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A two-step strategy to expand primary human hepatocytes in vitro with efficient metabolic and regenerative capacities

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Abstract

Background Primary human hepatocytes (PHHs) are highly valuable for drug-metabolism evaluation, liver disease modeling and hepatocyte transplantation. However, their availability is significantly restricted due to limited donor sources, alongside their constrained proliferation capabilities and reduced functionality when cultured in vitro. To address this challenge, we aimed to develop a novel method to efficiently expand PHHs in vitro without a loss of function.

Methods By mimicking the in vivo liver regeneration route, we developed a two-step strategy involving the de-differentiation/expansion and subsequent maturation of PHHs to generate abundant functional hepatocytes in vitro. Initially, we applied SiPer, a prediction algorithm, to identify candidate small molecules capable of activating liver regenerative transcription factors, thereby formulating a novel hepatic expansion medium to de-differentiate PHHs into proliferative human hepatic progenitor-like cells (ProHPLCs). These ProHPLCs were then re-differentiated into functionally mature hepatocytes using a new hepatocyte maturation condition. Additionally, we investigated the underlying mechanism of PHHs expansion under our new conditions.

Results The novel hepatic expansion medium containing hydrocortisone facilitated the de-differentiation of PHHs into ProHPLCs, which exhibited key hepatic progenitor characteristics and demonstrated a marked increase in proliferation capacity compared to cells cultivated in previously established expansion conditions. Remarkably, these subsequent matured hepatocytes rivaled PHHs in terms of transcriptome profiles, drug metabolizing activities and in vivo engraftment capabilities. Importantly, our findings suggest that the enhanced expansion of PHHs by hydrocortisone may be mediated through the PPARα signaling pathway and regenerative transcription factors.

Conclusions This study presents a two-step strategy that initially induces PHHs into a proliferative state (ProHPLCs) to ensure sufficient cell quantity, followed by the maturation of ProHPLCs into fully functional hepatocytes to guarantee optimal cell quality. This approach offers a promising means of producing large numbers of seeding cells for hepatocyte-based applications.

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Keywords Primary human hepatocytes, Hepatocyte proliferation, Regenerative transcription factors, Hydrocortisone, PPARα

Introduction

Liver disease is a devastating disease, accounting for over two million deaths and 4% of global mortality annually [1]. Hepatocytes play a crucial role in treating liver diseases through their applications in drug screening, disease modeling, and clinical cell transplantation. Various strategies have been developed for generating hepatocytes in vitro, including differentiation from pluripotent stem cells or mesenchymal stem cells, and trans-differentiation from fibroblasts etc. [2, 3]. Despite their utility, in vitro hepatocytes fail to fully replicate the molecular and functional characteristics of their in vivo counterparts. Human primary hepatocytes (PHHs) are widely considered the preferred cell source for these applications. [4, 5]. However, the use of PHHs is significantly limited by the scarcity of available liver donors [4, 5].

The in vitro expansion of PHHs offers a promising avenue to obtain a significant number of functional hepatocytes. However, it has traditionally been challenging to expand these cells while maintaining their functions [6–8]. Recent advances utilizing small molecules and growth factors have presented a potential way to expand PHHs in vitro [9–15]. However, these expansion conditions often demonstrate limited efficacy in expanding adult hepatocytes [12–15], and the resulting hepatocytes exhibit variable proliferation and functionality across PHHs sourced from different liver donors. Consequently, there still holds a great demand for developing more robust and universally effective methods for PHHs expansion.

Liver exhibits remarkable regenerative capacity upon injuries, primarily due to the rapid de-differentiation of hepatocytes into proliferative progenitors, followed by their extensive expansion, and eventual re-differentiation into functionally mature hepatocytes to restore the liver [16–18]. The process of de-differentiation and expansion process is governed by a cascade of regenerative transcription factors (TFs) that initially prompt hepatocytes to transition from a quiescent, functional state into a proliferative, progenitor state [19]. After injury cessation, the de-differentiated, progenitor-like cells revert to functional hepatocytes under the influence of endogenous signals [16]. Inspired by liver regeneration routes in vivo, we supposed that activating regenerative TFs to initiate the de-differentiation of PHHs into proliferative progenitor-like cells, followed by employing specific signaling mimics to guide progenitor-like cells differentiate into mature hepatocytes, presents a viable strategy for generating large amounts of functional hepatocytes in vitro.

Utilizing small molecule compounds to specifically activate regenerative TFs in PHHs presents a straightforward and economical approach. However, this method poses significant challenges. The traditional library screening approach is both labor-intensive and inefficient [20]. We have recently introduced a novel platform, SiPer, based on single-cell RNA sequencing. This platform is specifically designed for predicting chemical perturbagens that can facilitate cellular conversion by targeting a specific set of TFs [21]. By employing SiPer, we were able to efficiently identify candidate chemical compounds that are capable of converting PHHs into proliferative hepatic progenitors by specifically targeting key regenerative TFs.

To re-differentiate the progenitor-like cells into functional hepatocytes, the use of an appropriate hepatic maturation medium is crucial. Currently, most hepatic maturation media exhibit limitations in generating fully mature hepatocytes such as inefficient drug-metabolizing activities and low engraftment rates in vivo, when compared to PHHs [10, 11, 13, 22]. We have previously developed a robust 5-chemical (5C) condition for the direct in vitro culture of PHHs, capable of supporting the long-term functional maintenance of PHHs for over a month [23]. We have also demonstrated key chemicals within the 5C formulation can induce the maturation of reprogrammed hepatic progenitor-like cells into functionally mature hepatocytes [24]. Therefore, leveraging the 5C formulation allows for the highly functional maturation of hepatic progenitor-like cells.

Here, we developed a two-step system for the de-differentiation and expansion of PHHs, followed by the subsequent re-differentiation into functionally mature hepatocytes to meet the demands for large numbers of applicable hepatocytes. We first established a new hepatic expansion medium containing hydrocortisone, which effectively converted PHHs into proliferative hepatic progenitor-like cells (ProHPLCs). PPARα signaling pathway and regenerative TFs XBP1, EGR1, EPAS1 and FOS were identified as potential key players of hydrocortisone-induced hepatocyte expansion. Following expansion, ProHPLCs underwent maturation in an optimized 6C medium, resulting in 6C-cultured hepatocytes (6C_HHs) that closely replicate the molecular and functional attributes of original PHHs. Taken together, our two-step strategy offers promising novel cell sources for hepatocyte-based applications.

Materials and methods

Primary human hepatocyte isolation and culture

Human hepatocytes were isolated from excess liver tissue of surgical liver resections. A modified two-step collagenase perfusion procedure was used to isolate primary human hepatocytes from these tissues [25]. Briefly, the liver tissue was perfused with PB (perfusion buffer: 0.15 M NaCl; 5 mM KCl; 25 mM $NaHCO_3$; 5 mM Glucose; 20 mM Hepes) to remove residual blood cells, followed by PBE (perfusion buffer plus 1 mM EDTA) perfusion. The tissue was then perfused with PBC (perfusion buffer plus 1 mg/mL collagenase type IV, 5 mM $CaCl_2$, Gibco). All buffer solutions were prewarmed to 37 °C prior to the isolation process. The hepatocyte suspension was collected and washed with Williams' Medium E (Gibco), then filtered through a 40 μm Nylon cell strainer [26]. PHH donor information is shown in Table S1.

The isolated hepatocytes were cryopreserved or plated afterwards. The isolated hepatocytes were then seeded on cell culture plates coated with collagen type I (Rat tail collagen type I, Gibco) at a density of $2.5\times10^5/cm^2$ for 5C direct culture and $2.5\times10^4/cm^2$ for NHEM expansion in Null culture medium. The medium was changed into corresponding 5C/NHEM the next day. For resuscitated hepatocytes, the density will be adjusted according to the cell viability of different donors.

The formulation of Null and 5C was used as described in Xiang et al. [23]. Null: Williams' medium E (Gibco, 35050061) containing B27 (50×, Gibco, 17502048), Glutamax (Gibco, 35050061) and Penicillin Streptomycin (PS, Gibco, 15140122). 5C: Null supplemented with Forskolin (20 μM), SB431542 (10 μM), IWP2 (0.5 μM), DAPT (5 μM), and LDN193189 (0.1 μM). 6C: 5C supplemented with100 μg/ml heparin.

De-differentiation/expansion and maturation of PHHs

Fresh or resuscitated PHHs were plated in a low density around $2.5\times10^4/cm^2$ in HCM or Null medium. The next day the medium was changed into expansion medium. The HEM for initial expansion test was formulated as: 50% DMEM/F12 and 50% William's E Medium supplemented with 1% PS, 2% B27 (without vitamin A), 5 mM Nicotinamide, 200 μM 2-phospho-L-ascorbic acid (pVc), 3 μM CHIR99021, 5 μM SB431542, 0.5 μM Sphingosine-1-phosphate (S1P), 5 μM Lysophosphatidic acid [27], 50 ng/mL EGF. The information of small molecule compounds for expansion screening were listed in Table S2. NHEM was formulated as: 50% DMEM/F12 and 50% William's E Medium supplemented with 1% PS, 2% B27 (without vitamin A), 200 μM 2-phospho-L-ascorbic acid (pVc), 3 μM CHIR99021, 5 μM SB431542, 50 ng/mL EGF, 20 ng/mL HGF and 5 μM hydrocortisone. NHEM was changed every two days in the first 6 days and changed every day in the rest of days. Upon confluence of ProHPLCs in NHEM, cells were dissociated with Accutase and replated in a ratio around 1:3 in NHEM with CEPT (MCE). Confluent ProHPLCs were cultured in 6C (5C supplemented with 100 μg/ml heparin) for 7–10 days for maturation. The formulations of other media reported for PHHs expansion were listed in Table S3.

Animals and cell transplantation

All animal experiments have been reported in line with the ARRIVE guidelines 2.0 [28] and approved by IACUC Committee of Beijing Vitalstar Biotechnology. Tet-uPA/Rag2−/−/γc−/− (URG) mice on a BALB/c background were purchased from Beijing Vitalstar Biotechnology. Male URG mice aged between 8 and 10 weeks, weighing 25 ± 2 g, were selected for the experiments. Cell transplantation and mice care were conducted at Beijing Vitalstar Biotechnology. 8 weeks post transplantation, the mice were euthanized by decapitation and transferred to the laboratory for further detection and analysis.

For transplantation, cells were suspended as single cells in HCM™ medium. A total of 2×10^6 cells in 200 μL of suspension were injected into the mouse liver via the splenic vein. For details, URG mice were injected intraperitoneally with 0.15 mg/kg Doxycycline to induce liver injury 48 h before surgery. After anesthesia with an avertin solution, an abdominal incision was made to expose the spleen, which was then carefully pulled out of the body using ophthalmic forceps. Then hepatocyte suspension was injected into the splenic vein of the spleen with a 1 mL syringe, after which the splenic head was quickly ligated to prevent cell outflow. The spleen was gently placed back to the abdominal cavity, and the incision was sutured closed. Following recovery from anesthesia, the mice were housed in an SPF environment, with 0.1–0.5 mg/mL Doxycycline added to their drinking water. PHHs, ProHPLCs and 6C_HHs were transplanted into 6, 13, 7 URG mice respectively, with PHHs serving as the positive control. The sample size of the mouse cohort for transplantation was determined based on data from published studies, previous experience, and cost considerations. No criteria were set for including or excluding animals and no data points were excluded from the analysis. The mice selected for transplantation of each cell type were randomly chosen with an age variance of no more than two weeks (8–10 weeks) and had similar body weights. No specific strategy was employed to minimize potential bias. For blinding purposes, the investigator responsible for the cell transplantation was unaware of the cell type being transplanted.

Blood samples were collected at 5, 6, 7, 8 weeks post transplantation from the retro-orbital plexus with a

capillary glass tube puncture. Human albumin levels in the mouse blood samples were quantified using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories). The livers of recipient mice were fixed with 4% paraformaldehyde and dehydrated with 30% sucrose solution followed by embedding in OCT compound (Sakura) and frozen in liquid nitrogen. Cryo-sections were generated using cryostat (Leica) and were subjected to immunofluorescent staining as outlined in the supplementary methods section. To identify areas of the liver where human hepatocytes successfully integrated, we identified $hALB^{+}$ regions as the repopulation region. For quantification of hALB-positive cells, images were captured with Vectra Polris (PerkinElmer) and then analyzed by Image J.1.51. The repopulation rate of each humanized mouse liver was calculated using 3–6 random sections.

Statistical analysis

Experiments were independently replicated at least twice, and representative data are shown. *P* values for the purpose of group comparisons were calculated using multiple *t*-test, one-way ANOVA or two-way ANOVA. Correlations were evaluated using Pearson correlation coefficients. The level of significance in all graphs is represented as follows: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Unless described otherwise, standard statistical analyses were performed with GraphPad Prism 7 using default parameters. All the error bars represent SEM.

Results

Optimization of the culture system for PHHs expansion using SiPer

Based on our two-step de-dedifferentiation/expansion and maturation strategy (Fig. 1A), we first sought to test and optimize our previously established hepatic expansion medium (HEM) [24], which was designed for inducing and expanding hepatic progenitor-like cells from fibroblasts along with reprogramming TFs, for PHHs dedifferentiation and expansion. We found that HEM could induce an EMT in PHHs and turn them into a proliferative state, however the expansion efficiency was limited (Fig. S1A). To identify the fundamental factors in HEM for PHHs expansion, we first conducted a "HEM minus 1" experiment. We observed that the removal of nicotinamide (NICO) significantly promoted PHHs expansion (Fig. 1B, Fig. S1A), which is consistent with reported study [9]. Interestingly, the removal of LPA or S1P, inhibitors of the HIPPO signaling pathway, had negligible effects on the expansion of PHHs (Fig. 1B, Fig. S1A). Further analysis revealed that the simultaneous exclusion of LPA and S1P from HEM had a minimal impact on PHHs expansion and hepatic identity maintenance (Fig. 1C–D, Fig. S1B), suggesting dispensable role of LPA and S1P in PHHs expansion. Consequently, this led us to identify an optimized HEM for PHHs expansion incorporating the essential factors EGF, SB43 and CHIR. Based on this medium, we further evaluated a range of reported small molecule compounds and growth factors for their potential in promoting PHHs expansion [9–14, 29]. Our findings highlighted HGF as a consistent enhancer of PHHs proliferation across different donors, whereas other factors showed little or negative effect (Fig. 1E, and Fig. S1C). Consequently, we formulated an optimized version of the expansion medium incorporating HGF, termed as basic hepatic expansion medium (BHEM), which demonstrated improved expansion capabilities of hepatocytes. Despite the enhanced performance of BHEM, its efficiency and robustness for hepatocyte expansion remained insufficient. This necessitates further investigation into chemical compounds that could more effectively foster hepatocyte proliferation.

Upon injury, hepatocytes de-differentiate into a highly proliferative progenitor state, and then re-differentiate to compensate the lost or injured liver during liver regeneration. A recent comprehensive study revealed 234 genes that were upregulated in response to liver injury induced by both acetaminophen (APAP) and partial hepatectomy (PHx) [30], among which these genes were identified as potential regenerative TFs: KLF6, HNF4A, BACH1, PAS1, STAT3, XBP1, EGR1, FOS, PROX1 (Fig. S1D). We then sought to activate these TFs in PHHs in vitro to induce them into a proliferative progenitor state. Utilizing single-cell RNA sequencing data of PHHs and the potential regenerative TFs as inputs for SiPer, a prediction platform, combined with a thorough literature review on cell proliferation-promoting compounds, we obtained a list of 30 promising candidate chemical compounds for subsequent experimental validation (Table S2).

Hydrocortisone promotes PHHs expansion in vitro

With the 30 selected chemical compounds, we performed 2 rounds of screening to assess the expansion potential of PHHs from two donors. We introduced each of the candidate chemical compounds to the PHHs cultures separately based on BHEM (BHEM plus one). Our initial screening revealed that 15 of these compounds exhibited a more pronounced ability to foster hepatocyte expansion than the control after 9 days of culture (Fig. 1F). To avoid potential donor-related variability, we subjected these promising candidates from the first screening round to further evaluation using PHHs from an additional donor (Fig. 1G). Hydrocortisone and FPH2 distinguished themselves by demonstrating the most effective PHHs expansion across two donors (Fig. 1F–G). Subsequently, we investigated the potential synergistic effect of combining hydrocortisone and FPH2. Our results showed that

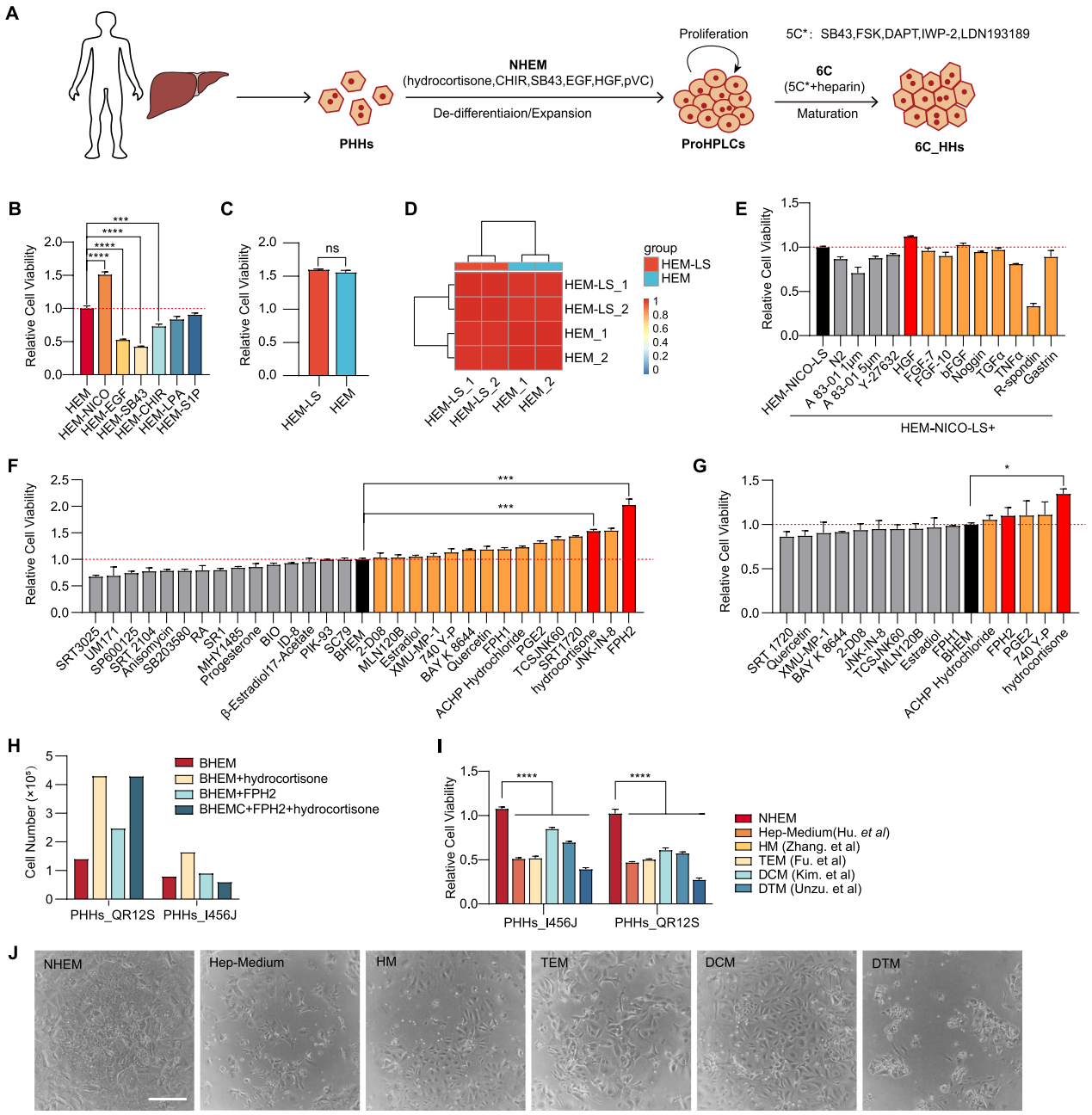


Fig. 1 Optimization of Medium for Expanding Primary Human Hepatocytes. **A**. Schematic diagram of the two-step strategy of de-differentiation/expansion and maturation of PHHs in vitro. **B**. Relative cell viability of PHHs cultured in hepatic expansion medium (HEM) compared to HEM without NICO, EGF, SB43, CHIR, LPA, or S1P over a 9-day period measured by CCK8 analysis. n = 3. P values were determined by one-way ANOVA. **C**. Relative cell viability of PHHs cultured in HEM with or without LPA and S1P (HME-LS) for 9 days measured by CCK8 analysis. n = 3. P values were determined by one-way ANOVA. **D**. Transcriptome correlation analysis of PHHs cultured in HEM with or without LPA and S1P (HME-LS) for 9 days. **E**. Relative cell viability of hepatocytes cultured in HEM-NICO-LS (HEM without NICO, LPA and S1P) with the addition of various reported small molecules or growth factors for 7 days measured by CCK8 analysis. n = 2. **F** Round 1 screening of candidate small molecule compounds for PHHs expansion based on BHEM through analyzing the cell viability of PHHs_ C567D cultured for 9 days measured by CCK8 analysis. Compounds with better effects were designated as round 1 hits (orange and red columns). n = 3. P values were determined by one-way ANOVA. **G**. Round 2 screening of round 1 hits for PHHs expansion based on BHEM through analyzing the cell viability of PHHs_NO45P cultured for 9 days measured by CCK8 analysis. Compounds with better effects were designated as round 2 hits (orange and red columns). n = 2. P values were determined by one-way ANOVA. **H**. Cell number of PHHs cultured in BHEM with FPH2 and/or hydrocortisone for 9 days. **I**. Comparison of the cell viability of PHHs cultured in NHEM and previously reported expansion media (Hep-Medium, HM, TEM, DCM and DTM) for 9 days measured by CCK8 analysis (n = 4 for PHHs_I456J, n = 2 for PHHs_QR12S). P values were determined by two-way ANOVA. **J**. Representative images of PHHs cultured in NHEM and reported expansion media for 9 days. Scale bar, 100 μm. All data were presented as mean ± SEM

hydrocortisone alone was superior in facilitating PHHs expansion compared to FPH2 alone or the combination of hydrocortisone and FPH2, as evidenced by the cell number of proliferating cells originated from two hepatocyte donors after 7 days of culture (Fig. 1H).

We next compared our expansion medium containing hydrocortisone with reported PHHs de-differentiation/expansion medium [9, 11–14] regarding the effect on PHHs expansion. Through the analysis of cell number and cell viability, we found that our expansion medium significantly outperformed other media in terms of its capacity to expand PHHs across two donors (Fig. 1I–J, and Fig. S1E). The superior performance of our expansion medium was maintained after passage (Fig. S1F). In all, we developed a new hepatic expansion medium outperforming previously-reported conditions for PHHs expansion, and we termed this new hepatic expansion medium of BHEM supplemented with hydrocortisone as NHEM.

Characterization of NHEM-induced ProHPLCs

Next, we examined the molecular identity of proliferative cells cultured in NHEM from PHHs. We observed that a typical hepatocyte morphology of PHHs began to change into mesenchymal morphology from day 3, reaching a fully confluent state at around day 9 (Fig. 2A and Fig. S2A). By day 7, most cells had turned into a $Ki67^+$ proliferative state (Fig. 2B). Notably, NHEM-cultured cells showed increased expression of hepatic progenitor-associated signature genes and TFs which were reported to be upregulated in hepatic progenitors during liver development and regeneration [31] (Fig. 2C). The upregulated genes in NHEM culture included *CK19*, *EPCAM*, *CD24*, *SOX9*, *HNF1B*, and *TBX3* compared to PHHs (Fig. 2C). Additionally, the upregulation of cyclin-dependent kinases *CDK2* and *CDK4* indicated the replicative state of NHEM-cultured cells (Fig. 2C). Consistently, IF staining further revealed that nearly all hepatocytes exhibited positive markers for hepatic progenitors, including SOX9, CK19, and TBX3 (Fig. 2D). Moreover, flow cytometry analysis showed that NHEM-cultured cells had high proportions of ALB/AFP (97.7%), ALB/EPCAM (96.3%), CK19/EPCAM (87.92%), CK19/SOX9 (79.17%) and CK19/TBX3 (89.66%) double-positive population (Fig. 2E), with all of the above being hepatic progenitor specific markers.

To further characterize the progenitor characteristics of NHEM-cultured cells, we compared their transcriptomic profiles with that of progenitor-like human fetal liver cells (hFLCs) and PHHs. PCA and correlation analysis of global genes revealed that NHEM-cultured hepatocytes shared closer similarities with hFLCs (Fig. 2F–G). We then found that NHEM-cultured hepatocytes and hFLCs showed similar high expression pattern of a panel of hepatic progenitor genes [32], while PHHs showed little expression of these genes (Fig. 2H), indicating that NHEM-cultured hepatocytes acquired a progenitor identity. Furthermore, we found that 3505 genes were upregulated during the conversion of PHHs into a proliferative progenitor-like state (Fig. S2B). Further analysis highlighted that these genes were enriched in pathways critical for cell replication and liver regeneration (Fig. S2C). These results collectively underscore the capacity of NHEM to induce a progenitor-like state in PHHs. Consequently, we refer to NHEM-cultured PHHs as proliferative human hepatic progenitor-like cells (ProHPLCs).

We further evaluated whether ProHPLCs could expand long-term. We passaged ProHPLCs in NHEM with the CEPT cocktail to enhance their survival and expansion. Remarkably, ProHPLCs demonstrated the capacity to undergo more than 7 passages (Fig. 2I), with a doubling time around of 51 h estimated by the growth curve, and maintained a high proportion of Ki67 positive rate at passage 7 (P7) (Fig. 2J and Fig. S2D). qRT-PCR analysis further revealed that key hepatic progenitor markers, such as *EPCAM* and *SOX9*, retained their expression levels up to passage 7 (Fig. 2K). Consistently, IF staining also confirmed the expression of key progenitor markers EPCAM, SOX9 and HNF4A at passage 7 (Fig. S2D). Moreover, the expression of *CDK2* and *CDK4* increased from passage 1 (P1) to P7, indicating ongoing cell cycle progression during passaging (Fig. 2K). Collectively, these findings

(See figure on next page.)

Fig. 2 Characterization of ProHPLCs. **A**. Bright field images of PHHs_QR12S cultured in NHEM at day 0, 3, 6, and 9. Scale bar, 100 μm **B**. IF staining of Ki67 in PHHs cultured in NHEM for 7 days. Scale bar, 100 μm. **C**. RT-qPCR analysis of the expression of hepatic progenitor-associated signature genes and cyclin-dependent kinase genes in NHEM-cultured PHHs for 9 days compared with fresh PHHs. n = 4. P values were determined by multiple t-tests. **D**. IF staining of hepatic progenitor-associated markers SOX9, CK19, TBX3 and ALB in PHHs cultured in NHEM for 9 days. Scale bar, 50 μm. **E**. Flow cytometric analysis of hepatic progenitor-associated protein expression of PHHs cultured in NHEM for 9 days. **F**. Transcriptome correlation analysis of PHHs cultured in NHEM (n = 6), hFLC (n = 2), and fresh PHHs (n = 4). **G**. Principal components analysis of PHHs cultured in NHEM (n = 6), hFLC (n = 2), and fresh PHHs (n = 4). **H**. Heatmap of hepatic progenitor gene expression in PHHs cultured in NHEM (n = 6), hFLC (n = 2), and fresh PHHs (n = 4). **I**. Representative images of ProHPLCs at passage 1 (P1), passage 3 (P3), passage 5 (P5), and passage 7 (P7) in NHEM. Scale bar, 50 μm. **J**. The growth curve of ProHPLCs at passage 7. n = 3. **K**. qRT-PCR analysis of the expression of *EPCAM, SOX9, CDK2,* and *CDK4* in ProHPLCs at P1, P4, and P7 in NHEM. n = 4. P values were determined by one-way ANOVA. All data were presented as mean ± SEM

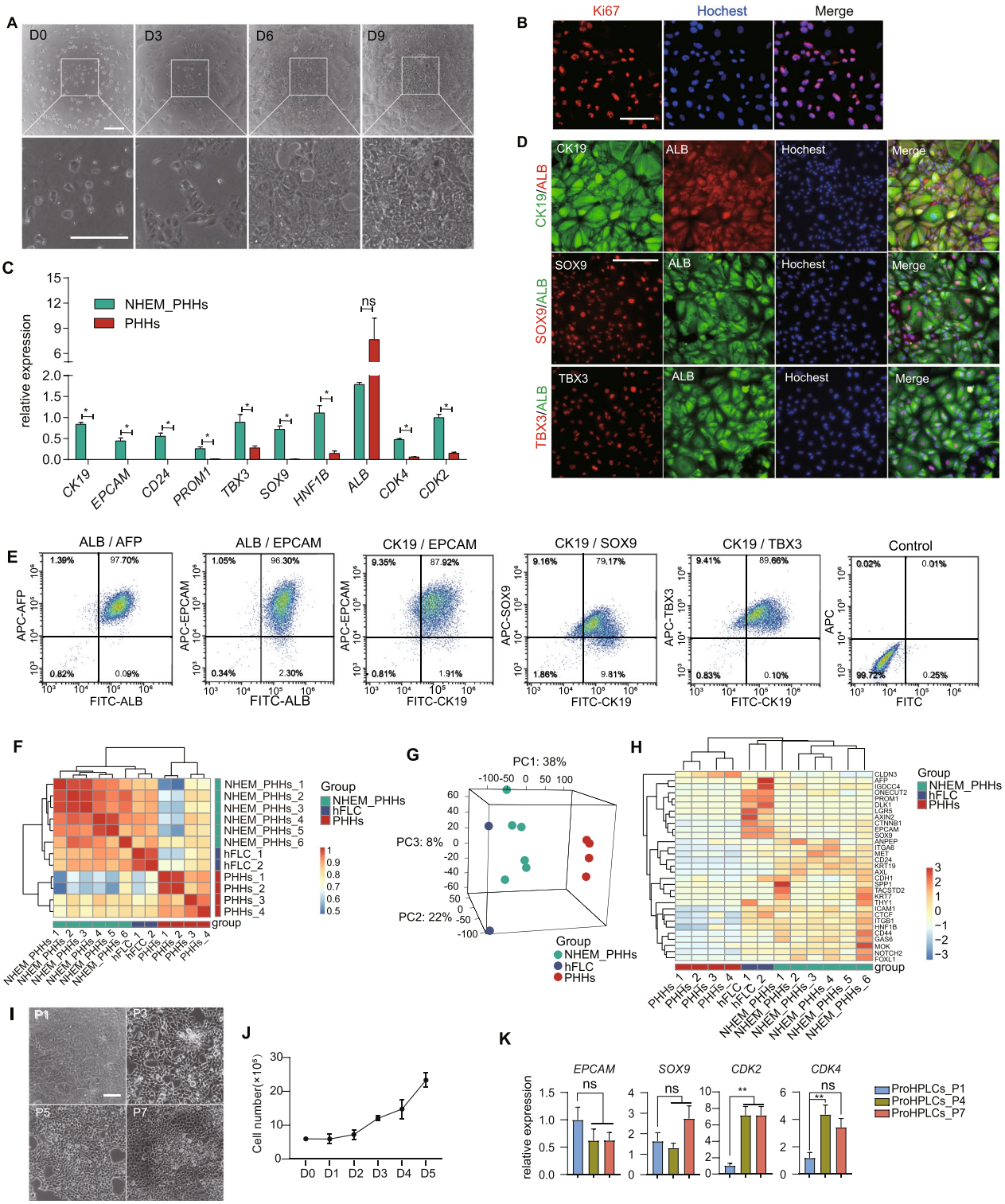


Fig. 2 (See legend on previous page.)

suggest that ProHPLCs not only retain their capacity for expansion but also preserve their fundamental progenitor characteristics during extended in vitro passaging.

Maturation of ProHPLCs with 6C

We have previously established a 5C medium for the robust long-term culture and function maintenance

of PHHs for more than 1 month without a de-differentiation/expansion stage. However, when tested for re-differentiating ProHPLCs into mature hepatocytes, 5C displayed the induction of cells (5C_HHs) with a compromised cellular morphology and gene expression profiles not fully comparable with those of PHHs (Fig. 3A and Fig. S3A). Therefore, we sought to optimize the 5C medium to better accommodate with ProHPLCs maturation. A previous study on induced hepatocyte maturation indicated that heparin would promote hepatocyte maturation [33]. By integrating heparin into the 5C medium, we created a modified medium named "6C". When culturing confluent ProHPLCs for an additional 7 days in 6C (6C_HHs), we found that the expression of a set of key hepatocyte function genes such as *CYP3A4, CYP2C9, CYP2B6, CYP2A6, UGT1A1, UGT1A3, NTCP* and *ASL* was boosted by the addition of heparin (Fig. S3B). Correspondingly, ALB secretion and urea synthesis levels were also enhanced in 6C_HHs compared with 5C_HHs (Fig. S3C), which is consistent with the upregulated expression of those involved genes. Together, these results suggest that we have developed a novel 6C maturation medium which could effectively differentiate ProHPLCs into mature human hepatocytes.

Previous study demonstrated that 5C cultured PHHs (5C_PHHs) are good alternatives for PHHs in hepatocyte-based application. To evaluate whether 6C_HHs have similar potential for application, we first investigated the characteristics of 6C_HHs regarding morphology and gene expression in comparison with 5C_PHHs and fresh PHHs. 5C_PHHs exhibited typical polygonal hepatocyte morphology but showed gradual cell death. In contrast, 6C_HHs, resuscitated from the same donor, displayed superior hepatocyte morphology with a distinct cellular border (Fig. 3A). IF staining showed that a high proportion of cells expressed key hepatocyte markers ALB, AAT, HNF4A, CEBPA, and PROX1 in 6C_HHs (Fig. 3B). Further comparative analysis of gene expression between 6C_HHs, 5C_PHHs, and fresh_PHHs revealed that 6C_HHs and PHHs shared similar or slightly higher expression levels of critical hepatocyte functional genes and TFs, including secreted proteins *ALB* and *AAT,* cytochrome P450 enzymes (*CYP3A4, CYP2C9, CYP2C19, CYP1A2, CYP2B6, CYP2D6* and *CYP2C8*), UDP glucuronosyltransferases (*UGT2B7, UGT2B15*), flavin-containing monooxygenase 3 (*FMO3*), monoamine oxidase A (*MAOA*), coagulation factors (*F2, F10, F12*), ammonia metabolism enzymes (*CPS1, GLUL*), the bile acid transporter *NTCP*, and key hepatic nuclear receptors and TFs (*HNF4A, PROX1, FXR,* and *RXR*) (Fig. 3C). In contrast, the 5C_PHHs exhibited a marked decline in the expression of *ALB*, *ARG1*, *CYP2D6* and *CYP2C8* after 7 to 14 days of culture (Fig. 3C).

We next examined whether the global gene expression pattern of 6C-HHs resembled that of PHHs by RNA-seq. PCA revealed that 6C-HHs were closer with PHHs than 5C_PHHs (Fig. 3D). Moreover, 6C-HHs expressed a set of hepatocyte genes involved in glucose metabolism, lipid cholesterol metabolism, fatty acid metabolism and drug metabolism in a pattern similar to that of PHHs (Fig. 3E). Overall, these results indicated that 6C-HHs possess a gene expression network of mature hepatocytes, closer to fresh PHHs than 5C_PHHs.

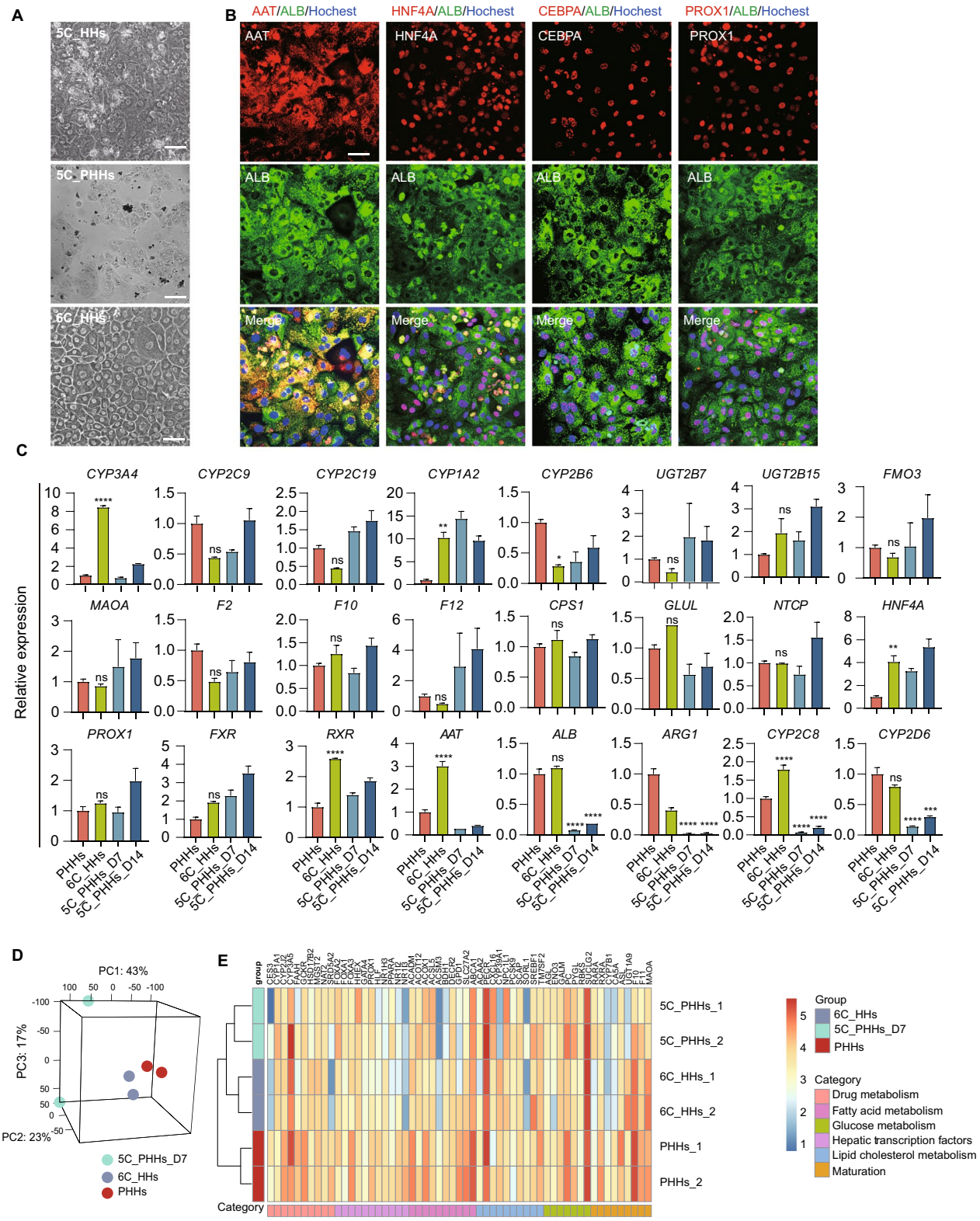
6C-HHs displayed hepatocyte function comparable to PHHs

We further evaluated the hepatocyte function of 6C-HHs. The accumulation of fat droplets and glycogen synthesis in 6C-HHs were observed by Oil Red O and Periodic Acid-Schiff (PAS) staining, respectively (Fig. 4A–B). Functional characterization of 6C_HHs showed that they were competent for low-density lipoprotein (LDL) uptake (Fig. 4C). CDCFDA staining indicated that 6C_HHs could form bile canaliculi, a polarized structure of hepatocytes (Fig. 4D). Next, we compared the biosynthesis function of 6C_HHs with 5C_PHHs, and found that 6C-HHs showed higher albumin secretion and urea synthesis compared to 5C_PHHs (Fig. 4E), which was consistent with the higher expression of *ALB* and *ARG1* expression in 6C-HHs than in 5C_PHHs (Fig. 3C).

Drug-metabolizing capacity is one of the most important features of mature human hepatocytes. Therefore, we sought to examine the drug-metabolizing ability of 6C-HHs. In accordance with mRNA expression (Fig. 3C), the protein expression of key CYP450s (CYP3A4, CYP1A2, CYP2C9, CYP2C19, CYP2C8 and CYP2E1) in 6C_HHs was confirmed by IF staining (Fig. 4F). We further performed liquid

(See figure on next page.)

Fig. 3 Maturation of ProHPLCs in 6C medium. **A**. Representative bright field images of 5C_HHs (ProHPLCs cultured in 5C medium for 7 days), 6C_HHs (ProHPLCs cultured in 6C medium for 7 days), and 5C_PHHs (PHHs cultured in 5C medium for 7 days). Scale bar, 100 μm. **B**. IF staining images of mature hepatocyte markers in 6C_HHs. Scale bar, 200 μm. **C**. qRT-PCR analysis of the expression of hepatocyte functional genes and TFs in PHHs, 6C_HHs, and 5C_PHHs cultured in 5C for 7 days (5C_PHHs_7D) and 14 days (5C_PHHs_14D). n = 3. P values were determined by one-way ANOVA (compared with PHHs). **D**. Principal components analysis of global genes in 6C_HHs (n = 2), 5C_PHHs (n = 2) and PHHs (n = 2). **E**. Heatmap of hepatocyte functional gene sets expression in 6C_HHs (n = 2), 5C_PHHs (n = 2) and PHHs (n = 2). All data were presented as mean ± SEM



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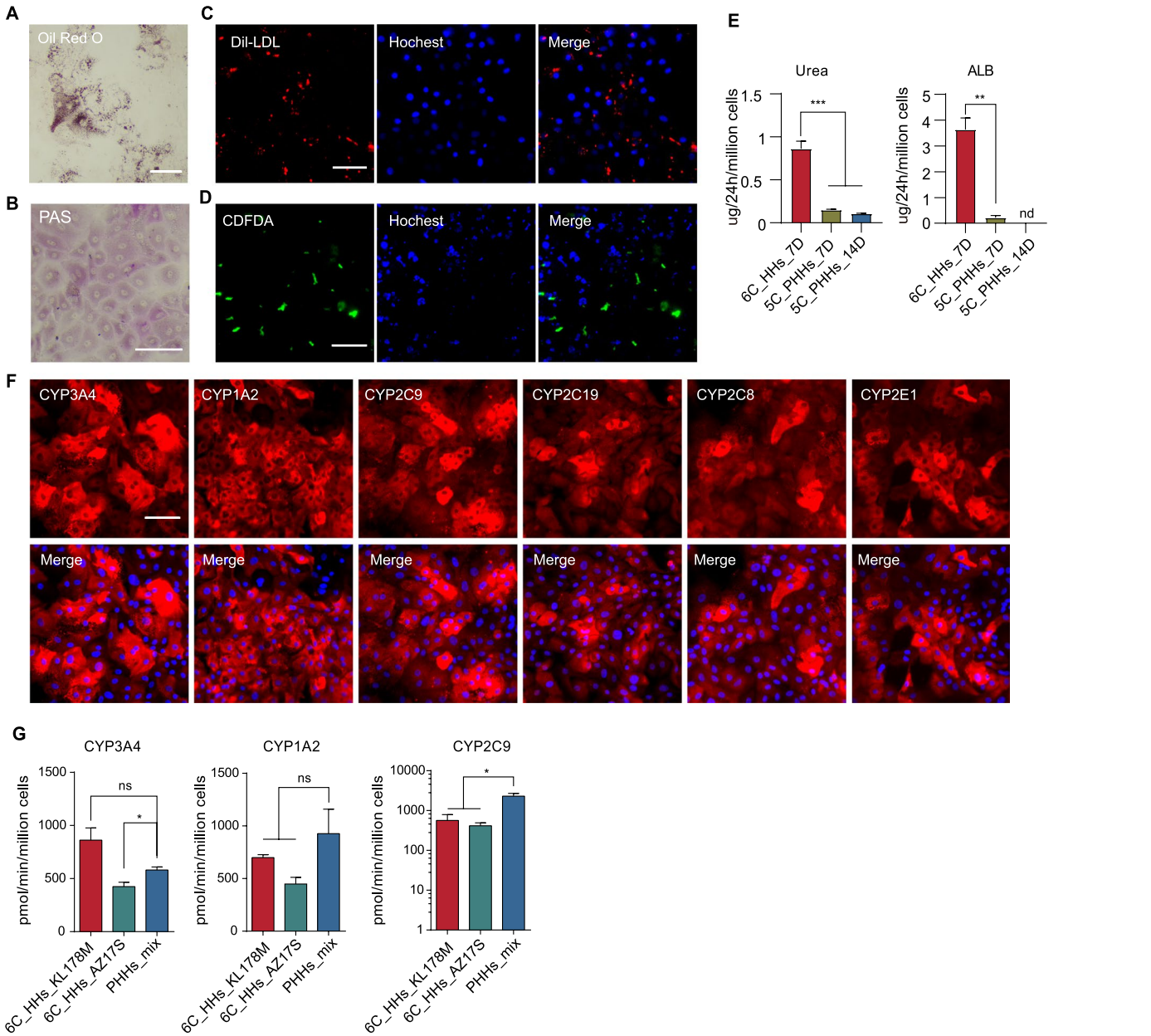

Fig. 4 Functional characterization of 6C_HHs. **A**. Lipid accumulation analysis in 6C_ HHs by oil red O staining. Scale bar, 50 μm **B**. Glycogen storage analysis in 6C_HHs by periodic acid- Schiff staining (PAS). Scale bar, 50 μm. **C**. Dil-LDL uptake analysis in 6C_HHs. Scale bar,100 μm. **D**. CDFDA staining images of 6C_HHs. Scale bar, 100 μm. **E**. The albumin secretion and urea biosynthesis in 6C_HHs (n = 7) and 5C_PHHs cultured for 7 days (n = 3) and 14 days (n = 3), respectively. P values were determined by one-way ANOVA. **F**. IF staining of major CYP450 proteins in 6C_HHs cultured in 6C for 7 days. Scale bar, 50 μm. **G**. LC–MS/MS analysis of drug-metabolizing activities of core CYP450 enzymes in 6C_HHs_KL178M, 6C_HHs_AZ17S and PHHs_mix (1:1 mixture of PHHs_KL178M and PHHs_AZ17S) (n = 3 for 6C_HHs, n = 6 for PHHs). P values were determined by one-way ANOVA. All data were presented as mean ± SEM

chromatography tandem mass spectrometry (LC/MS/MS) to analyze the drug-metabolizing activity of 3 major CYP450s (CYP3A4, CYP1A2, and CYP2C9), which are responsible for most of CYP450-metabolized commercial drugs. The results indicated that the drug-metabolizing activity of CYP3A4 and CYP1A2 was comparable between 6C_HHs and PHHs, whereas the CYP2C9 activity was slightly reduced in 6C_HHs compared to PHHs. (Fig. 4G).

ProHPLCs and 6C-HHs repopulated mouse liver efficiently after transplantation

We further transplanted ProHPLCs and 6C-HHs into URG (Tet-uPA/Rag2−/−/γc−/−) liver injury mouse model to assess their ability to engraft in vivo. Human ALB secretion in the serum of these mice was monitored from weeks 5 to 8 post-transplantation. The levels of human ALB secretion in mice transplanted with ProHPLCs and 6C-HHs were similar throughout the study period and marginally lower compared to those transplanted with PHHs (Fig. 5A). To evaluate engraftment efficiency more directly, we counted the number of human ALB-positive cells using IF staining at 8 weeks post-transplantation. The engraftment efficiency was comparable among the three cell types with 36.2% ± 1.9% for 6C-HHs, 31.6% ± 0.96% for ProHPLCs, and 36.8% ± 4.16% for PHHs (Fig. 5B–C), demonstrating that ProHPLCs and 6C-HHs can engraft and repopulate the mouse liver as effectively as PHHs, which are considered the gold standard cell source for transplantation.

We next examined whether the transplanted cells could function within the mouse liver at 8 weeks post-transplantation. IF staining revealed that livers transplanted with ProHPLCs and 6C-HHs expressed a range of functional hepatocyte markers, such as AAT, CYP2A3, CYP1A2, CYP2D6, CYP2C9 and HNF4A (Fig. 5D–E). Moreover, most ProHPLCs lost expression of the progenitor marker EPCAM after transplantation (Fig. 5D). These results indicated the in vivo maturation of ProHPLCs and the functioning of matured ProHPLCs and 6C-HHs after transplantation. Interestingly, despite ProHPLCs, 6C-HHs, and PHHs originating from the same liver donor, the variability in human ALB secretion and engraftment efficiency was greater in mice transplanted with PHHs (Fig. 5A–B). This suggests that ProHPLCs and 6C-HHs represent more stable and reliable cell sources than PHHs for transplantation.

Mechanisms underlying hydrocortisone-promoted hepatocyte expansion

Hydrocortisone is a glucocorticoid that has been reported to induce mouse liver enlargement and promote human hepatocyte engraftment in injured mouse livers [34, 35]; however, its role in PHHs expansion in vitro remains unclear. We found that other glucocorticoids like dexamethasone and meprednisone exhibited similar effects to hydrocortisone on PHHs expansion (Fig. 6A), suggesting hydrocortisone may work through classic glucocorticoid receptors (GR) to boost human hepatocyte expansion. To explore the underlying mechanism, we analyzed the differentially expressed genes between PHHs cultured in BHEM with or without hydrocortisone. We identified 103 genes that were upregulated in PHHs cultured in BHEM with hydrocortisone (NHEM) (Fig. 6B–C). KEGG analysis highlighted the upregulation of pathways related to hepatocyte metabolism, biosynthesis, and the PPAR signaling pathway following hydrocortisone treatment (Fig. 6D). GSEA also confirmed the upregulation of the PPAR signaling pathway (Fig. 6E).

The PPAR signaling pathway has been identified as a target of glucocorticoids [36, 37] and is associated with hepatocyte proliferation during mouse liver regeneration [38–40]. To investigate whether the PPAR signaling pathway acts downstream of hydrocortisone, we examined the effects of PPARα and PPARγ agonists or antagonists on PHHs expansion in the presence or absence of hydrocortisone. Initially, we blocked PPARα and PPARγ signaling pathways by introducing the corresponding antagonists, GW6471 and GW9662, respectively, to PHHs cultured in NHEM. The PPARα antagonist GW6471 significantly inhibited hepatocyte expansion, whereas the PPARγ antagonist GW9662 had minimal inhibitory effects (Fig. 6F). Subsequently, we evaluated the impact of PPARα agonists Fenofibrate and GW7647, along with the PPARγ agonist troglitazone, in BHEM which did not contain hydrocortisone. Our findings indicated that Fenofibrate and GW7647 promoted hepatocyte expansion to a level comparable to hydrocortisone, while the PPARγ agonist did not exhibit a similar promotional effect on PHHs expansion (Fig. 6G). We also conducted additional testing of the PPARα antagonist (GW6471) and agonists (Fenofibrate and GW7647) using hepatocytes from another donor. Our findings revealed that PPARα agonists significantly enhanced the expansion of primary human hepatocytes (PHHs) in terms of cell confluence, cell viability, and Ki67 immunofluorescence staining, while the antagonist had the opposite effect, inhibiting PHHs expansion (Fig. S4A-C). Furthermore, our gene expression analysis demonstrated that PPARα agonists activated downstream genes such as *APOA1, APOA2, ACOX1, CYP7A1, CYP8B1, CYP2C9*, and *CYP2B6*, as well as proliferation-related markers *CDK4* and *PCNA* (Fig. S4D). Additionally, they down-regulated the cellular senescence marker *P21* (Fig. S4D). Conversely, the PPARα antagonist exhibited an opposite pattern, down-regulating the aforementioned genes while up-regulating *P21* (Fig. S4D). These results indicated that PPARα acts as the main PPAR isoform downstream of the hydrocortisone in promoting hepatocyte expansion. To further confirm the role of PPARα as a downstream factor of hydrocortisone, we carried out PPARα knockdown experiments in PHHs cultured in NHEM, and observed a reduction in hepatocyte expansion in the PPARα knockdown group (Fig. 6H). Collectively, these findings provide strong evidence that PPARα functions as a downstream mediator of hydrocortisone-induced PHHs expansion.

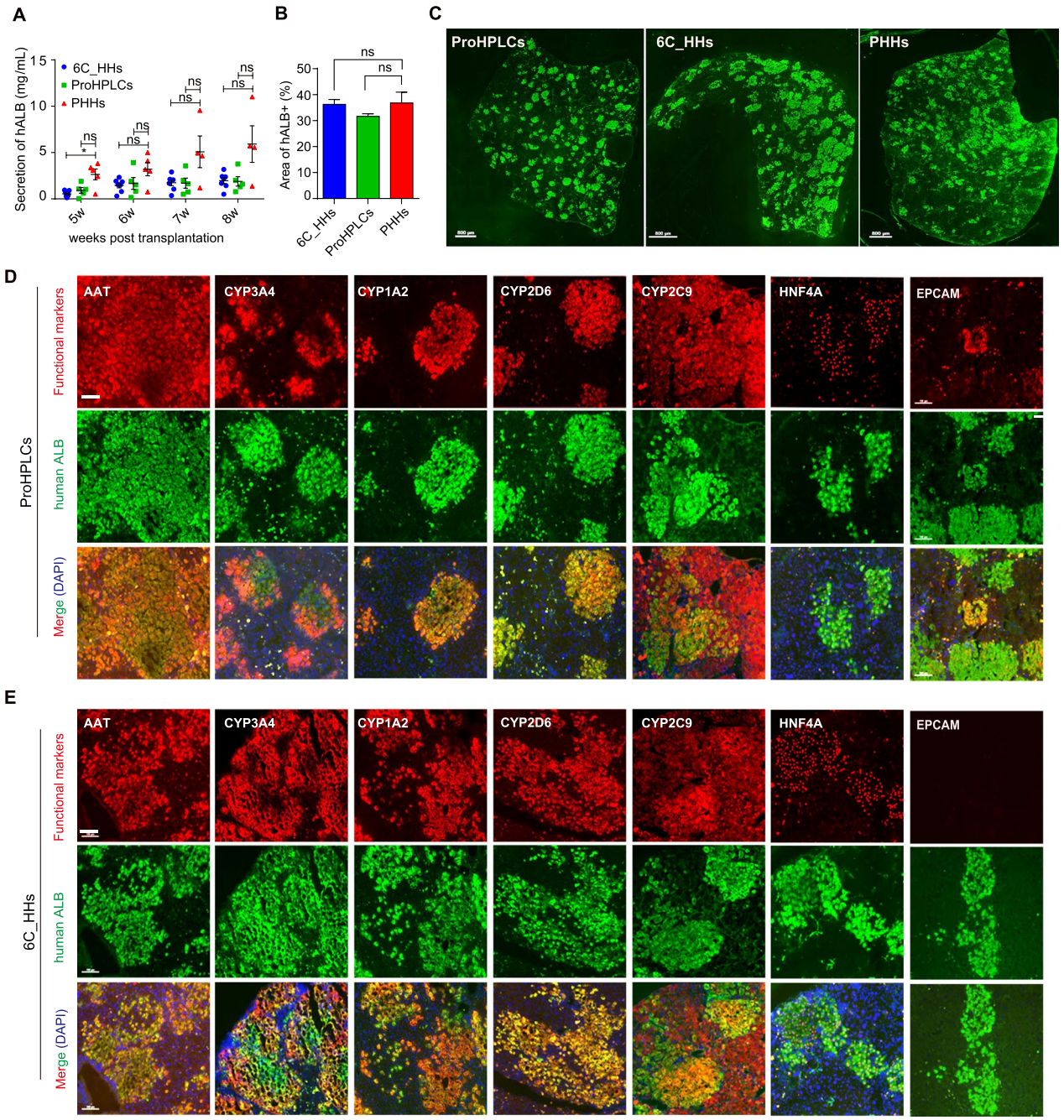

Fig. 5 Characterization of cells transplanted into URG mouse livers. **A**. Secretion of human ALB in mouse serum from 5 to 8 weeks post-transplantation in mice transplanted with 6C_HHs (n = 7), ProHPLCs (n = 5), and PHHs (n = 5 for 5w and 6w, n = 4 for 7w and 8w). P values were determined by multiple t-tests. **B**. Engraftment rate of transplanted 6C_HHs, ProHPLCs, and PHHs measured by percentage of hALB-positive cells in mouse livers at 8 weeks post-transplantation. Around 3–6 sections of each mouse liver were chosen for analysis, and a total of 26 sections were chosen for each cell type. n = 26. P values were determined by one-way ANOVA. **C**. Representative IF images of whole liver of mice transplanted with 6C_HHs, ProHPLCs, and PHHs at 8 weeks post-transplantation. Scale bar, 800 μm. **D**–**E**. IF staining of functional hepatocyte markers AAT, CYP3A4, CYP1A2, CYP2D6 and CYP2C9, a key hepatocyte TF HNF4A, and hepatic progenitor marker EPCAM in mouse livers transplanted with ProHPLCs (D) and 6C_HHs (E) at 8 weeks post-transplantation, Scale bar, 100 μm

We have posited that SiPer could potentially identify small molecule compounds capable of activating the 9 regenerative TFs to facilitate the de-differentiation and expansion of PHHs. In line with this, we conducted an analysis to determine if hydrocortisone could enhance the expression of these TFs. Our qRT-PCR results demonstrated a significant increase in the expression of FOS, EPAS1, EGR1, and XBP1 upon treatment with hydrocortisone and PPARα agonists (Fig. 6I, and Fig. S4D). Collectively, these findings suggest that hydrocortisone may activate the PPARα signaling pathway to elevate the expression of regenerative TF networks, thereby promoting the de-differentiation and expansion of PHHs.

Discussion

In this research, we introduced a novel two-step strategy focused on the de-differentiation/expansion and subsequent maturation process to produce a significant quantity of functional human hepatocytes from primary human hepatocytes (PHHs) in vitro. These PHHs can robustly de-differentiate into ProHPLCs and expand by approximately 16,000 folds in 30 days, and then re-differentiate into functionally mature hepatocytes with metabolic and regenerative capacities comparable to those of PHHs. This presents a promising strategy to generate large quantities of human hepatocytes for use in drug screening, disease modeling, and cellular transplantation.

We have developed a unique hepatic expansion medium, named NHEM, tailored for the de-dedifferentiation and expansion of PHHs. Initially, by excluding NICO, LPA, and S1P, and incorporating HGF, we enhanced the effect of our previously established HEM on PHHs expansion. Utilizing SiPer, we identified the pivotal role of hydrocortisone and subsequently developed the new hepatic expansion medium NHEM, which outperforms existing media in both efficiency and stability for hepatocyte expansion (Fig. 1H–J). Importantly, NHEM is serum-free, making it a more reliable choice for minimizing the variability between PHH donor batches and facilitating stable, large-scale expansion. The robustness of NHEM has been consistently demonstrated across PHHs from 8 adult hepatocyte donors as well as 2 pediatric donors (Table S1; Fig. 2F and Fig. 6B). A recent study highlighted the potential of treating liver failure using encapsulated progenitor-like proliferating human hepatocyte organoids derived from pediatric donors [15]. Likewise, employing a similar encapsulation technique with NHEM-cultured ProHPLCs holds considerable promise for treating liver diseases.

To generate functional hepatocytes from ProHPLCs, we formulated a novel 6C medium that includes the addition of heparin to the existing 5C formula. 6C_HHs displayed a more authentic hepatocyte identity, characterized by higher levels of a range of key hepatocyte function genes such as *ALB, CYP450s, UGTs, NTCP, NAGS* and *ASL*, and enhanced albumin secretion and urea synthesis compared with their 5C_HH counterparts (Fig. S3B-C). Furthermore, 6C_HHs demonstrated a global gene expression pattern, drug-metabolizing activities and in vivo engraftment rate that were comparable to that of PHHs. A previous study demonstrated that 5C cultured PHHs (5C_PHHs) showed good hepatocyte identity maintenance over the long-term. However, culturing 5C_PHHs requires high initial cellular viability and confluency, which are difficult to achieve in most cases, thereby resulting in gradual cell death and loss of function (Fig. 3A, 3C–E and Fig. 4E). In contrast, 6C_HHs showed superior hepatocyte identity in terms of morphology and gene expression compared to 5C_PHHs. One possible explanation is that the proliferative progenitor-like state of ProHPLCs before 6C treatment ensures the initial cell viability and confluency, contributing to the better hepatocyte identity of 6C_HHs compared to 5C_PHHs. Collectively, our two-step strategy generates functionally mature 6C_HHs that can serve as an alternative to PHHs or 5C_PHHs for hepatocyte application.

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Fig. 6 Hydrocortisone promotes human hepatocytes proliferation via PPARα signaling pathway. **A**. Cell viability of PHHs cultured with different glucocorticoids based on BHEM for 6 days measured by CCK8 analysis. n = 3. P values were determined by one-way ANOVA. **B**. Heatmap depicting the top 50 differentially expressed genes in PHHs treated with or without hydrocortisone based on BHEM for 9 days. **C**. Volcano plot displaying differentially expressed genes in PHHs treated with or without hydrocortisone based on BHEM for 9 days. The horizontal dashed line represents an adjusted P value of 0.05, while the vertical dashed lines are fold changes of -1 and 1. **D**. KEGG analysis of activated signaling pathways in PHHs treated with or without hydrocortisone based on BHEM for 9 days. **E**. GSEA of PPARα signaling pathway in PHHs treated with or without hydrocortisone based on BHEM for 9 days. **F**. Cell viability of PHHs cultured in NHEM, and NHEM with PPARγ inhibitor GW9662, PPARγ agonist troglitazone, PPARα inhibitor GW6471, and PPARα agonists fenofibrate and GW7647 from day 2 to day 12 measured by CCK8 analysis. n = 2. P values were determined by two-way ANOVA. **G**. Cell viability of PHHs cultured in BHEM, BHEM with PPARγ inhibitor GW9662, PPARγ agonist troglitazone, PPARα inhibitor GW6471, and PPARα agonists fenofibrate and GW7647 from day 2 to day 12 by CCK8 analysis. n = 2. P values were determined by two-way ANOVA. **H**. Cell viability analysis of PPARα knockdown in PHHs cultured in NHEM for 10 days measured by CCK8 analysis. n = 3. P values determined by two-way ANOVA. **I**. Gene expression of regenerative TFs in PHHs cultured in BHEM with and without hydrocortisone. n = 3. P values were determined by one-way ANOVA. All data were presented as mean ± SEM

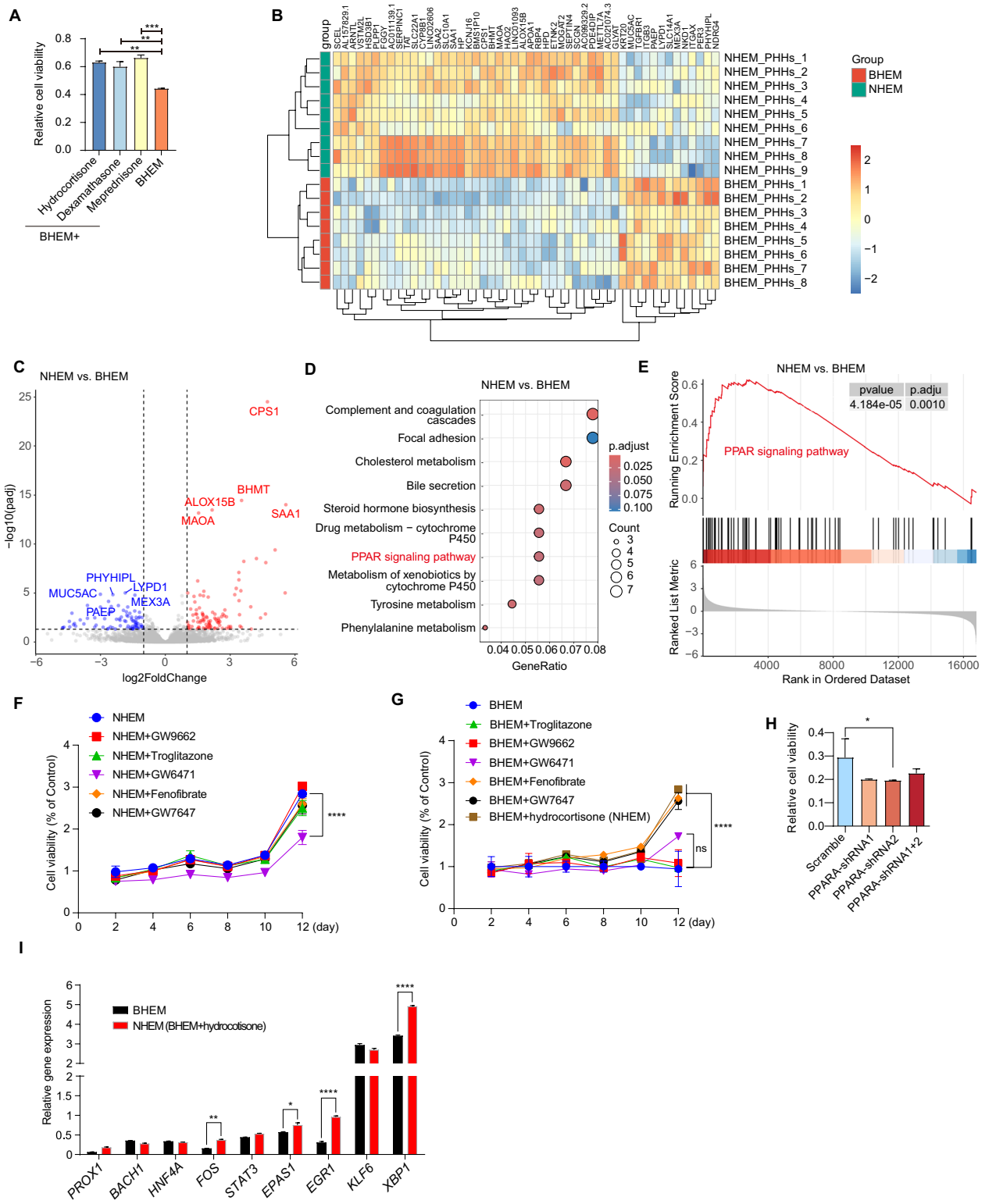

Fig. 6 (See legend on previous page.)

The study highlights a significant advance in understanding the mechanisms underlying hepatocyte expansion through regenerative TFs. Regenerative TFs play a fundamental role to repair the liver upon injuries. However, the intricacies of the TF regulatory network guiding liver regeneration remain largely unexplored, particularly in humans. Through the identification of potential regenerative TFs from mouse data and SiPer prediction, we have pinpointed XBP1, EGR1, EPAS1 and FOS as key regenerative TFs that may play pivotal roles in the de-differentiation/expansion of PHHs in vitro. (Fig. 4I–J). Notably, we identified hydrocortisone for the activation of these regenerative TFs and PPARα signaling pathway may act as the key mediator. This is consistent with studies that hydrocortisone acts through GR to stimulate PPARα expression in rat hepatocytes [36], and PPARα activators induce DNA synthesis in primary cultures of rat hepatocytes [37]. Our findings of the activation of PPARα signaling pathway and regenerative TFs by hydrocortisone could pave the way for innovative treatments for liver injuries, offering new avenues for therapeutic interventions.

Conclusion

In conclusion, our novel two-step system of de-differentiation/expansion followed by maturation of PHHs offers a strategic approach for producing large quantities of hepatocytes in vitro. This approach holds significant potential for a range of applications involving hepatocytes, both in vitro and in vivo. Additionally, the discovery of hydrocortisone, PPARα and regenerative TFs as key factors in promoting PHHs expansion may provide new insights into the liver regeneration regulatory mechanism, which is beneficial for liver disease treatment.

Abbreviations

PHHs	Primary human hepatocytes
ProHPLCs	Proliferative human hepatic progenitor-like cells
6C	6-Chemical
5C	5-Chemical
6C_HHs	6C cultured human hepatocytes
5C_PHHs	5C cultured primary human hepatocytes
HEM	Hepatocyte expansion medium
NHEM	New hepatocyte expansion medium
BHEM	Basic hepatocyte expansion medium
SiPer	A single cell-based computational platform
IF	Immunoflurorescence
LDL	Low-density lipoprotein
PAS	Periodic acid-schiff
CDFDA	5(6)-Carboxyfluorescein diacetate
PHx	Partial hepatectomy
APAP	Acetaminophen
RNA-seq	RNA sequencing
TFs	Transcription factors
UGT	UDP-glucuronosyltransferase
HCM	Hepatocyte culture medium
GO	Gene ontology
GSEA	Gene set enrichment analysis
KEGG	Kyoto encyclopedia of genes and genomes
PCA	Principal component analysis
URG	Tet-upa/Rag2–/–/γc–/–
EMT	Epithelial-mesenchymal transition
CEPT	Chroman 1, emricasan, trans-ISRIB and polyamine solution
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03911-0.

Additional file 1: Supplementary Materials and Methods

Additional file 2: Supplementary Figures and legends

Additional file 3: Supplementary Tables

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Author contributions

Huangfan Xie, Guangya Li and Yunxi Fu designed, performed the experiments, interpreted the results, and wrote the manuscript. Nan Jiang performed RNA-seq data analysis. Simeng Yi and Xi Kong performed experiments for hepatocyte function analysis. Jihang Shi conducted the isolation of PHHs from liver tissues. Shigang Yin, Jianhua Peng, and Yong Jiang assisted the research design and manuscript writing. Shichun Lu provided liver tissues and designed the research. Hongkui Deng supervised this project and designed the research. Bingqing Xie supervised this project, designed the research, interpreted the results, and wrote the manuscript.

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Availability of data and materials

All data are available in the Article and its Supplementary Information. The next-generation sequencing datasets have been deposited to the Gene Expression Omnibus (GEO) with the accession GSE261962 and GSE261965. The public datasets we used can be accessed at NCBI GEO database under accession numbers GSE112330 (PHHs, hFLCs).

Declarations

Ethical approval and consent to participate

All research using Tet-uPA/Rag2–/–/γc–/– (URG) mice was approved by IACUC Committee of Beijing Vitalstar Biotechnology Co., Ltd, titled "Planning research and experimental procedures on animals" (Ethical approval No.: VST-SY-2023101501, approval date: 28 Oct 2023). All research using primary human hepatocytes was approved by the Research Ethics Committee of Chinese PLA General Hospital (Ethical approval No.: S2020-137-01, renewed on 30 April 2020) with informed written consent, titled "Research on obtaining primary human hepatocytes from discarded human liver tissue after surgical resection".

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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