TISSUE ENGINEERING

Long-term functional maintenance of primary human hepatocytes in vitro

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The maintenance of terminally differentiated cells, especially hepatocytes, in vitro has proven challenging. Here we demonstrated the long-term in vitro maintenance of primary human hepatocytes (PHHs) by modulating cell signaling pathways with a combination of five chemicals (5C). 5C-cultured PHHs showed global gene expression profiles and hepatocyte-specific functions resembling those of freshly isolated counterparts. Furthermore, these cells efficiently recapitulated the entire course of hepatitis B virus (HBV) infection over 4 weeks with the production of infectious viral particles and formation of HBV covalently closed circular DNA. Our study demonstrates that, with a chemical approach, functional maintenance of PHHs supports long-term HBV infection in vitro, providing an efficient platform for investigating HBV cell biology and antiviral drug screening.

Terminally differentiated cells are functionally stable in vivo, a state that is highly dependent on precise spatiotemporal regulation by microenvironmental signals. Hence, isolated primary adult cells are commonly observed to lose their core gene expression signatures and specific functions, resulting in a demand for long-term stabilization of cell identity and functionality of cells, such as primary human hepatocytes (PHHs) (*1*). PHHs are considered ideal for drug-metabolism evaluation and modeling hepatotropic infectious diseases, including HBV infection; however, their long-term utility is restricted by limited viability and unstable functional maintenance in vitro (*1*–*3*). Considering that long-term maintenance of cell function requires a sophisticated signaling regulatory network, to maintain PHHs in vitro for an extended period of time, we developed a chemical strategy using small-molecule combinations, which confers the advantage of synergistically orchestrating innate signals to achieve spatiotemporal modulations of specific cellular targets (*4*).

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First, we compared the transcriptome profiles of plateable freshly isolated PHHs (F-PHHs) with those of 24-hour cultured hepatocytes (24h-PHHs). We found that 24h-PHHs showed a down-regulation of 15 important hepatocyte-functional genes by an average of 10-fold and an up-regulation of major components of the TGF-β signaling pathway, epithelial-mesenchymal transition (EMT) inducers causing PHH dysfunctions (fig. S1) (*5*). Therefore, we used transforming growth factor β (TGF-β) inhibitor SB431542 (SB43) to block TGF-β signaling and observed an epithelial morphology with E-cadherin expression in PHHs after a 2-week culture (fig. S2A). However, SB43 treatment failed to suppress the expression of EMT-associated transcription factors and to rescue hepatocyte-functional gene expression (fig. S2B), indicating that blocking TGF-β signaling was insufficient to maintain PHHs.

Next, we tested a pool of small molecules and found that forskolin (FSK), an adenylate cyclase (AC) activator, down-regulated EMT marker gene expression and sustained hepatocyte-functional gene expression (fig. S3). When combined with SB43, FSK-SB43 showed synergistic effects on hepatocyte maintenance (fig. S4). On the basis of FSK-SB43, we identified DAPT (Notch inhibitor), IWP2 (Wnt inhibitor), and LDN193189 (BMP inhibitor) to further block the expression of EMT marker genes and support hepatocyte-functional gene expression (Fig. 1A and figs. S5, A to C, and S6). This minimal condition, consisting of the five chemicals (5C) FSK, SB43, DAPT, IWP2 and LDN193189, effectively supported the maintenance of PHHs, which showed polarization and expressed surrogate hepatocyte-functional markers after a 4-week culture (Fig. 1, B and C, and figs. S5D and S7). Albumin secretion and urea synthesis in 5C-cultured PHHs (5C-PHHs) were also maintained on nine non-preselected batches of PHHs for at least 3 weeks (Fig. 1D and figs. S8 and S9). PHHs with high viability could be functionally maintained for 2 months under the 5C condition (fig. S9).

We then performed RNA sequencing analysis to compare the transcriptome of 5C-PHHs and PHHs cultured in a sandwich configuration, an often-used hepatocyte culture condition (*1*). After a 4-week culture, only 5C-PHHs were clustered with F-PHHs and human liver tissues, whereas sandwich-cultured PHHs were clustered with Null and DMSO (dimethyl sulfoxide) groups, a result that was reproducible on four independent batches of PHHs (Fig. 1E and fig. S10). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) confirmed that the 5C condition effectively supported the expression of hepatocyte-functional genes and key transcription factors while suppressing mesenchymal marker gene expression (fig. S11). These data indicated that the 5C condition maintained the global gene expression pattern of human hepatocytes over the long term in vitro.

Hepatocytes are crucial for metabolizing and detoxifying exogenous drugs; however, the metabolizing activities of cytochrome P450 (CYP) of hepatocytes are rapidly lost during culture (fig. S1A) (*1*). RNA sequencing and qRT-PCR analysis revealed that long-term cultured 5C-PHHs efficiently maintained the expression of phase I/II enzymes and phase III transporters (figs. S12 and S13). We then applied liquid chromatography–mass spectrometry (LC-MS) to compare metabolizing activities of key CYP enzymes of 5C-PHHs and F-PHHs, including CYP3A4, CYP1A2, CYP2C9, CYP2D6, and CYP2B6, which are responsible for 80 to 90% of clinical drug metabolism (*6*). The metabolizing activities of 2-week-cultured 5C-PHHs were comparable to those of F-PHHs (Fig. 1F and fig. S14).

Long-term functional maintenance of PHH by 5C permitted persistent expression of *NTCP*, which encodes a functional hepatocyte receptor for HBV, suggesting the potential for modeling HBV infection in vitro (Fig. 2A) (*7*). By inoculation with HBV-infected patient plasma, HBV infection in 5C-PHHs was successfully achieved, with consistently secreted HBV products (HBsAg, HBeAg, and DNA) detected in the supernatant and expression of intracellular HBV RNA for at least 4 weeks (Fig. 2, B and E). Notably, the formation of HBV covalently closed circular DNA (cccDNA) was detectable by Southern blot and qPCR in 5C-PHHs for ~4 weeks after infection (Fig. 2, F and G). HBV infection in 5C-PHHs was reproduced on more than five different batches of PHHs with various sources of HBV, even when the multiplicity of infection (MOI) was reduced to 10 (figs. S15 to S18 and tables S5 to S7). Notably, PHHs that had been cultured in 5C for 3 to 4 weeks could still support HBV infection (fig. S19). Collectively, these data suggested that 5C-PHHs provide an efficient, long-term HBV infection system.

Next, we harvested and enriched HBV particles from the supernatant of HBV-infected 5C-PHHs, which successfully infected naïve PHHs indicated by the production of HBeAg and HBV DNA in newly infected PHHs (Fig. 2, H and I).

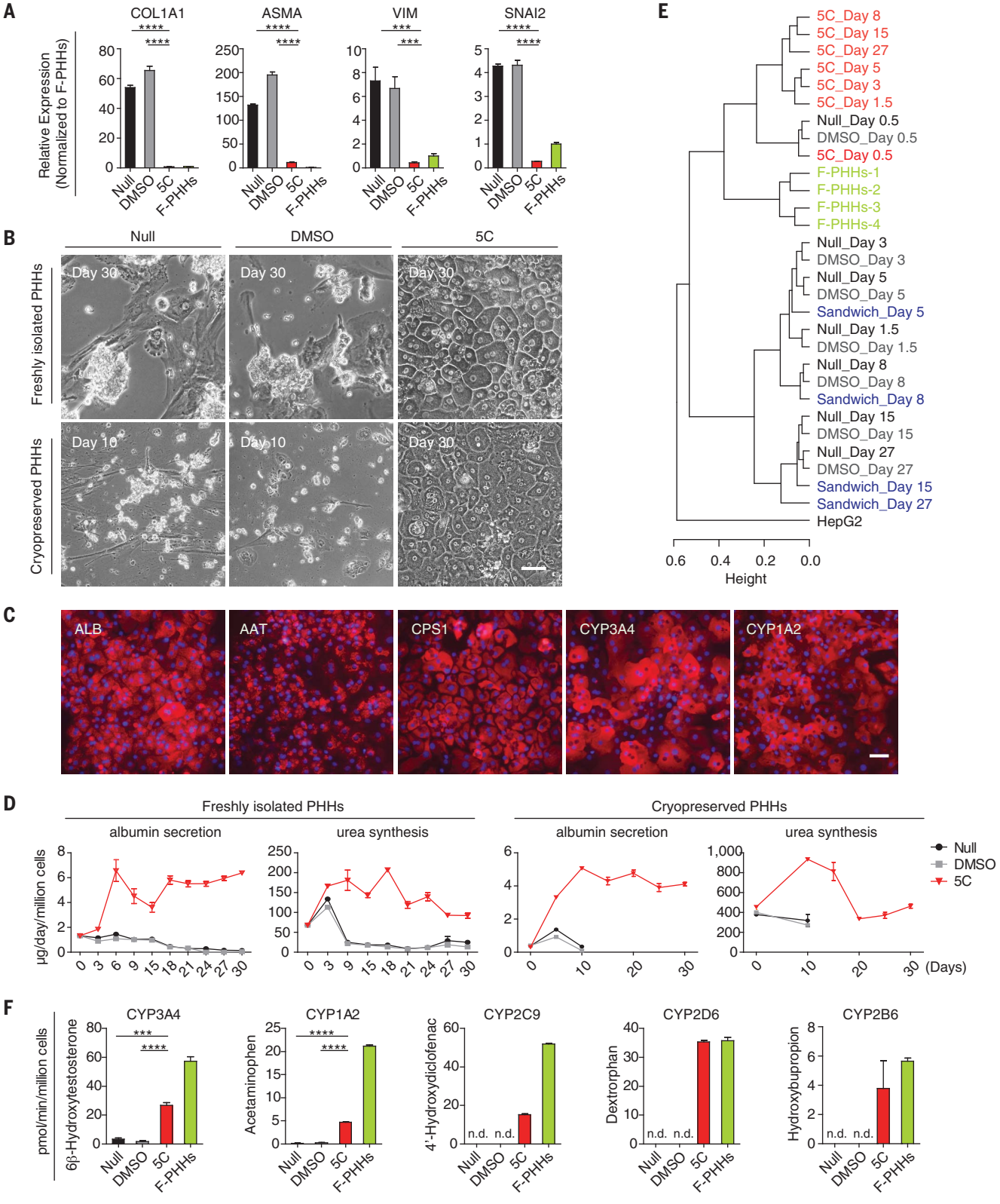

Fig. 1. The 5C culture condition enables long-term maintenance of PHHs. (**A**) qRT-PCR analysis of mesenchymal markers of cryopreserved PHHs (Cryo-PHHs) cultured in hepatocyte culture medium (Null), vehicle control condition (DMSO), and the 5C condition for 2 weeks. $n \geq 3$ biological replicates. (**B**) Representative images of cultured PHHs. (**C**) Immunofluorescence of hepatocyte-functional markers in 4-week cultured 5C-PHHs. (**D**) Analysis of albumin secretion and urea synthesis of cultured PHHs. $n = 3$. Detection in Cryo-PHHs ended at day 10 in control groups because of severe cell death. (**E**) Hierarchical clustering for global gene expression profiles of cultured PHHs. (**F**) Drug-metabolizing activities of five core CYP450 enzymes as detected by LC-MS. $n = 3$. n.d., not detected. Scale bar, 50 μm.

The results suggested that 5C-PHHs could support the entire HBV life cycle to generate infectious HBV particles. Additionally, HBeAg levels and HBcAg–positive cell proportions were significantly decreased when neutralizing antibody was added to 5C-PHHs after HBV infection, suggesting HBV spread in a polyethylene glycol 8000 (PEG)–independent manner in 5C-PHHs (Fig. 2, J and K, and fig. S20).

The 5C-PHHs infection model was sensitive to clinical anti-HBV drugs interferon-α (IFN-α) and nucleoside reverse transcriptase inhibitors entecavir and lamivudine (fig. S21) (*3*). Furthermore, in 5C-PHHs, expression of interferon-stimulated genes (ISGs) was significantly up-regulated by IFN-α treatment, but not by HBV infection, consistent with previous reports of HBV evasion from innate immunity of hepatocytes (fig. S22) (*8*). Additionally, when compared with other reported PHHs culture conditions, 5C showed advantages in the long-term maintenance of hepatocyte functions and the stable support of HBV infection (figs. S23 and S24) (*2*, *9*, *10*). Moreover, the 5C condition could be downscaled to the microwell format, amenable to high-throughput applications (fig. S25). The main results of the 5C condition in supporting long-term hepatocyte maintenance and HBV infection were also efficiently repeated with an additional four batches of commercial cryopreserved human hepatocytes (fig. S26 and table S5).

Aside from HBV infection, we exposed 5C-PHHs to infectious HCV particles Jc1G and observed viral replication in 5C-PHHs, which could be effectively blocked by daclatasvir (fig. S27, A and B) (*11*). Unlike HBV, HCV induced robust

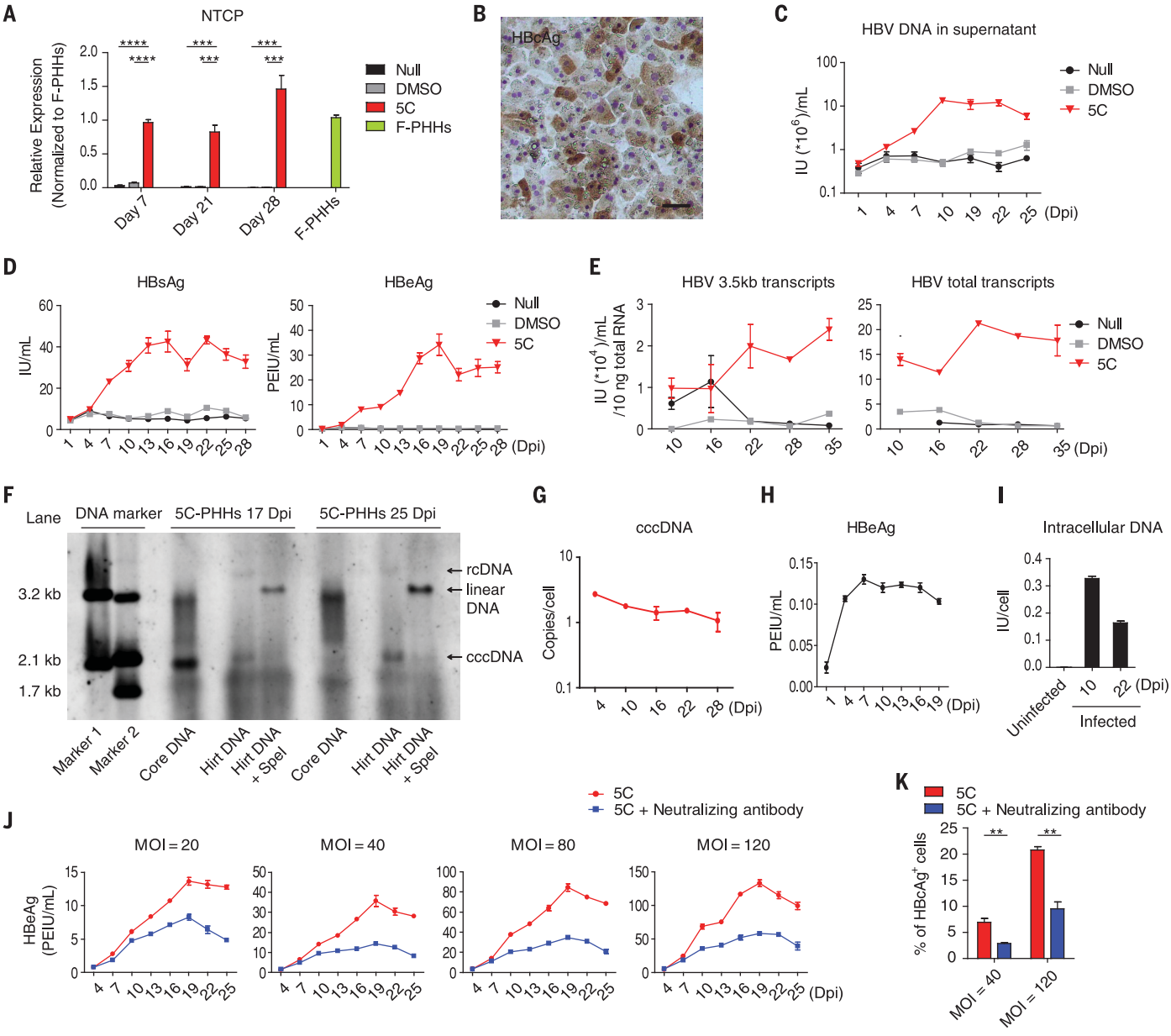

Fig. 2. 5C-PHH–based HBV infection model. (**A**) Gene expression analysis of *NTCP*. *n* = 3. (**B**) Immunostaining of HBcAg in HBV-infected 5C-PHHs at 28 days postinfection (Dpi). Scale bar, 100 μm. (**C** to **E**) The formation of secreted HBV-DNA (*n* = 3) (C), HBV antigens (*n* = 3) (D) and HBV-RNA (*n* = 2) (E) in infected PHHs cultured under different conditions. (**F** and **G**) HBV cccDNA detected in infected 5C-PHHs by Southern blot (F) and qPCR (G). (**H** and **I**) Detection of HBeAg (*n* = 3) (H) and intracellular HBV DNA (*n* = 2) (I) in PHHs inoculated with the concentrated supernatant of HBV-infected 5C-PHHs. (**J**) Analysis of HBeAg of 5C-PHHs infected at various MOIs. Neutralizing antibody (anti-HBsAg) was added from 1 Dpi. *n* = 3. (**K**) Quantification of HBcAg-positive cells at 28 Dpi. *n* = 3.

ISG expression in 5C-PHHs, and virus clearance was observed in prolonged culture (fig. S27, C to E) (*8*). Collectively, these results suggested 5C-PHHs as an effective model for investigating hepatotropic infection and antiviral strategies.

In this study, we used a chemical approach to maintain the functionality of PHHs over the long term, preserving the global transcriptional profile of mature hepatocytes. Using this platform, we demonstrated persistent HBV infection covering the entire viral life cycle and effective HBV spread in PHHs in vitro. Furthermore, HBV cccDNA was captured in PHHs, which is essential for HBV to reenter the viral life cycle and induce relapse. This platform is simple and easily applied to high-throughput screening for devising new antiviral strategies. Methodologically, given that the in vivo physiological homeostasis is highly dependent on precise regulations of molecular networking in the microenvironment, we infer that small-molecule combinations could provide analogous regulatory functions in a tunable manner to stabilize functions of other cell types in vitro.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S27
Tables S1 to S7
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Keep hepatocytes fresh

Freshly isolated primary human hepatocytes (PHHs) quickly lose their identity and function in vitro, limiting their application in modeling infectious liver diseases and screening drugs. Xiang *et al.* describe a simple and effective five-chemical culture condition that can maintain the mature function of cultured PHHs for an extended period of time. PHHs cultured in this way can support the entire viral life cycle and recapitulate long-term infection of the hepatitis B virus. This system created a useful drug-screening platform for developing new antiviral strategies.

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