transporter function for biliary excretion and cholestasis risk assessment. Both ProliHH-P and ProliHH-M could be used to evaluate cytotoxicity. ProliHH-P has the potential to be developed into a screening tool to detect cytotoxicity. Therefore, a novel strategy combined 3D and sandwich culture make it feasible for ProliHH to support drug candidate screening in hepatic metabolism, disposition and toxicity.

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Author Contributions

Participated in research design: Shida Qiao, Sisi Feng, E Tian and Guoyu Pan

Conducted experiments: Shida Qiao, Sisi Feng, He Ting, Chen Ma and Zhaoliang Peng

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Performed data analysis: Shida Qiao, Sisi Feng, He Ting, Chen Ma and Zhitao Wu

Wrote or contributed to the writing of the manuscript: Shida Qiao, Sisi Feng, Zhitao Wu and Guoyu Pan

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Footnotes:

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Competing interests:

There is no conflict of interests.

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Figures

Figure 1 ProliHH were proliferative in hypoxia and HM conditions and revert back to maturation status in normoxia and 3D culture environment.

(A) The schematic overview showed the protocol for proliferation and maturation of ProliHH. (B) Phase microscopy showed representative morphologies of ProliHH at day 0, day 28 and day 38 (after 10 days 3D culture). Scale bars, 100 μm. (C-E) RT-qPCR analyses of progenitor-associated genes (C), hepatic nuclear receptor genes (D) and secretion protein genes (E) in PHH and ProliHH (n=3). CK7 and Sox17 were normalized to ProliHH-P and others were normalized to PHH. (F) Albumin secretion in ProliHH-P at indicated passages and in ProliHH-M at days 0, 2 and 8. All error bars indicate ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ProliHH-P, proliferative status of ProliHH; ProliHH-M, maturation status of ProliHH; ProliHH derived from donor QIE.

Figure 2 ProliHH-M showed CYP450 expression, enzyme function similar to those of PHH.

(A) Comparison of CYP450 mRNA expression determined at PHH, ProliHH-P (proliferative status, passage 4), and ProliHH-M (maturation status) from donor QIE by qPCR. (B) Comparison of CYP450 enzyme activities of PHH, ProliHH-P, and ProliHH-M. CYP1A2, 2B6, and 3A4 measured by phenacetin, bupropion, and testosterone, respectively. ProliHH were derived from donor MRW, 17905A, and 15101. (C) Comparison of *in vitro* clearance rates of phenacetin, bupropion, and testosterone measured in ProliHH-P and ProliHH-M with their native PHH source. ProliHH were derived from donor MRW, 17905A, and 15101. All error bars indicate ± s.d. The correlation between groups was evaluated by the Pearson correlation coefficient (r). *P < 0.05; **P < 0.01; ***P < 0.001; ProliHH-P, proliferative status of ProliHH; ProliHH-M, maturation status of ProliHH.

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Figure 3 Comparison of CYP450 metabolic capacities between ProliHH-M and PHH.

Correlation of absolute enzyme activity levels determined in ProliHH-M and PHH for CYP1A2, CYP2B6, CYP3A4, CYP2C19, CYP2D6, CYP2C9, and CYP2E1. All error bars indicate ± SEM. Clearance measured in ProliHH-M from donor MRW, 17905A, and 15101. The correlation between ProliHH-M and PHH was evaluated by the absolute average fold error (AAFE). The solid and dashed lines represent conformity and 3-fold error range, respectively. ProliHH-M, maturation status of ProliHH.

Figure 4 Bile canaliculi formation and polarization of ProliHH.

(A) The schematic overview showed the protocol for sandwich culture of PHH, ProliHH-P and ProliHH-M. (B) Phase microscopy showed morphology of SC-ProliHH-M similarly to SC-PHH. (C) CDFDA staining: CDFDA fluorescence intensity tend to converge to the junction of ProliHH. The bile canaliculi structure marked with a red arrow. ProliHH-P, proliferative status of ProliHH; ProliHH-M, maturation status of ProliHH; SC, sandwich culture; ProliHH derived from donor MRW. Scale bars, 100 μm.

Figure 5 Transporter expression and function of ProliHH in sandwich culture system.

(A) RT-qPCR analyses of major transporter genes in ProliHH at indicated stages, gene expressions are normalized to SC-PHH. (B) Four different compounds were used to determine the bile efflux index (BEI) of SC-PHH and SC-ProliHH. ProliHH were generated from donor MRW. *P < 0.05; **P < 0.01; ***P < 0.001; SC-ProliHH-P, sandwich-cultured proliferative status of ProliHH; SC-ProliHH-M, sandwich-cultured maturation status of ProliHH.

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Fig. 6 Comparable toxicity prediction ability of ProliHH with PHH.

(A) Comparison of the TC_{50} values in PHH and ProliHH-P with 12 compounds. (B) Comparison of the TC_{50} values in PHH and ProliHH-M with 12 compounds. ProliHH were derived from donor 15101. ProliHH-P, proliferative status of ProliHH; ProliHH-M, maturation status of ProliHH. The solid and dashed lines represent conformity and 3-fold error range, respectively. The TC_{50} values in both PHH and ProliHH higher than 5000 μM was marked as 5000 μM in the figure.

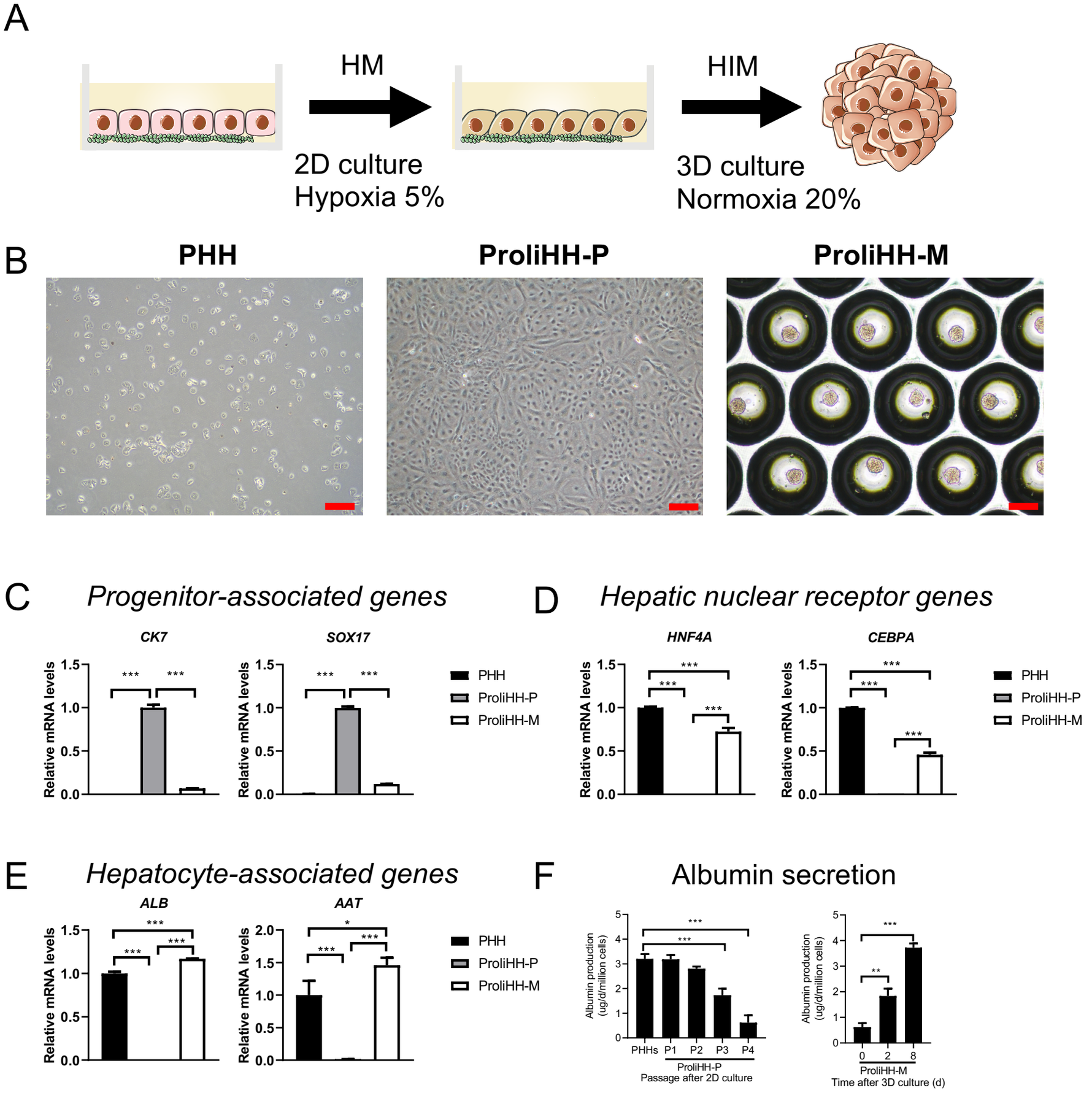
A
HM
2D culture
Hypoxia 5%
HIM
3D culture
Normoxia 20%
B
PHH
ProliHH-P
ProliHH-M
C
Progenitor-associated genes
CK7
SOX17
Relative mRNA levels
PHH
ProliHH-P
ProliHH-M
D
Hepatic nuclear receptor genes
HNF4A
CEBPA
E
Hepatocyte-associated genes
ALB
AAT
F
Albumin secretion
Albumin production (ug/d/million cells)
PHHs P1 P2 P3 P4
ProliHH-P
Passage after 2D culture
0 2 8
ProliHH-M
Time after 3D culture (d)

Figure 1

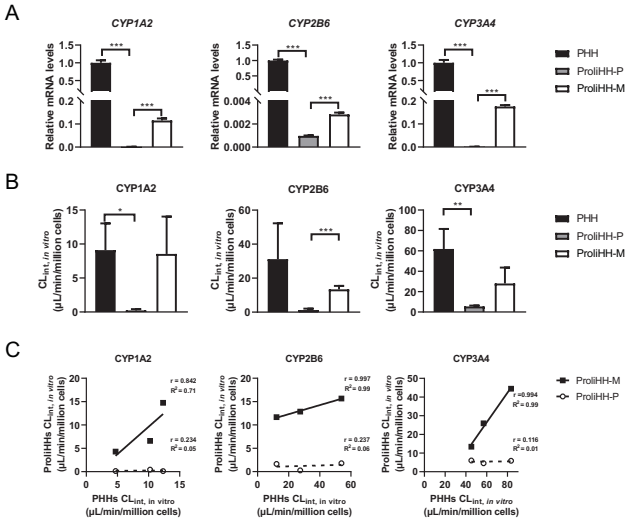

Figure 2

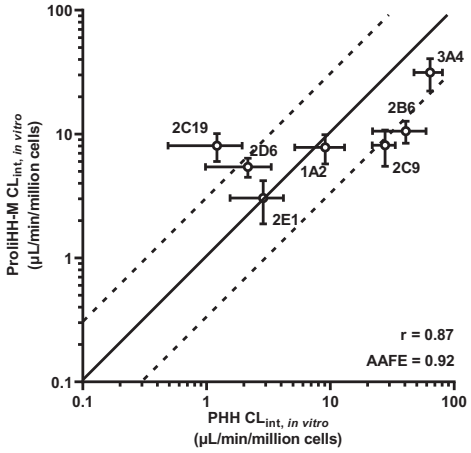

Figure 3

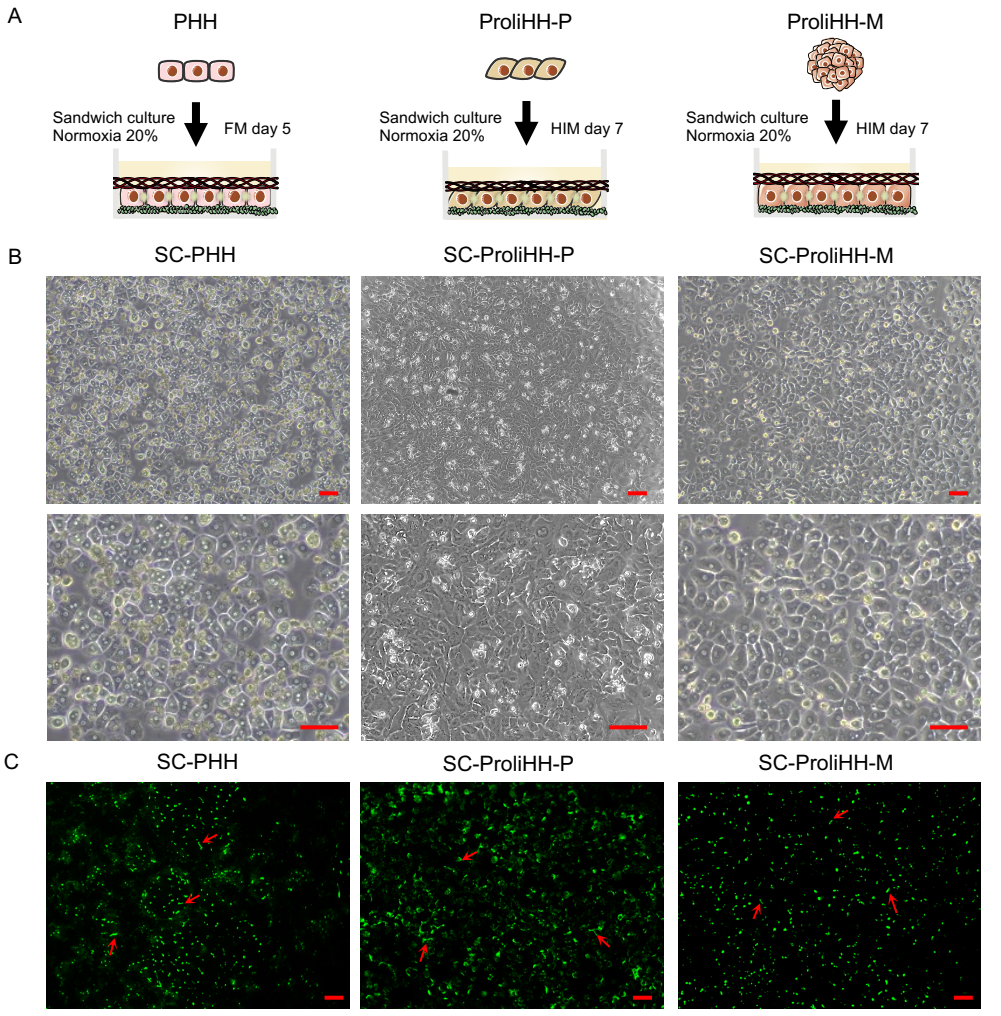
A
PHH
Sandwich culture
Normoxia 20%
FM day 5
ProliHH-P
Sandwich culture
Normoxia 20%
HIM day 7
ProliHH-M
Sandwich culture
Normoxia 20%
HIM day 7
B
SC-PHH
SC-ProliHH-P
SC-ProliHH-M
C
SC-PHH
SC-ProliHH-P
SC-ProliHH-M

Figure 4

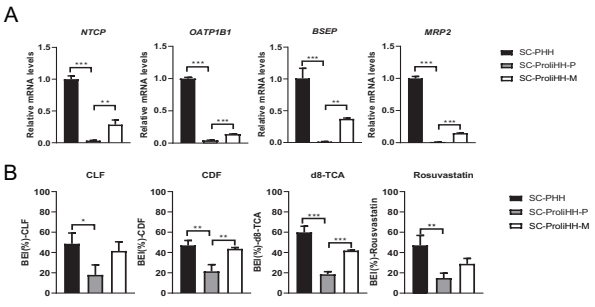

Figure 5

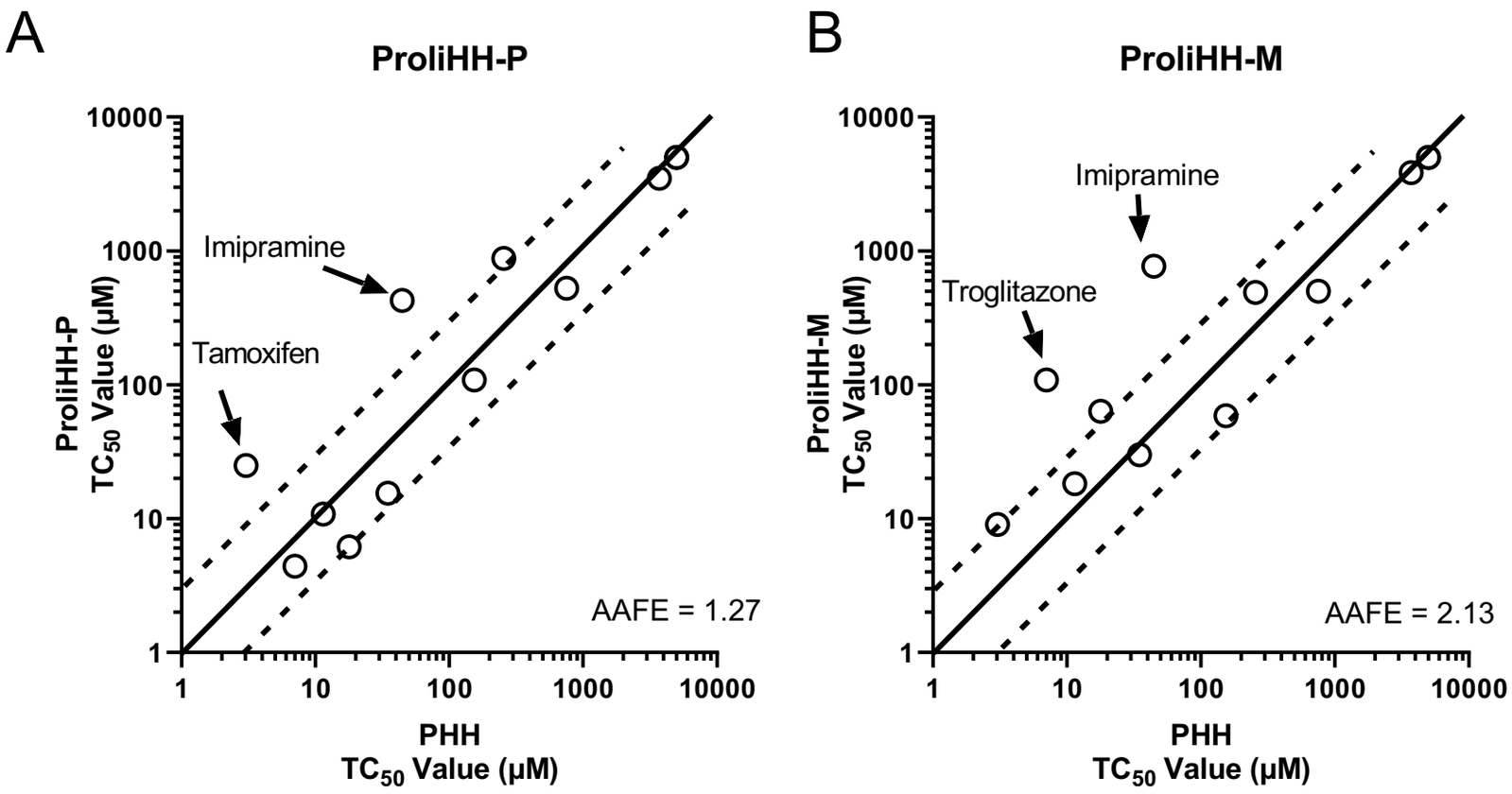
A
ProliHH-P
10000
1000
100
10
1
Imipramine
Tamoxifen
AAFE = 1.27
ProliHH-P
TC50 Value (μM)
1
10
100
1000
10000
PHH
TC50 Value (μM)
B
ProliHH-M
10000
1000
100
10
1
Imipramine
Troglitazone
AAFE = 2.13
ProliHH-M
TC50 Value (μM)
1
10
100
1000
10000
PHH
TC50 Value (μM)

Figure 6