

# Identification of small molecules for human hepatocyte expansion and iPS differentiation

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**Cell-based therapies hold the potential to alleviate the growing burden of liver diseases. Such therapies require human hepatocytes, which, within the stromal context of the liver, are capable of many rounds of replication. However, this ability is lost *ex vivo*, and human hepatocyte sourcing has limited many fields of research for decades. Here we developed a high-throughput screening platform for primary human hepatocytes to identify small molecules in two different classes that can be used to generate renewable sources of functional human hepatocytes. The first class induced functional proliferation of primary human hepatocytes *in vitro*. The second class enhanced hepatocyte functions and promoted the differentiation of induced pluripotent stem cell-derived hepatocytes toward a more mature phenotype than what was previously obtainable. The identification of these small molecules can help address a major challenge affecting many facets of liver research and may lead to the development of new therapeutics for liver diseases.**

Chronic liver disease affects more than 500 million people worldwide<sup>1</sup>. Most treatments are palliative; the only therapy shown to directly alter outcome and prevent mortality is organ transplantation, but its utility is limited by a persistent shortage of donor organs<sup>2</sup>. Cell-based therapies, such as cell transplantation, engineered hepatocellular tissue constructs and bioartificial liver devices, have long held promise as alternatives to whole-organ transplantation<sup>3</sup>. Such therapies require human hepatocytes because of substantial species-specific differences between animal and human hepatocellular functions, including apolipoprotein expression, metabolic regulation of cholesterol and phase I detoxification enzymes<sup>4,5</sup>.

Human hepatocytes, within the stromal context of the liver *in vivo*, are capable of extensive expansion. After two-thirds partial hepatectomy, the residual mature cell populations consisting mainly of hepatocytes are able to proliferate and replace lost liver mass. *In vitro*, hepatocytes, particularly human ones, lose this proliferative ability<sup>6,7</sup>. Various attempts have been made in the last several decades to harness the innate replication potential of hepatocytes *ex vivo*. Investigations have yielded a number of different culture conditions that can support moderate expansion of rodent hepatocytes<sup>8-13</sup>, including a multifactor medium formulation that expands rat hepatocytes through a dedifferentiated bi-potential intermediate<sup>11</sup>. However, translation of these findings to human cultures has not been reported. To overcome the growth limitations of primary human cells, human hepatocyte cell lines have been developed that were derived from tumor cells or generated through introduction of the SV40 immortalizing gene<sup>14,15</sup>. Although these cell lines are growth competent, their use is limited by safety concerns and abnormal amounts and repertoires of hepatic functions, including notable divergences in cytochrome P450 (CYP450) activity<sup>15,16</sup>.

Human stem cells are an attractive alternative cell source to primary human hepatocytes and immortalized cell lines and hold great promise due to their ability to self renew without limit and differentiate along many lineages, including hepatocytes. Induced pluripotent stem (iPS) cells additionally create the possibility of establishing patient-specific cell types, thus facilitating *in vitro* modeling of rare diseases and enabling personalized medicine. Human iPS cells are generated from somatic cells by forced expression of reprogramming factors and can be differentiated toward hepatocyte-like (iHep) cells in a stepwise manner using defined factors<sup>17-19</sup>. However, the resulting iHep cells show an immature hepatic phenotype that resembles fetal hepatocytes more than adult hepatocytes. Notably, iHep cells persistently express fetal markers such as  $\alpha$ -fetoprotein (AFP) and lack key mature hepatocyte functions, as reflected by the markedly reduced activity (0.1%) of many detoxification enzymes (for example, CYP2A6 and CYP3A4)<sup>17-19</sup>. These key differences between iHep cells and adult hepatocytes have limited the use of stem cells as a renewable source of functional human hepatocytes, especially for *in vitro* applications.

For decades, human hepatocyte sourcing has been a bottleneck for many fields of research and clinical therapies. To overcome this limitation, we used a small-molecule screening approach and identified factors that can induce either the proliferation of mature primary human hepatocytes or the maturation of human iHep cells.

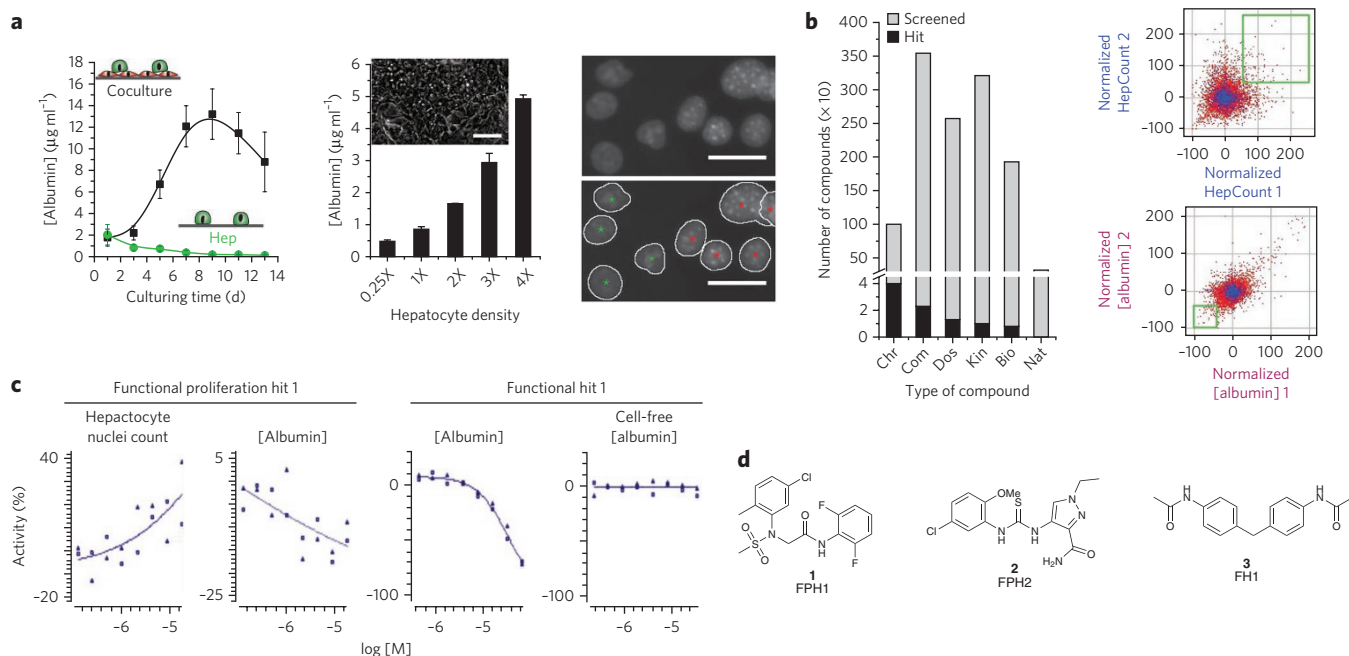
## RESULTS

### Human hepatocyte screening platform

To identify factors that permit renewable sourcing of functional human hepatocytes, we developed a high-throughput liver platform that enables unbiased chemical screening using primary human

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**Figure 1 | High-throughput identification of small molecules that induce proliferation and enhance the functions of primary human hepatocytes.**

(a) High-throughput liver platform. Primary human hepatocytes (green) were seeded on a feeder layer of confluent J2-3T3 fibroblasts (red) in 384-well plates. The line graph shows the representative rate of albumin secretion in screening cocultures and hepatocyte-only cultures (Hep; green) over time. The bar graph shows albumin secretion as a function of hepatocyte density in screening cultures. The inset above the bar graph is a phase-contrast image showing the morphology of the feeder-layer cocultures (scale bar, 100  $\mu\text{m}$ ). Far right, Hoechst staining of screening cocultures showing that hepatocyte nuclei (green asterisks) have a uniform texture, whereas fibroblast nuclei (red asterisks) are punctate (scale bars, 50  $\mu\text{m}$ ). An automated high-content imaging assay identifies and classifies individual nuclei. (b) Chemical screening. The bar graph shows categories of screened and hit compounds. The scatter plots display replicates of the screen, shown separately for the image-based proliferation and competitive ELISA functional readouts. Blue and red data points represent DMSO and experimental small molecules, respectively. The green boxed regions indicate hit zones. Chr, chromatin-biased compounds; Com, commercially available compounds; Dos, products of diversity-oriented synthesis; Kin, kinase-biased compounds; Bio, compounds with previously documented bioactivity; Nat, natural products; HepCount, count of hepatocyte nuclei. (c) Hit validation. All primary hits were retested in an eight-point dose-response curve. Active hits had increasing curves of hepatocyte nuclei counts and/or decreasing curves of competitive albumin concentrations with flat curves of cell-free albumin concentrations. (d) Chemical structures of the hit compounds FPH1, FPH2 and FH1. All data are presented as the mean  $\pm$  s.d.

hepatocytes to avoid species-specific differences and reliance on cell lines and their attendant mutations. So far, chemical screening on such cells has been prohibited by their limited availability in large quantities and precipitous decline in viability and liver-specific functions *in vitro*. Recent advances in cryopreservation technologies have enabled the storage of sufficiently large batches of human hepatocytes from single donors. On the basis of extensive previous characterizations, we have demonstrated that some cryopreserved primary human hepatocytes show phenotypes that approach those of fresh hepatocytes and are thus very useful for *in vitro* liver studies<sup>20–22</sup>. To maintain these cells in culture, we cocultivated them with mouse embryonic J2-3T3 fibroblasts, which have been shown to transiently stabilize hepatocytes *in vitro*<sup>23,24</sup>. We designed the screening platform to contain a sparse population of hepatocytes on top of a confluent feeder layer of J2-3T3 cells within 384-well plates (Supplementary Results and Supplementary Fig. 1a). This design enabled fibroblast-mediated hepatocyte stabilization (Fig. 1a) without hepatocyte crowding, thus offering both time and space for hepatocyte expansion.

To assess cell fates in this platform, we developed two separate high-throughput readouts. The functional readout evaluated the phenotype of treated cells using competitive ELISA. This biochemical assay measured the amount of secreted albumin as a marker for protein synthesis functions of the cultured hepatocytes (Fig. 1a).

The proliferation readout detected hepatocyte proliferation using a customized, automated, high-content imaging protocol. In brief, the assay quantified the number of hepatocyte nuclei using nuclear morphologies to distinguish the hepatocyte and fibroblast

subpopulations that coexist within the screening platform. When visualized with Hoechst stain, hepatocyte nuclei are more uniform in texture, whereas fibroblast nuclei are punctate (Fig. 1a). We developed automated image analysis pipelines to identify all nuclei in each Hoechst-stained image of the screening cultures and measure various characteristics (for example, shape, size, intensity or texture) of each nucleus using the open-source CellProfiler software<sup>25</sup>. We used these characteristics to train machine-learning algorithms to identify and count the number of hepatocyte nuclei in each image using CellProfiler Analyst (Supplementary Fig. 2)<sup>26,27</sup>. Assay validation data showed that this image-based readout can confidently ( $Z' > 0$ ) detect doublings in hepatocyte nuclei numbers with low variance (coefficient of variation <20%) and good reproducibility. In addition to quantifying hepatocyte nuclei that have completed mitosis, we also found and quantified the number of nuclei in the process of mitosis. Two additional analysis pipelines were built to detect nuclear morphologies consistent with cells undergoing metaphase and anaphase.

### High-throughput identification

Using the high-throughput liver platform with both readouts, we screened 12,480 small molecules supplied by the Broad Institute. Screen details are provided in Supplementary Tables 1 and 2. Briefly, the collection included commercially available compounds, known bioactives and natural products (Fig. 1b). To avoid donor-to-donor variability, human primary hepatocytes from a single donor (donor a) were preconditioned for 7 d with J2-3T3 fibroblasts in collagen-coated 384-well plates (Supplementary Fig. 1b). After stabilization

*in vitro*, cultures were treated with individual molecules from the chemical library for 48 h. All compounds were tested in duplicate at a single concentration of  $\sim 15 \mu\text{M}$ . After compound treatment, media supernatants were collected for functional analyses using competitive ELISA, and cultures were fixed in 4% paraformaldehyde for proliferation analyses by imaging (Supplementary Tables 1 and 2).

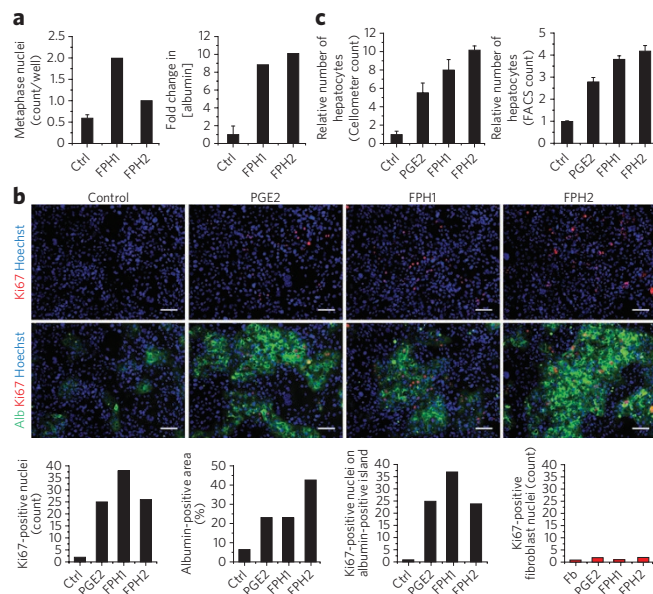
To identify proliferation hits, we integrated the three image-based readouts (the number of nuclei in metaphase or anaphase and the number of hepatocyte nuclei in interphase). Functional hits were separately identified on the basis of the ELISA readout. Ninety-three compounds met all hit selection criteria, thereby qualifying as functional proliferation hits (FPHs; Fig. 1b shows the types of compounds that comprised this set of hits). In addition to these 93 FPHs, we also identified proliferation-only and function-only hits. A total of 400 primary hits across these classes were retested in eight-point dose-response curves using cryopreserved hepatocytes from a different donor (donor b) for biological diversity (Supplementary Fig. 1c). The remaining hits were further tested using a cell-free counter assay to eliminate compounds that interfered with the ELISA assay chemically. Ultimately, we obtained 12 confirmed hits (Fig. 1c and Supplementary Fig. 3).

We divided these confirmed hits into three separate classes of compounds: proliferation hits, functional hits and functional proliferation hits (Supplementary Table 3). Each of the three classes enables a distinct approach toward generating renewable sources of functional human hepatocytes, and they may synergize when used in combination. We chose to focus on candidates within the FPH and functional hit categories only (Supplementary Fig. 1d). FPHs induced functional proliferation of hepatocytes *in vitro* (FPH1 (1) and FPH2 (2); Fig. 1d) and thus may be useful for expanding mature human primary hepatocytes. Functional hits enhanced the functions of cultured hepatocytes (FH1 (3); Fig. 1d), prompting us to hypothesize that these molecules may promote the differentiation of iPS cell-derived hepatocytes toward a phenotype that is more mature than what has been obtainable so far.

We characterized the top hits by LC/MS,  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  (Supplementary Note 2). To determine whether any structure-activity relationships were present for the three strongest hits (FH1, FPH1 and FPH2), we searched the remaining 12,477 compounds that had been screened for analogs (similarity score  $\geq 0.8$ ). Using this criterion, FH1 and FPH2 were found to be singletons, and 21 analogs of FPH1 were found. These analogs all contained the same *N*-phenyl-2-(*N*-phenylmethylsulfonamido)acetamide core (4) as FPH1 but had varying substitutions around the sulfonamide and amide phenyl rings (Supplementary Fig. 4). The key driver of compound activity in the FPH1 series was the presence of a 5-chloro-2-methyl substitution on the sulfonamido phenyl ring and a small functional group at the *para* position of the phenylamide ring. FPH1 and analog 5 were proliferation hits, analog 6 was a weak hit, and analogs 7 and 8 were inactive (Supplementary Fig. 4). Analogs 9 and 10 illustrate that low steric bulk at the *para* position of the phenylamide ring alone is not sufficient to drive the compound activity of the FPH1 series. Ultimately, although the structure-activity relationship around FPH1 makes it the most suitable for chemical optimization, the three strongest hits (FH1, FPH1 and FPH2) were each selected for follow up in downstream assays on the basis of their ability to increase the function and proliferation of primary human hepatocytes.

### Expansion of human primary hepatocytes

We assayed the ability of our strongest FPHs to expand human primary hepatocytes *in vitro*. As a first step, we tested prostaglandin E2 (PGE2) as a putative positive control, as it has been shown to promote liver regeneration in zebrafish and mouse through Wnt signaling<sup>28,29</sup>. We tested PGE2 on human primary hepatocytes in



**Figure 2 | Expansion of primary human hepatocytes.** (a) Primary screening data for FPH1 and FPH2. The data are presented as the mean  $\pm$  s.d. Ctrl, control. (b) Ki67 (red) and albumin (green) immunofluorescent staining after 6 d of culture. The bar graphs show CellProfiler quantification of the images above. Alb, albumin; Fb, fibroblasts. (c) FACS (right) and Cellometer (left) Automated Cell Counter analyses. Fibroblasts were labeled with CM-DiI before the initiation of culture to allow for the identification of hepatocytes by negative selection. FACS cell counting was further enabled by fluorescent-counting beads. Control cultures were treated with empty vehicle (DMSO). The data are presented as the mean  $\pm$  s.e.m.

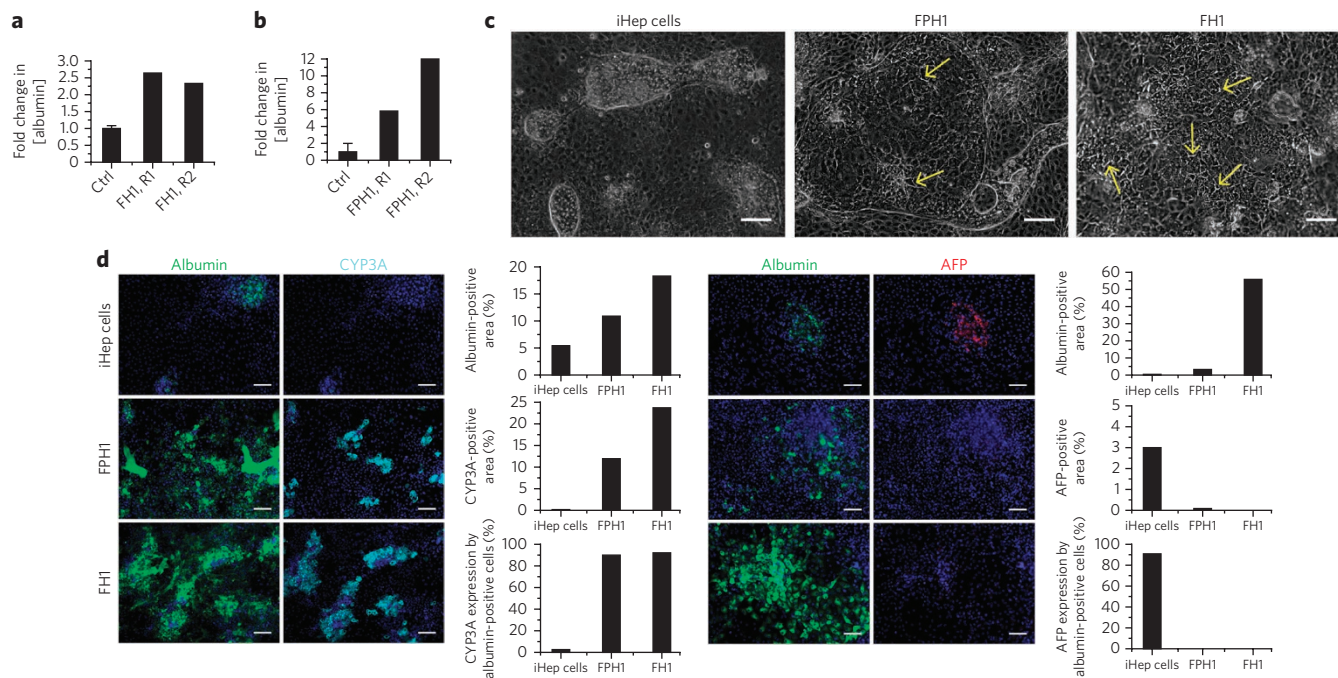
our high-throughput liver platform and found it to indeed perform as an FPH in this system (Supplementary Fig. 3). Two other strong FPHs (FPH1 and FPH2), identified through unbiased screening, were also tested. Both induced an increase in hepatocyte nuclei count, elevated the number of nuclei undergoing mitosis during primary screening or both (Fig. 2a and Supplementary Fig. 5a), and these effects on hepatocytes were concentration dependent (Fig. 1c). Cells treated with these FPHs also maintained their liver-specific functions (Figs. 1c and 2a).

To further characterize the effects of FPH1 and FPH2, we stained for the proliferation marker Ki67, counted hepatocyte cell numbers and evaluated the morphology, gene expression and functional output of treated hepatocytes. Human primary hepatocytes were cultured in standard 12-well tissue culture plates on top of a feeder layer of growth-arrested J2-3T3 fibroblasts. A single FPH was supplemented into the culture medium on days 1 and 5 at a concentration of 20  $\mu\text{M}$  for FPH1 or 40  $\mu\text{M}$  for FPH2. Treated hepatocyte colonies increased in area over time, with more hepatocytes populating each colony (Supplementary Fig. 5b).

Immunofluorescent staining showed that FPH treatment increased Ki67 staining, which colocalized with Hoechst stains for cell nuclei and also with human albumin stains for hepatocytes. Quantitative image analysis showed an up to 6.6-fold increase in the area of albumin-positive colonies after small-molecule treatment (Fig. 2b). The vast majority of Ki67-positive nuclei showed hepatocyte nuclear morphologies, which is consistent with the lack of proliferating cells in fibroblast-only cultures treated with FPHs (Fig. 2b and Supplementary Fig. 5c). These results strongly indicate that human primary hepatocytes can be induced to proliferate *in vitro* using FPHs.

To characterize the degree and kinetics of proliferation, we quantified the number of hepatocytes in culture using both an automated





**Figure 3 | Functional enhancement of human primary hepatocytes and iHep cells.** (a) Primary screening data of FH1. R1, replicate 1; R2, replicate 2. (b) Primary screening data of FPH1. Control cultures (Ctrl) were treated with empty vehicle (DMSO). All data in **a** and **b** are presented as the mean  $\pm$  s.d. (c) Morphology and colony size of FPH1- and FH1-treated iHep cells in six-well plates 9 d after the initiation of treatment. Untreated iHep cells (left) are shown for comparison (scale bars, 100  $\mu$ m). The yellow arrows point to select bile canaliculi. (d) Albumin, CYP3A and AFP staining of iHep cells after 9 d of culture (scale bars, 100  $\mu$ m). The bar graphs show the quantifications of the images.

cell counter and fluorescence-activated cell sorting (FACS) analysis. The results showed a substantial, up to tenfold increase in the number of hepatocytes when treated with various FPHs (Fig. 2c and Supplementary Fig. 5d). The strongest proliferation inducer was FPH2. Over 7 d, FPH2 induced hepatocyte doublings at a rate that is consistent with reported liver regeneration kinetics *in vivo*<sup>30</sup>.

To generalize our findings across multiple donors, we obtained primary human hepatocytes from six additional cell sources and treated them with FPH1 and FPH2. Immunofluorescent staining for Ki67 and albumin, along with FACS-mediated cell counting, revealed that hepatocytes from all sources examined expanded after small-molecule exposure (Supplementary Fig. 6). These results, together with the primary screening and validation data obtained using cells from donors a and b, suggest that the FPHs are active across a wide range of genetically diverse individuals.

To assess the phenotype of the treated hepatocytes, we performed phase-contrast imaging, gene expression profiling and biochemical analyses. The imaging showed that normal hepatocyte morphology was maintained throughout the treatment period (Supplementary Fig. 5b). Gene expression profiling demonstrated that there were no substantial differences between FPH-treated and untreated primary human hepatocytes (Supplementary Fig. 7a). Urea synthesis, a surrogate marker of nitrogen metabolism, and albumin secretion were both stable throughout FPH treatment (Supplementary Fig. 7c). Metabolic functions were assessed by examinations of CYP450 activity and canalicular transport. The results showed active MRP2 transport, which is consistent with intact bile canaliculi (Supplementary Fig. 7b), and also that CYP450 activity was not compromised by small-molecule treatment (Supplementary Fig. 7c). These data agree with published findings of sustained liver functions throughout liver regeneration<sup>31</sup>.

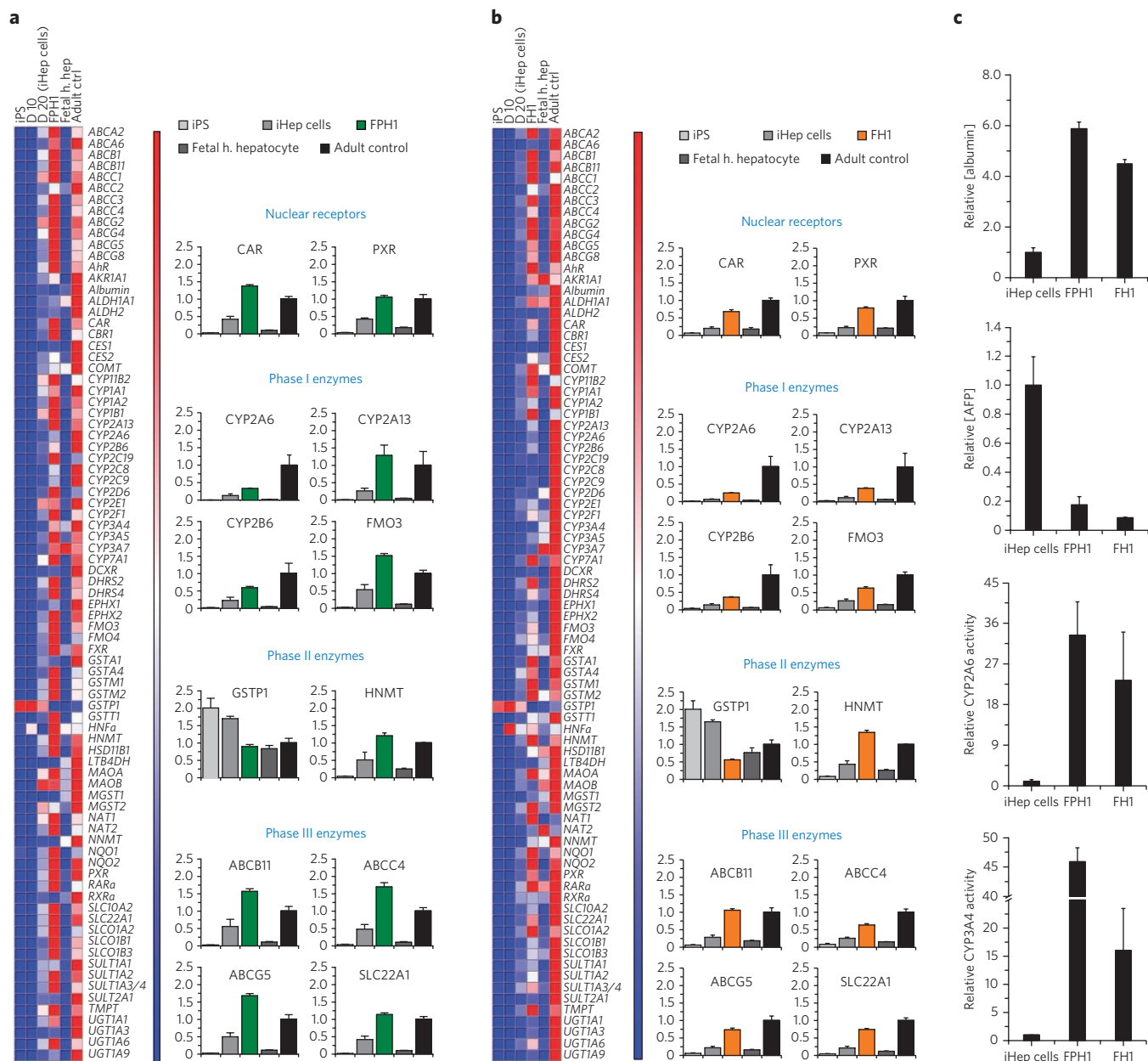
### Maturation of human iHep cells

We tested the ability of hit molecules to promote the differentiation of iPS cells toward a hepatic lineage and induce the maturation of iHep

cells toward a more adult-like liver phenotype. Undifferentiated iPS cells were cultured on Matrigel, supported by conditioned medium from primary mouse embryonic fibroblasts and differentiated into iHep cells as previously described<sup>18</sup>. In brief, iPS cells were cultured in differentiation medium with sequential addition of growth factors (activin A, BMP-4, bFGF, HGF and OSM) to guide differentiation, first into endoderm, then into hepatic-specified endoderm, then into hepatic progenitor cells and finally into iHep cells (Supplementary Fig. 8). Small molecules were added to the basal medium (Lonza) without OSM (Figs. 3 and 4 and Supplementary Fig. 9) or with OSM (Supplementary Fig. 10) on day 21 after the initiation of differentiation and acted over a period of 9 d. One functional hit (FH1) and one FPH (FPH1) were used to treat iHep cells. FH1 doubled albumin secretion during the primary screening (Fig. 3a), and the effects were dose responsive (Fig. 1c). FPH1 had a similar effect on hepatocyte functions (Fig. 3b), which was much more pronounced in the younger donor (donor a, age 1 year).

Cultures treated with FH1 and FPH1 contained larger colonies of iHep cells, which showed more pronounced hepatocyte morphologies, including polygonal cell shapes, visible nuclei and more noticeable bile canaliculi between hepatocytes (Fig. 3c). We subsequently examined the maturity of treated iHep cells using gene expression profiling, immunofluorescent staining and various biochemical assays probing protein secretion and enzyme activity.

Gene expression profiles showed that treated iHep cells more closely resembled mature hepatocytes than untreated cells (Fig. 4a,b). Euclidian clustering analyses grouped untreated cells with fetal hepatocytes and treated cells with adult hepatocytes. Of particular interest were the expression levels of various ABC transporters and GSTP1. ABC transporters are known to mature after birth. The expression level of ABCB11, also known as bile-salt export pump (BSEP), increased approximately four-fold with FH1 treatment, reaching 100% of the levels in adult hepatocytes. In contrast, GSTP1 expression, whose levels decrease with maturity, remained low with small-molecule treatment.



**Figure 4 | Maturation of human iHep cells.** (a, b) Gene expression profiling of FPH1-treated (a) and FH1-treated (b) iHep cells. Left, heat maps of the Luminex analyses for 83 liver-specific genes, shown separately for the independent experiments. The columns of the heat map are averaged values of replicate ( $n \geq 3$ ) loadings of mRNA extracted from various populations of iPS cells (250 ng total RNA per replicate). mRNA expression was determined relative to the average of the control gene transferrin, and heat maps are row normalized. Hep, hepatocytes. The bar graphs show select gene sets comparing the relative mRNA expression of small molecule-treated hepatocytes (colored bars) to iPS cells (light gray), untreated iHep cells (gray bars) and fetal human (h.) hepatocytes (dark gray) normalized to control (black) for nuclear receptors, phase I, phase II and phase III drug metabolism genes. Adult control refers to adult cryopreserved human primary hepatocytes stabilized by micropatterned coculture (Supplementary Note 1). The data (a, b) are presented as the mean  $\pm$  s.e.m. of Luminex-loaded replicates. (c) ELISA assays for secreted albumin and AFP and quantitative CYP3A4 and CYP2A6 activity assays. For all analyses, iHep cells were cultured for 9 d after differentiation in six-well plates ( $n = 3$ ). All data are presented as the mean  $\pm$  s.e.m.

To examine the effects of FH1 and FPH1 at the protein level in iHep cells, we visualized the amounts of AFP, albumin and CYP3A in culture using immunofluorescent staining (Fig. 3d and Supplementary Fig. 9). Images showed marked increases in the amount of albumin with both FH1 and FPH1 treatment, although the effects of FH1 were more pronounced. This pattern is in agreement with morphological findings. Consistent with previous work<sup>17–19</sup>, untreated cultures contained islands that were strongly positive for both albumin and the fetal marker AFP but showed minimal staining for the mature protein CYP3A. In contrast, treated islands

double stained strongly for albumin and CYP3A, and AFP was largely absent. This more mature phenotype was stable for at least 1 week after the removal of small-molecule treatment (Supplementary Fig. 10).

To confirm the staining results, we measured secreted amounts of albumin and AFP by ELISA and CYP450 activities through isoenzyme-specific substrates with fluorescent or luminescent metabolites. The ELISA results verified that small-molecule treatment both increased albumin secretion and decreased AFP secretion (Fig. 4c). CYP3A4 activity was found to increase by 16 and

45 times after treatment with FH1 and FPH1, respectively. The activity of CYP2A6, another mature CYP450, was also found to increase markedly as a result of small-molecule treatment (Fig. 4c). We considered enzyme induction as a possible explanation for these elevations in CYP450 activity. However, this is unlikely. Although human hepatocytes treated with specific inducers do show elevated CYP450 activity, such elevations typically revert by 24 h after removal of the inducer (Supplementary Fig. 11). A period of at least 48 h separated small-molecule treatment and the measurement of CYP450 activity, thus the iHep cells would be expected to have recovered from any general elevations in CYP450 activity.

## DISCUSSION

Developing new cell-based therapies for liver disease requires human hepatocytes because of substantial species-specific differences<sup>4,5</sup>. Human hepatocytes, in their native environment, have remarkable regenerative capabilities<sup>30,32–35</sup> that are lost *ex vivo*. Consequently, for decades, human hepatocyte sourcing has been a bottleneck for many fields of research and clinical therapies.

In traditional cultures of other cells, small molecules have shown promising roles in modulating a wide range of complex cell phenotypes. These outcomes include stem cell self-renewal and differentiation and the proliferation of normally quiescent mature adult cells such as pancreatic beta cells and cardiomyocytes<sup>36–38</sup>. Compounds can act through a variety of mechanisms to induce cell division, including activation of developmental signaling pathways such as Wnt<sup>28</sup> or recruitment of guanine nucleotide exchange factors to the plasma membrane for RAS-MAPK pathway activation<sup>38</sup>. However, such systems have limited applicability to primary human hepatocytes because of the rapid loss of hepatocyte phenotype and viability *in vitro*. So far, high-throughput screens related to the liver have been restricted to hepatocyte cell lines or hepatocyte extracts, neither of which offers the full repertoire of hepatocellular functions and responses shown by primary human hepatocytes. Such deviation from clinically relevant biology has been a major cause of the high attrition rates currently troubling drug discovery. The high-throughput platform developed here leveraged stromal interactions, which are known to be important *in vivo*, to enable screening using primary human hepatocytes. Such a platform enables the study of many previously inaccessible aspects of liver biology and small-molecule bioactivity. In this work we used the platform to address two long-standing issues: lack of a renewable human hepatocyte source and incomplete differentiation of human stem cells.

Early work on hepatocyte expansion focused on the *in vitro* addition of growth factors, hormones, serum and vitamins<sup>8,10,11,39</sup> and has led to a medium formulation for the expansion of rodent hepatocytes<sup>11</sup>. Our study contributed to this progress through the identification of small molecules that can induce the proliferation of human hepatocytes *in vitro*. With this advance, we foresee the potential to extend our findings to generate renewable materials (functional human hepatocytes) for various cell-based therapies and *in vitro* liver models and assays, including tissue-engineered liver constructs, cell transplantation, ADME (absorption, distribution, metabolism and excretion) or toxicity screening and humanized mice for *in vivo* disease modeling. Our future work will determine the maximum expansion potential and functionality of treated hepatocytes using animal rescue experiments and explore the use of hepatocytes expanded from patient biopsies for cell transplantation therapy and other forms of personalized medicine. Such outcomes can contribute to our understanding of liver regeneration and may have implications for regenerative medicine.

An alternative cell source to primary human hepatocytes is human iPS cells, which can be differentiated toward iHep cells<sup>17,18,40</sup>. Among the factors used to drive differentiation is OSM. It has been observed that continued supplementation of OSM beyond the first

20 d of differentiation promotes survival of the resulting hepatocyte-like cells but suppresses the late stages of hepatocyte maturation. Thus, to isolate the effect of hit molecules on maturation (rather than differentiation), OSM was excluded beyond the 20-d point in some of our experiments (Figs. 3 and 4 and Supplementary Fig. 9). Under these conditions, a small percentage of hepatocyte-like cells were derived; however, the few that were obtained showed marked improvements in maturation. When OSM was maintained throughout the treatment period (Supplementary Fig. 10), high efficiencies of differentiation to hepatocyte-like cells were observed. Interestingly and contrary to our expectations, the hit molecules were also able to mature these hepatocyte-like cells; however, the amounts of maturation as judged by CYP2A6 and CYP3A4 activity were not as robust as in the absence of OSM.

The efficiency of differentiation is generally stochastic, yielding some highly efficient differentiations and others that are far less efficient—even in the same laboratory. The variables that underlie this stochasticity are poorly understood but may include variability in other cell types that emerge over time during differentiation, effectively creating a variable coculture environment. Even the most robust cultures of differentiated iHep cells persistently express fetal markers (for example, AFP) and lack key mature hepatocyte functions (for example, CYP2A6 and CYP3A4)<sup>17–19</sup>, an outcome that presents a major hurdle to the application of these cells in both therapeutic and basic research assays. Our iPS cell results suggest that FH1 and FPH1 are able to promote the maturation of well-differentiated cultures of iHep cells beyond what has been obtainable so far, thus potentially alleviating a major obstacle to the use of iPS cells as a renewable source of functional human hepatocytes. We found that this effect was generalized across two pluripotent cell lines; nonetheless, we did observe stochasticity in the efficiency of maturation akin to the variability observed in response to existing directed differentiation protocols.

On the basis of tetraploid complementation experiments with mice, it is clear that mouse iPS cell-derived progeny are capable of differentiating into mature, adult liver cells when provided with the correct *in vivo* context and developmental cues<sup>41,42</sup>. As it is not possible to conduct the parallel experiment with human cells, it remains to be seen whether similar conditioning will be sufficient to promote complete maturation to a comparable, fully mature hepatocyte. The evidence that human iHep cells mature when xenografted in a 'gold standard' adult, *in vivo* environment is mixed<sup>43,44</sup>; the remaining donor cells still seem to have low function on a per-cell basis<sup>18,40,45–47</sup>. This apparent defect could indicate that the cells are truly blocked from the late stages of tissue-specific maturation or that the preconditioning protocols provided *in vitro* are deficient in some way—for example, they may lack specific cues that the human embryo experiences in the third trimester of development. Our data suggest that human iPS cells are indeed capable of further maturation *in vitro*, which is consistent with their observed potential in the mouse system. In light of similar challenges in other cell types<sup>48</sup>, our platform and its resulting compounds offer a roadmap for testing the generality of this finding in other tissues and for the identification of regulatory networks that are influenced by small-molecule exposure.

Through a high-throughput screen of 12,480 small molecules on primary human hepatocytes, we identified compounds in two separate classes that can be used to establish renewable sources of functional human hepatocytes. One class, FPHs, induced proliferation of mature human primary hepatocytes. The second class, functional hits, helped differentiate human iPS cells toward more mature, adult hepatocytes. The identification of these small molecules may have an impact on several areas of research, including maturation of other iPS-derived cell types, expansion of other 'terminally' differentiated cell types and the translational potential of the resultant hepatocytes and other cell types.



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## METHODS

Methods and any associated references are available in the [online version of the paper](#).

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## Author contributions

J.S. and S.N.B. designed all experiments. J.S. performed and analyzed all experiments. D.J.L. helped analyze image-based experiments. N.T.R. helped perform and analyze screening experiments and analyzed chemical characterization data. R.E.S. helped design, perform and analyze iPS experiments. D.T. developed and implemented the Luminex system. T.E.N., S.A.D., W.G. and A.E.C. contributed reagents and expertise. J.S., S.N.B. and N.T.R. wrote the manuscript. S.N.B. supervised the project.

## Competing financial interests

The authors declare no competing financial interests.

## Additional information

Supplementary information and chemical compound information is available in the [online version of the paper](#). Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Correspondence and requests for materials should be addressed to S.N.B.

## ONLINE METHODS

**High-throughput screen.** First, 384-well screening plates (Corning) were incubated with a solution of type I collagen in water (100 µg/ml; BD Biosciences) for 1 h at 37 °C. A feeder layer of J2-3T3 fibroblasts (gift from H. Green) was robotically plated onto the collagen at a density of 8,000 cells per well (designated as day -2) and allowed to reach confluency over 48 h, when their growth became contact inhibited. Primary human hepatocytes were plated onto the fibroblasts on day 0 at a density of 2,000 cells per well and maintained under standard culture conditions with daily replacement of the hepatocyte medium (the detailed composition of which is listed in **Supplementary Note 1**) for 7 d. Primary human hepatocytes were purchased in cryopreserved suspension from Celsis *In vitro* Technologies (donor a) or Invitrogen (donor b) and pelleted by centrifugation at 50g for 10 min. The supernatant was discarded before resuspension of cells in hepatocyte medium. A library of 12,480 compounds was added on day 7 at a final concentration of ~15 µM and allowed to incubate for 48 h. On day 9, culture supernatants were collected for automated ELISA analysis, and cells were fixed in 4% paraformaldehyde for imaging analysis. Hepatocyte functions were determined by competitive ELISA (MP Biomedicals) using horseradish peroxidase detection and chemiluminescent luminol (Pierce) as a substrate. The cell-free counter assay involved ELISAs on fresh medium incubated for 48 h with the compounds of interest (without cells). Hepatocyte proliferation was assessed through customized, automated high-content imaging protocol (**Supplementary Fig. 2**). Fixed cells were permeabilized with 0.1% Triton-X, and nuclei were visualized with Hoechst stain (Invitrogen) and robotically imaged (Thermo, Molecular Devices) at 21 dispersed sites per well. Images were digitized and analyzed using CellProfiler and CellProfiler Analyst (Broad Institute). To identify functional proliferation hits, we integrated all four readouts using *P* values. FPHs were selected using the criterion  $P_{\text{prod}} < 1 \times 10^{-6}$  and  $P_{\text{max}} < 0.25$  and  $P_{\text{alb}} < -0.05$ , where  $P_{\text{prod}} = P_{\text{HepInterphase}} \times P_{\text{metaphase}} \times P_{\text{anaphase}}$ ,  $P_{\text{max}} = \max(P_{\text{HepInterphase}}, P_{\text{metaphase}}, P_{\text{anaphase}})$  and  $P_{\text{alb}} = \text{ELISA } P \text{ value}$ . Additional proliferation-only hits were selected on the basis of  $Z > 3$  in image-based assays; function-only hits were selected by an ELISA  $Z < -4$ . Compounds with ELISA  $Z > 3$  were eliminated as toxic.

**Cell counting.** J2-3T3 fibroblasts were covalently labeled with Vybrant CM-DiI (Invitrogen) before the initiation of coculture. For FACS analysis, cells were treated with trypsin, or collagenase then accutase, and suspended in PBS and 0.2% FBS. Cell suspensions were supplemented with 50,000 fluorescent-counting beads (CountBright, Invitrogen) per sample. Data were acquired with a four-color flow cytometer (FACSCalibur, BD Biosciences) and analyzed with

CellQuest (BD Biosciences). For Cellometer analysis, cells were trypsinized and placed on cell-counting chambers (Nexcelom) for automated cell counting.

**Luminex analysis.** Cells were lysed using RLT buffer (Qiagen) or TRIzol (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). Gene expression was determined using Luminex analysis, as previously described<sup>22</sup>. Briefly, total RNA was immobilized on a Qiagen turbo capture 384-well plate and reverse transcribed using oligo(dT) priming. A biotinylated FlexMap tag sequence unique to each gene of interest and a phosphorylated downstream probe were then added to resulting cDNAs to generate biotinylated FlexMap-tagged amplicons. Universal PCR was then performed for 35 cycles using a biotinylated T7 forward primer and a T3 reverse primer in buffer with dNTPs and Taq polymerase. FlexMap microsphere beads conjugated with anti-tag oligonucleotides were then added and allowed to hybridize. Amplicons were captured by streptavidin-phycoerythrin, and 100 events per bead were analyzed for internal bead color and phycoerythrin reporter fluorescence on a Luminex FlexMap three-dimensional analyzer. Data for replicate loadings, expressed as the mean fluorescent intensity of at least 100 beads per sample, were scaled to the human transferrin gene and row normalized for heat map representation using GeneE open software (Broad Institute).

**Biochemical assays.** Culture media were collected and either processed immediately or frozen at -20 °C until analysis. Albumin content was measured through competitive or sandwich ELISA assays (MP Biomedicals, Fitzgerald, Bethyl Laboratories) using horseradish peroxidase detection and 3,3',5,5'-tetramethylbenzidine (TMB; Fitzgerald Industries) as a substrate. Urea concentration was determined colorimetrically using diacetylmonoxime with acid and heat (Stanbio Labs)<sup>24</sup>. To quantify CYP450 activity at 48 h after small-molecule exposure, iHep cell cultures were incubated with substrates (coumarin from Sigma for CYP2A6 or luciferin-IPA from Promega for CYP3A4) for 4 h at 37 °C; primary human hepatocyte cultures were incubated with 7-benzyloxy-4-trifluoromethylcoumarin (BFC; BD Gentest) at 50 µM for 1 h at 37 °C in phenol red-free medium. Many different CYP450 isoforms process BFC into its fluorescent product of 7-hydroxy-4-trifluoromethylcoumarin (7-HFC). The incubation medium was collected, and metabolite concentrations were quantified by luminescence or fluorescence after hydrolyzation of potential metabolite conjugates by β-glucuronidase/arylsulfatase (Roche, IN).

**Cell culture.** All cells were cultured under standard tissue culture conditions. Details of the specific platforms can be found in **Supplementary Note 1**.