Enhancing the Functional Maturity of Induced Pluripotent Stem Cell–Derived Human Hepatocytes by Controlled Presentation of Cell–Cell Interactions In Vitro

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Induced pluripotent stem cell–derived human hepatocyte-like cells (iHeps) could provide a powerful tool for studying the mechanisms underlying human liver development and disease, testing the efficacy and safety of pharmaceuticals across different patients (i.e., personalized medicine), and enabling cell-based therapies in the clinic. However, current in vitro protocols that rely upon growth factors and extracellular matrices (ECMs) alone yield iHeps with low levels of liver functions relative to adult primary human hepatocytes (PHHs). Moreover, these low hepatic functions in iHeps are difficult to maintain for prolonged times (weeks to months) in culture. Here, we engineered a micropatterned coculture (iMPCC) platform in a multiwell format that, in contrast to conventional confluent cultures, significantly enhanced the functional maturation and longevity of iHeps in culture for at least 4 weeks in vitro when benchmarked against multiple donors of PHHs. In particular, iHeps were micropatterned onto collagen-coated domains of empirically optimized dimensions, surrounded by 3T3-J2 murine embryonic fibroblasts, and then sandwiched with a thin layer of ECM gel (Matrigel). We assessed iHep maturity by global gene expression profiles, hepatic polarity, secretion of albumin and urea, basal cytochrome P450 (CYP450) activities, phase II conjugation, drug-mediated CYP450 induction, and drug-induced hepatotoxicity. Conclusion: Controlling both homotypic interactions between iHeps and heterotypic interactions with stromal fibroblasts significantly matures iHep functions and maintains them for several weeks in culture. In the future, iMPCCs could prove useful for drug screening, studying molecular mechanisms underlying iHep differentiation, modeling liver diseases, and integration into human-on-a-chip systems being designed to assess multiorgan responses to compounds. (HEPATOLOGY 2015;61:1370–1381)
could provide a nearly unlimited supply of cells for: (1) building sustainable and high-throughput platforms to enable a better understanding of interindividual susceptibility to drugs; (2) modeling liver development and diseases; and (3) enabling cell-based therapies (i.e., hepatocyte transplantation, extracorporeal bioartificial liver devices, and implantable cell-laden engineered constructs). 7-10

Several groups have generated iHeps using temporal delivery of molecular cues inspired from liver organogenesis, 7,10-18; however, some key issues prevent routine and widespread iHep utility in the aforementioned applications. For instance, iHep functions are commonly compared to PHH monolayers after 24-72 hours in culture, by which time liver functions are severely down-regulated. 6 Thus, comparisons to a suboptimal PHH phenotype often bias the functional maturity in favor of iHeps. Even so, it is widely accepted that in vitro differentiation protocols need to be improved to induce functions in iHeps closer to adult PHHs. 7,10 Additionally, an abundant supply of iHeps cultured in a reproducible culture format is not routinely available to investigators interested in applying these cells to downstream applications. Alleviating such issues is likely to help spur widespread use of iHeps in different arenas.

Coculture with liver- and non-liver-derived stromal cells from multiple species can stabilize the phenotype of primary hepatocytes from human and animal livers in vitro. 5,19 For instance, Khetani and Bhatia utilized semiconductor-derived microfabrication tools to develop a micropatterned coculture (PHH-MPCC) platform with 3T3-J2 murine embryonic fibroblasts that led to long-term liver functions in PHHs 6 and was shown to be a better predictor of clinical drug outcomes, as compared to pure PHH monolayers. 20,21 Yet, the effects of stromal cells on iHep differentiation and functional stabilization have not been fully elucidated, even though such heterotypic interactions are critical in liver embryology and physiology. A handful of groups have cocultured iHeps with stromal cells; however, demonstrating improvement of coculturing on long-term and diverse categories of iHep functions as well as comparisons to stable cultures of PHHs have been lacking. 22-25

Here, we hypothesized that, similar to what was observed with the aforementioned PHH-MPCCs, controlled presentation of both homo- and heterotypic cell–cell interactions to iHeps could induce, in these cells, higher functional maturity and stabilize liver functions for prolonged times in culture than observed in conventional pure iHep monolayers. In particular, iHeps were micropatterned onto collagen-coated domains of empirically optimized dimensions, surrounded by 3T3-J2 fibroblasts, and then sandwiched with a Matrigel overlay to create a hybrid model using both the MPCC and extracellular matrix (ECM) sandwich techniques. Furthermore, the micropatterned cocultures containing iHeps and 3T3-J2 murine embryonic fibroblasts with a Matrigel overlay (iMPCCs) were created in industry-standard multiwell plates to enable future drug screening and higher-throughput biological investigations. We characterized the maturity of iMPCCs by global gene expression profiles, hepatic polarity, secretion of albumin and urea, basal cytochrome P450 (CYP450) activities, phase II conjugation, drug-mediated CYP450 induction, and drug-induced hepatotoxicity. Finally, results in iMPCCs were compared to conventional confluent cultures of iHeps and benchmarked against multiple donors of PHHs.

Materials and Methods

Culture of iHeps. Following protocols from Cellular Dynamics International (CDI), iHeps (commercially available as iCell Hepatocytes) were diluted to a density of 8×10^5 cells/mL (1×10^6 cells/mL for cryopreserved cells) and seeded in rat tail collagen I-coated wells (500 μL/well) of tissue culture polystyrene 24-well plates (Corning Life Sciences, Tewksbury MA) to create conventional confluent cultures (iCCs). After attachment over 4 hours, Matrigel was added to the cultures (0.25 mg/mL in culture medium). Maintenance medium was replaced after the first 24 hours and every other day thereafter.

To create micropatterned cocultures (iMPCCs), 24-well or 96-well plates were subjected to soft-lithography to micropattern circular collagenous islands (500 μm diameter, 1200 μm center-to-center spacing) (Fig. 1). iHeps were seeded at a density of ~8×10^5 cells/mL.
(~1×10^6 cells/mL for cryopreserved cells) in 300 µL per well (or 50 µL per well for 96-well plate format). After allowing 4-5 hours for cells to fill the islands, wells were washed 3x in culture medium to remove unattached iHeps. A single well contained ~25,000 iHeps spread over ~90 total islands (or ~4500 iHeps spread over ~14 islands in 96-well format). 3T3-J2 murine embryonic fibroblasts were seeded at a density of ~4×10^5 cells/mL within 24 hours and allowed to fill the remaining bare areas not covered by iHeps. A thin Matrigel overlay (0.25 mg/mL) was applied to iMPCCs two days after fibroblast seeding, once the monolayer had become confluent. Culture medium was changed every other day.

**Cell Staining.** For immunofluorescent staining of intracellular albumin, fixed cells (4% paraformaldehyde or PFA, Alfa Aesar, Ward Hill, MA) were permeabilized using 0.1% triton X-100 (Amresco, Solon, OH) for 10 minutes followed by 3x PBS (phosphate buffered saline, Corning Life Sciences) rinses. Samples were incubated at 37°C for 30 minutes in 20% goat serum (Pierce Thermo Scientific, Rockford, IL) in PBS (blocking solution). Rabbit anti-human albumin antibody (Rockland Immunochemicals, Limerick, PA) was added to blocking solution 1:100 and incubated for 1 hour at 37°C. Cultures were then washed 3x in PBS and incubated with rhodamine-conjugated goat anti-rabbit IgG antibody (Rockland Immunochemicals), diluted 1:100 in blocking solution, for 1 hour at 37°C. For the final 15 min of incubation, DAPI (4',6-diamidino-2-phenylindole, MP Biomedicals, Solon, OH) was added at 300 nM. After 3x PBS washes, cultures were imaged using an EVOS FL microscope (Life Technologies, Grand Island, NY).

For staining functional bile canaliculi, cultures were washed 3x with phenol red-free culture medium, then incubated at 37°C with 2 µg/mL CDF (5-[and-6]-carboxy-2',7'-dichlorofluorescein diacetate [Life Technologies] for 10 min and washed 3x again prior to fluorescence microscopy. Periodic acid-Schiff staining (Sigma-Aldrich, St. Louis, MO) was used to assess cellular glycogen. Briefly, cultures were fixed with 4% PFA, washed 3x with PBS, and incubated for 7 min at room temperature (RT) with periodic acid solution. Cultures were washed 3x with PBS and incubated with a 1:1 solution of Schiff’s Reagent and PBS for 5 min at RT. Finally, cultures were washed 10x with PBS and imaged using bright-field microscopy. Low density lipoprotein (LDL) uptake was assessed using DiI-LDL (Life Technologies). Cultures were washed 3x with phenol red-free culture medium, then incubated with 20 µg/mL DiI-LDL in serum-free culture medium for 3 hours at 37°C. Cells were washed with PBS to remove unbound LDL and were imaged in phenol red-free culture medium.

**Biochemical Assays.** Culture supernatants were assayed for albumin levels using a competitive enzyme-linked immunosorbent assay (MP Biomedicals, Santa Ana, CA) with horseradish peroxidase detection and 3,3',5,5'-tetramethylbenzidine (Rockland Immunochemicals) as the substrate. Alpha-fetoprotein was quantified using a sandwich ELISA (R&D Systems, Minneapolis, MN). Urea production was measured using a colorimetric assay with diacetylmonoxime, acid, and heat (Stanbio Labs, Boerne, TX). Luminescence-based assays (Promega, Madison, WI) for CYP2C9 (luciferin-H), CYP3A4 (luciferin-IPA), and the combined activity of CYPs 1A1, 1A2, 2B6, and 2D6 (luciferin-ME-EGE) were used to measure CYP450 activity. Briefly, cultures were rinsed in phenol red-free culture medium and incubated with luminescent substrates, diluted in the same medium, for 1 hour (3 µM luciferin-IPA, 10 µM luciferin-ME-EGE) or 3 hours (100 µM luciferin-H) at 37°C. Following incubation, supernatants were processed according to manufacturer instructions, and luminescence was measured using a luminometer (BioTek, Winooski, VT).

FDA-approved CYP450 substrates (Sigma-Aldrich, St. Louis, MO) included bupropion HCl (Bup), coumarin (Cou), dextromethorphan (Dex), phenacetin (Phe), (S)-mephénytoïn (S-Me), testosteron (Tes), and tolbutamide (Tol). Cultures were incubated with substrates (500 µM Bup, 50 µM Cou and Tol, 16 µM Dex, 100 µM Phe and S-Me, 200 µM Tes) for 3 hours at 37°C. Amounts of metabolites of the respective substrates, as provided in a previous publication, were quantified via liquid chromatography/mass spectrometry by Integrated Analytical Services (Berkeley, CA). Phase II metabolites, 7-hydroxycoumarin-glucuronide and 7-hydroxy-coumarin-sulfate, were quantified after incubating cells with 50 µM Cou.

**Drug Dosing.** Drugs were purchased from Sigma-Aldrich or Cayman Chemicals (Ann Arbor, MI). For CYP450 induction studies, cultures were treated in serum-free culture medium with rifampicin (25 µM) or phenobarbital (1 mM) or 0.1% vol/vol DMSO control dissolved in culture medium for 4 days, followed by quantitation of CYP3A4 and CYP2C9 activities using the luminescent assays described above. Analogously, cultures were treated with omeprazole (25, 50, and 100 µM) for 4 days followed by quantitation of CYP1A2 activity though the 1A2-mediated O-dealkylation of 7-ethoxyresorufin into fluorometric resorufin. For drug toxicity studies, iMPCCs were dosed in serum-free culture medium every 2 days for 8 days with multiple concentrations (12.5x, 25x, 50x, and
100x) of a drug’s $C_{\text{max}}$ (maximum human plasma concentration)\textsuperscript{21} or 0.1–0.2% vol/vol DMSO controls.

**Statistical Analysis.** Experiments were repeated 2–3 times with duplicate or triplicate samples for each condition. Data from representative experiments are presented, whereas similar trends were seen in multiple repeats. Statistical significance was determined using Student $t$ test (GraphPad Prism, La Jolla, CA) where shown. All error bars represent standard deviation.

Additional methods can be found in the Supporting Information.

**Results**

**Engineering the iMPCC Platform.** Characterization of liver gene expression and functions indicated that commercial batches of iCell Hepatocytes from Cellular Dynamics International (CDI; Madison, WI) were reproducibly iHeps (Supporting Fig. 1). We applied polydimethylsiloxane (PDMS) masks to simultaneously micropattern rat tail type I collagen into all wells of a tissue culture polystyrene plate (Fig. 1). Subsequent to selective attachment of iHeps to collagen domains, 3T3-J2 murine embryonic fibroblasts were seeded in the surrounding areas within 24 hours to create iMPCCs. The island diameter and spacing were optimized for iMPCCs, with higher liver functions observed when less of the surface area in each well was occupied by iHeps, relative to the area available for fibroblast growth (Supporting Fig. 2). Overlaying iMPCCs with Matrigel, an ECM commonly utilized for PHH culture,\textsuperscript{1} further improved liver functions (Supporting Fig. 3).

The iHeps that attached to collagen domains were positive for both ALB and glycogen (Fig. 2A). In iMPCCs, iHep morphology improved (polygonal shape, bile canaliculi, and distinct nuclei/nucleoli) and was maintained for 4 weeks (Fig. 2B). In contrast, density-matched micropatterned iHep cultures without fibroblasts (iMPHs) displayed a dedifferentiated (i.e., spread-out) morphology. Additionally, iHeps in iMPCCs became polarized, as assessed by excretion of a fluorescence dye into the bile canaliculi between cells and uptake of fluorescent low-density lipoprotein (LDL) into the cytoplasm (Fig. 2C). The bile canaliculi network was not completely formed around all iHeps, which is also observed with PHHs,\textsuperscript{6} though the mechanisms of in vitro canaliculi formation are not fully known.

**Liver Gene Expression and Functions in iHep Cultures.** Next, we profiled gene expression in iMPCCs, relative to pure iHep conventional confluent
cultures, with a Matrigel overlay (iCCs). We measured human-specific transcripts representing liver maturation factors and CYP450 enzymes involved in drug metabolism and toxicity (Fig. 3A).\(^{10,26}\) We found iMPCCs to have significantly higher expression for most of the liver transcripts, compared to both iCCs and the starting material from CDI, thereby suggesting a more highly differentiated phenotype in iMPCCs. Additionally, expression levels of most genes were maintained for several weeks in iMPCCs. The two exceptions were arginase 1 (ARG1; urea cycle enzyme) and CYP2D6, which were down-regulated in both iMPCCs (\(~1.7\text{-fold for } ARG1\) and \(~1.9\text{-fold for } CYP2D6\)) and iCCs (\(~20\text{-fold for } ARG1\) and \(~2.9\text{-fold for } CYP2D6\)) by the third week of culture, relative to the starting material. However, the rate of decline of these liver markers was highly dependent on the culture method, as also observed with PHHs.\(^{27}\)

The magnitude of albumin and urea secretion in iMPCCs was significantly greater (5-20×) than in pure iHep culture formats (Fig. 3B). Rates of secretion reached steady state by the first week in culture and were maintained for 4 weeks in iMPCCs. Despite higher functional maturity, alpha-fetoprotein (AFP) was still detected in supernatants from iMPCCs, albeit the albumin/AFP ratio in iMPCCs increased over time (i.e., AFP declined over time whereas ALB remained relatively stable), suggesting an improvement in the maturation status of iHeps in iMPCCs (Supporting Fig. 4). Furthermore, iMPCCs displayed stability of CYP450 enzyme activities for at least 4 weeks (Fig. 4A-C). CYP3A4 activity also remained stable in pure iHeps without fibroblasts for 4 weeks, potentially owing to the stable expression of hepatocyte nuclear factor 6 (HNF6), a transcriptional regulator of CYP3A4,\(^{28}\) in all models tested (Fig. 3A). However, the activities of other major CYP450s declined in pure iHep cultures and were significantly lower (\(~7\%-30\%) than in iMPCCs, as assessed by luminescence-based and U.S. Food and Drug Administration (FDA)-approved CYP450 substrates (Fig. 4A-C and Supporting Fig. 5). Even micropatterning iHeps alone without coculture (i.e., iMPH) yielded higher activities of some CYP450s (i.e., CYP2C9) on a per-cell basis, as compared to iCCs (Supporting Fig. 6). We also detected coupled phase I (CYP2A6) and II (glucuronidation and sulfation) metabolism in iMPCCs at higher levels than in pure iHep cultures (Fig. 4D and Supporting Fig. 7).

In order to enable on-demand creation of cultures using the same iHep batch, we demonstrated stable functions of cryopreserved iHeps from two different donors (one derived from fibroblasts and another from peripheral blood mononuclear cells; see Supporting Methods) in iMPCCs (Supporting Figs. 8 and 9). Cryopreserved iHeps cultured in iCCs did not display stable CYP3A4 activity over time or the same CYP3A4 levels as those cultured in iMPCCs, which

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**Fig. 2.** Characterization of iHep morphology and polarity. (A) Phase contrast images of iHeps attached to collagen domains at different magnifications (4 hours postseeding). Glycogen and albumin staining in the iHep islands within 2 days of seeding. (B) Phase-contrast images of iHeps in micropatterned formats 1 and 4 weeks after culturing either without fibroblasts (iMPHs: micropatterned pure iHep cultures) or with fibroblasts (iMPCCs). (C) Functional bile canaliculi in iMPCCs as assessed by excretion of CDF [5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate] into the bile canaliculi between iHeps. Uptake of fluorescent LDLs (DiI-LDL) in iHep cytoplasm in iMPCCs. Images from 1-week-old cultures are shown. All scale bars are 250 \(\mu\text{m}.\)
was in contrast with the CYP3A4 data obtained in both culture models using fresh iHeps from the same donor (Fig. 4C vs. Supporting Fig. 8B). Ultimately, use of cryopreserved iHeps will enable more reproducible, convenient, and higher-throughput screening applications than freshly prepared cells.

**CYP450 Induction Studies.** Modulation of CYP450s underlies many drug-drug interactions (DDIs) with serious pharmacological or toxicological consequences.\(^{29}\) Here, prototypical inducers (rifampicin and phenobarbital) induced both CYP450 (3A4, 2C9) messenger RNA (mRNA) transcripts (Fig. 5A,B) and functions in iMPCCs (Fig. 5D,E). On the other hand, induction response was severely blunted in iCCs (gold standard for induction studies) created from the same donor, as also observed in another study using iHeps.\(^{30}\) Induction response was also not always consistent across gene expression and functional activities in iCCs. Such differences across culture models could be owing to the higher expression of key nuclear receptors in iMPCCs, as compared to iCCs (Fig. 5C). The same iMPCC wells could be reinduced with drugs over several weeks (Supporting Fig. 10), which may open up new avenues for investigating complex, clinically relevant DDI scenarios.

**Comparison With PHHs.** The transcriptome of iHeps in iMPCCs was relatively stable (linear regression analysis: \(R^2 = 0.96; \) slope = 1.07) over several weeks in culture, as assessed by Affymetrix whole genome microarrays (Fig. 6A). Gene expression in iMPCCs was also compared with previously published data from two donors of freshly isolated PHHs and when the same donors were stabilized in PHH-MPCCs, but without the Matrigel overlay, as is standard for such a model.\(^6,20,21\) We found that iMPCCs at multiple time points in culture exhibited strong correlations in global gene expression with fresh PHHs (\(R^2 = 0.51-0.6; \) slope = 0.68-0.84) and PHH-MPCCs.
(R² = 0.6-0.73; slope = 0.88-0.98; Fig. 6B,C and Supporting Figs. 11 and 12). Part of the variability observed in gene expression between iMPCCs and PHHs could likely be attributed to donor differences, as also observed when comparing gene expression between two PHH donors (R² = 0.79; slope = 0.94; Supporting Fig. 12A). Analysis of iMPCC microarrays by CellNet, a network biology platform used to evaluate the fidelity of engineered cells by measuring the establishment of tissue-specific gene regulatory networks (GRNs), exclusively classified iMPCCs as liver (classifications scores: 0.837-0.880; Supporting Fig. 13). Moreover, the liver-specific GRN establishment scores for iMPCCs (0.925-0.959) fell within the range of scores for the two PHH donors, both when freshly isolated (0.848-0.978) and when stabilized in PHH-MPCCs (0.924-1.005; Supporting Fig. 14).

Gene expression values of ~95 liver-specific transcripts within diverse categories were next compared between iMPCCs and 2 donors of freshly isolated PHHs and the same PHH donors stabilized in PHH-MPCCs. We found that the majority of liver transcripts in iMPCCs were expressed between 10% and 200% of the levels noted in fresh PHHs (Fig. 6D,E). A subset of transcripts was shown to be significantly up-regulated in iMPCCs, relative to PHHs, including AFP, which was ~100-fold greater, whereas another subset of transcripts was expressed at levels less than 10% of PHHs (Supporting Fig. 15).

Next, we compared liver functions in iMPCCs with cryopreserved PHHs (2 donors) that were cultured in both conventional confluent and PHH-MPCC formats (Fig. 7A). We found that PHH-MPCCs outperformed PHH conventional cultures for seven of eight functions measured, and for four of eight functions, PHH-MPCCs demonstrated increased activity levels over PHHs on day of seeding (day 0). For seven of eight functions measured, iMPCCs were found to function between 5% (sulfation) and 55% (CYP2C9) of day 0 PHH levels. Albumin production was substantially greater in iMPCCs and PHH-MPCCs than in day 0 PHHs and conventional PHH cultures, which is owing to the well-documented recovery of this function in more stable culture formats. We found that iMPCCs displayed ~2-fold more albumin production than observed in PHH-MPCCs; however, donor
dependent variations likely underlie these observed differences. In addition to using luminescence-based substrates, we also measured the activities of major CYP450s in iMPCCs and PHH-MPCCs using FDA-approved enzyme-specific substrates (Supporting Fig. 16). Activities of CYP450 enzymes in iMPCCs ranged from ~5% (CYP2C19) to ~70% (CYP1A2) of PHH-MPCC levels, averaged across several donors. Dosing with phenobarbital and rifampicin resulted in induction of CYP450 (3A4, 2C9) transcripts and functions in both iMPCCs and PHH-MPCCs (Fig. 7B,C). Whereas the drug-mediated fold induction of CYP450 transcripts in iMPCCs was similar (60%-130%) to that observed in PHH-MPCCs, induction in CYP450 functions was typically lower (44%-79%). Last, we observed induction of iMPCC CYP1A2 activity in a dose-dependent manner using β-naphthoflavone and omeprazole, with fold induction in iMPCCs reaching ~70% of the values observed in PHH-MPCCs (Supporting Fig. 17).

**Drug Toxicity Studies.** To assess potential utility of iMPCCs for drug toxicity screening, we treated iMPCCs for 8 days with multiple doses of known hepatotoxins (acetaminophen, amiodarone, tolcapone, and troglitazone) and nontoxins with respect to the liver (aspirin, dextromethorphan, diclofenac, and propranolol). Albumin and urea production, previously shown to be sensitive markers for detecting drug-induced hepatotoxicity in vitro, declined significantly in iMPCCs after exposure to increasing doses of the toxins only (Fig. 8A), whereas the nontoxins minimally affected these functions (Fig. 8B).

**Discussion**

It is widely believed that in vitro culture protocols need to be improved to further mature iHeps toward the adult PHH phenotype and sustain phenotypic functions for prolonged times in culture. Khetani and Bhatia have previously developed a micropatterned coculture (MPCC) technique that stabilizes the PHH phenotype for several weeks in vitro. Here, we created the iHep-based iMPCCs as a hybrid culture system between the MPCC and the ECM sandwich.
techniques given that the synergy resulted in higher iHep functions. Furthermore, in contrast to the use of stencils by Khetani and Bhatia for patterning collagen, we utilized a PDMS mask-based soft-lithographic process to create iMPCCs in industry-standard 24- and 96-well plates, which are more suitable for higher-throughput drug screening applications (i.e., compatible with robotic fluid handlers). Our characterization of iHeps in iMPCCs revealed an in vivo–like hepatocyte morphology and polarity, expression of several key liver transcripts, secretion of liver-derived factors, and activities of major drug metabolism enzymes for at least 4 weeks in vitro. Gene expression and functions of iHeps in iMPCCs were significantly closer to PHHs than observed in the starting iHep material and in conventional iHep cultures utilized in the field. Last,
to demonstrate potential utility in the drug development pipeline, we evaluated drug-mediated induction of major CYP450 enzymes in iMPCCs and dose-dependent toxicity of hepatotoxins after repeat drug dosing in vitro.

We chose 3T3-J2 fibroblasts as the supportive cell type because of ease of propagation, contact inhibition of growth in culture, lack of detectable liver functions, and induction of liver phenotype in primary hepatocytes from multiple species, as compared to liver stromal cells. Even in iHeps, Swiss-3T3 fibroblasts are known to induce higher liver gene expression, compared to endothelial cells. Here, use of 3T3-J2s enabled quantitative comparisons between iMPCCs and PHH-MPCCs. Interestingly, the architecture determined to have the highest functions for iMPCCs was the same as that determined previously for PHH-MPCCs, suggesting that similar mechanisms underly-
Some promising approaches include stimulation with small molecules that can reduce fetal markers in iHeps,\textsuperscript{38} utilizing iHeps derived from PHHs to maintain the native epigenetic modifications,\textsuperscript{39} and direct lineage reprogramming of fibroblasts into iHeps using key hepatic fate and maturation transcription factors.\textsuperscript{15,18} None of the aforementioned approaches provides for fully mature iHeps, including iMPCCs, but combining approaches with controlled homotypic and heterotypic cell–cell interactions may lead to continued iHep maturation. In the future, we anticipate that iMPCCs could provide a more mature and long-term culture platform for drug screening applications, studying molecular mechanisms underlying iHep differentiation, modeling liver diseases, and integration into human-on-a-chip systems being designed to assess multiorgan responses to compounds.

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References


Fig. 8. Assessment of drug toxicity in iMPCCs. (A) ALB and urea secretion in iMPCCs after treatment for 8 days with multiple doses of prototypical hepatotoxins. $C_{\text{max}}$ represents the maximum blood concentration observed in human plasma for a given drug.\textsuperscript{21,40} (B) Same experimental design as in (A), except that iMPCCs were dosed with nontoxins with respect to the liver.


Author names in bold designate shared co-first authorship.

**Supporting Information**

Additional Supporting Information may be found at http://onlinelibrary.wiley.com/doi/10.1002/hep.27621/supinfo.
SUPPORTING FIGURES AND METHODS

Enhancing the Functional Maturity of Induced Pluripotent Stem Cell-Derived Human Hepatocytes via Controlled Presentation of Cell-Cell Interactions In Vitro

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SUPPORTING FIGURES

Supporting Figure 1: Characterization of iHep batches received from Cellular Dynamics International (CDI, Madison, WI). Average albumin production, urea secretion, and CYP3A4 activity (luciferin-IPA from Promega, Madison, WI) across three independent batches of micropatterned iHeps without fibroblasts after 48 hours of culture. All error bars represent standard deviation (n=3). Gene expression analysis of iHep batches (n=3) also verified the presence of mature hepatocyte markers including ALB (albumin), HNF4A (hepatocyte nuclear factor 4-alpha), HNF6 (hepatocyte nuclear factor 6), A1AT (α1-antitrypsin, SERPINA10), OATP2 (solute carrier organic anion transporter family, member 1B1, SLCO1B1), TAT (tyrosine aminotransferase), and TDO2 (tryptophan 2,3-dioxygenase) (data not shown).

Supporting Figure 2: Effect of island geometry on iMPCC functions.
Different micropatterned geometries allowed iHeps to cover 9.2% (500 μm diameter, 1200 μm center-center spacing), 16.6% (500 μm diameter, 900 μm center-center), 21.3% (700 μm diameter, 1100 μm center-center), or 26.1% (700 μm diameter, 1000 μm center-center) of the available surface area in each well of a 24-well plate. All cultures were then surrounded by 3T3-J2 murine embryonic fibroblasts within 24 hours of iHep seeding. Albumin, urea, and CYP3A4 functions at a representative time point (day 15) are shown. Data was normalized to the 9.2% geometry (500 μm diameter, 1200 μm center-center spacing). All error bars represent standard deviation (n = 3).
Supporting Figure 3: Matrigel™ overlay enhances iHep liver functions in the iMPCC model. Day 6 of culture is shown, but trends were seen for several weeks. All error bars represent standard deviation (n = 3). *p<0.05.

Supporting Figure 4: Hepatic maturation of iHeps in iMPCCs. Stabilized albumin (Alb) production and declining alpha-fetoprotein (Afp) production in iMPCC supernatants collected from (a) fresh iHeps and (b) cryopreserved iHeps, both from the same donor #1 (see supporting methods for information on donors). All error bars represent standard deviation (n = 3).
Supporting Figure 5: CYP450 characterization of iHeps in iMPCCs and iMPHs using FDA-approved substrates requiring LC-MS/MS quantitation. Data from day 15 for iMPCCs and iMPHs (micropatterned iHeps without fibroblasts) are shown, yet trends were seen over multiple time-points. Enzyme, substrate, metabolite measured: 1A2, phenacetin, acetaminophen; 2B6, bupropion HCl, hydroxybupropion; and 2D6, dextromethorphan, dextrophan. All error bars represent standard deviation (n = 3).

Supporting Figure 6: CYP450 characterization of iHeps in iCCs and iMPHs using luminescent-based substrates. Time-course of (a) CYP3A4, (b) CYP2C9, and (c) non-specific CYP450 activities (cleaved by CYP1A1, 1A2, 2B6, and 2D6) in micropatterned pure iHep cultures without fibroblasts (iMPHs), and conventional confluent iHep cultures with Matrigel™ overlay (iCCs) via assessment with luminescent assays (Promega). All error bars represent standard deviation (n = 3).
Supporting Figure 7: Time-course of coupled phase-I and phase-II enzymatic activity in iMPCCs. iMPCCs were dosed with 50 µM coumarin and amounts of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin-sulfate were quantified in cell culture supernatants using LC-MS/MS (Integrated Analytical Solutions, Berkeley, CA). CYP2A6 first generates 7-hydroxycoumarin prior to the subsequent conjugation reactions. All error bars represent standard deviations (n = 3).

Supporting Figure 8: Prolonged in vitro culture of cryopreserved iHeps. (a) Albumin secretion and (b) CYP3A4 enzyme activity (luciferin-IPA substrate from Promega) in iMPCCs relative to conventional confluent cultures with Matrigel™ overlay (iCCs). All error bars represent standard deviation (n = 3). The donor used for this experiment was iPSC donor #1 (see supporting methods).
Supporting Figure 9: iMPCCs created from another donor of cryopreserved iHeps. (a) Albumin and urea secretion and (b) CYP3A4 enzyme activity (luciferin-IPA substrate from Promega) in iMPCCs over time. The donor used to collect this data is iPSC donor #2 (see methods). All error bars represent standard deviation (n = 3).

Supporting Figure 10: Repeat drug-mediated CYP450 induction in iMPCCs. Cultures were treated with inducers (rifampicin at 25 µM and phenobarbital at 1 mM) for 4 days followed by incubation with a CYP3A4-specific luciferin-IPA substrate from Promega (day 22). Following 6 more days of culture in drug-free maintenance culture medium, iMPCCs were induced again for 4 days and the aforementioned CYP450 assay repeated (day 32). All error bars represent standard deviation (n = 3). *p<0.05.
Supporting Figure 11: Global gene expression profiling of iMPCCs and freshly isolated primary human hepatocytes (PHHs). (a) Scatter plot comparing gene expression profiles (Affymetrix whole genome human microarray) in two donors of freshly isolated PHHs in order to demonstrate donor-to-donor variability. Data was acquired from a previous publication.\(^1\) (b) Scatter plot comparing gene expression intensities in iMPCCs to expression intensities in PHH donor 1 of freshly isolated PHHs. (c-d) Similar to panel ‘b’ but comparisons between iMPCCs (two time-points in culture, days 9 and 21) and PHH donor 2 is shown.
Supporting Figure 12: Global gene expression profiling of iHeps in iMPCCs and primary human hepatocytes (PHHs) stabilized in PHH-MPCCs. Scatter plot analysis comparing gene expression intensities (Affymetrix whole genome human microarray) in iMPCCs (day 9 and day 21) created from a single iPSC donor to expression intensities in PHH-MPCCs generated from 2 human donors (PHH donor 1 comparisons shown in panel a and PHH donor 2 comparisons shown in panels b-c).
Supporting Figure 13: CellNet cell type and tissue classification of microarray samples from iMPCCs, freshly isolated primary human hepatocytes (PHHs), and PHH-MPCCs. Cell and tissue (C/T) classification scores are displayed in heat map format. C/T scores indicate the probability that a given sample expresses gene regulatory network (GRN) genes to the same extent as those found in tissue specific training data. Liver classification scores for iMPCCs (0.880 for day 9 and 0.837 for day 21 cultures) were similar to scores observed in freshly isolated (0.967 for PHH donor 1 and 0.992 for PHH donor 2) and PHH-MPCCs (0.864 for PHH donor 1 and 0.975 for PHH donor 2). iHeps were classified exclusively as liver with C/T classification scores for all other tissue types ≤ 0.09. More details for the CellNet analysis schemes are provided in another publication.²

Supporting Figure 14: CellNet gene regulatory network (GRN) establishment scores from iMPCCs, freshly isolated primary human hepatocytes (PHHs) and PHH-MPCCs. Liver GRN scores represent the extent to which gene regulatory networks are established in a given set of samples as compared to liver-specific training data. Liver GRN scores for iMPCCs (0.959 for day 9 and 0.925 for day 21) fall between the scores provided for freshly isolated (0.978 for PHH donor 1 and 0.848 for PHH donor 2) and PHH-MPCCs (0.924 for PHH donor 1 and 1.005 for PHH donor 2). More details for the CellNet analysis schemes are provided in another publication.²
Supporting Figure 15: Liver-specific transcripts in iMPCCs with greater than 200% or less than 10% of the gene expression intensities observed in freshly isolated primary human hepatocytes (PHHs). RNA was extracted from 2 donors of freshly isolated PHHs prior to plating. PHHs were plated in the micropatterned co-culture format (PHH-MPCCs), and RNA was extracted at day 42 (PHH donor 1) or day 6 (PHH donor 2). iMPCCs were created using iHeps from iPSC donor 1 (see methods) (a) iMPCC transcripts with greater than 200% of PHH levels. (b) iMPCC transcripts with less than 10% of PHH levels. Ph. 1, phase I enzymes; Ph. II, phase II enzymes; TP, transporter proteins; NR, nuclear receptors; *, other liver-specific genes. All PHH gene expression levels (freshly isolated and PHH-MPCC) are the average of two donors.
Supp
taining Figure 16: CYP450 characterization of PHH-MPCCs and iMPCCs using FDA-approved substrates requiring LC-MS/MS analyses. CYP450 activities in PHH-MPCCs are averaged from 3-5 donors between 1 and 2 weeks of culturing when enzyme activities are known to be stable. For iHeps in iMPCCs, data from day 15 of culture is shown but trends were seen over multiple time-points. Enzyme, substrate, metabolite measured: 1A2, phenacetin, acetaminophen; 2A6, coumarin, 7-hydroxy-coumarin; 2B6, bupropion HCl, hydroxyl-bupropion; 2C8, paclitaxel, 6-alpha-hydroxy-paclitaxel; 2C9, tolbutamide, 4-hydroxy-tolbutamide; 2C19, s-mephenytoin, 4-hydroxy-s-mephenytoin; 2D6, dextromethorphan, dextorphan; and 3A4, testosterone, 6-beta-hydroxy-testosterone. All error bars represent standard deviation (n = 3-5).

Supporting Figure 17: CYP1A2 induction in iMPCCs and PHH-MPCCs via omeprazole and β-naphthoflavone. (a) Functionally stable iMPCCs were treated with omeprazole (25, 50 and 100µM) for 4 days followed by quantitation of CYP1A2 activity though the 1A2-mediated O-dealkylation of 7-ethoxyresorufin into fluorometric resorufin. (b) PHH-MPCCs were tested in a similar manner as described in panel 'a', but at a single dose of omeprazole (50µM). Induction levels in PHH-MPCCs were compared to those obtained in iMPCCs at the same dose administered for the same time duration. (c-d) Same as in panels 'a' and 'b', but iMPCCs and PHH-MPCCs were treated with β-naphthoflavone as a CYP1A2 inducer. All error bars represent standard deviation (n = 3).
SUPPORTING METHODS

Processing of iHeps

Cellular Dynamics International (CDI, Madison, WI) generated ~95% pure iPSC-derived human hepatocyte-like cells or iHeps (commercially available as iCell® Hepatocytes) as assessed via α1-antitrypsin using a proprietary differentiation protocol. Per information provided by CDI, donor 1 is a Caucasian female and iPSCs were reprogrammed from fibroblasts, while donor 2 is a Caucasian male and iPSCs were reprogrammed from peripheral blood mononuclear cells. All of the studies here were conducted with fresh or cryopreserved iHeps differentiated from donor 1 cells, except for Supporting Figure 9, which utilized donor 2. Fresh iHeps were processed according to manufacturer’s instructions. Briefly, fresh iHep aggregates were pelleted via centrifugation, dissociated with 0.5% trypsin-EDTA (Life Technologies, Carlsbad, CA), and cellular debris was removed using KryoThaw (SciKon Innovation, Durham, NC). iHeps were diluted in Roswell Park Memorial Institute 1640 medium (Life Technologies), containing 1 µM dexamethasone (Sigma-Aldrich, St Louis, MO), 2% v/v B27 (Life Technologies), 1% v/v penicillin/streptomycin (Cellgro, Manassas, VA), and 20 ng/mL oncostatin-M (R&D Systems, Minneapolis, MN). Cryopreserved iHeps were processed according to manufacturer protocols.

Culture of primary human hepatocytes (PHH)

Freshly isolated and induction-qualified cryopreserved PHHs were purchased from vendors permitted to sell products derived from human organs procured in the United States by federally designated Organ Procurement Organizations. These vendors included Triangle Research Labs (Research Triangle Park, NC) and BioreclamationIVT (Baltimore, MD). Cryopreserved PHH donor 1 was a Caucasian female, age 54 (lot HUM4055A), while cryopreserved PHH donor 2 was an African-American male, age 55 (lot HUM4056B). Cryopreserved hepatocytes were thawed, counted and viability was assessed as previously described.3 Micropatterned co-cultures of PHH and 3T3-J2 fibroblasts4 were created using similar protocols as described for iMPCCs but with a seeding density of 6.66 x 10^5 cells/mL and without the Matrigel™ overlay to remain consistent with the previous publication on PHH-MPCCs.1 Conventional PHH monolayer cultures were created as previously described.1,3 Briefly, ~350,000 hepatocytes were seeded in each collagen-coated (rat tail type I, Corning Biosciences) well of a 24-well plate in serum-supplemented hepatocyte medium. Monolayer cultures were maintained in serum-free culture medium (500 µL/well) with daily replacement of medium as previously described.1

Gene expression profiling

RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA), treated with Optizyme Recombinant DNase I to remove genomic DNA (Fisher Bioreagents, Pittsburgh, PA), and reverse transcribed into cDNA using random primers and dNTPs with MultiScribe Reverse Transcriptase (Life Technologies). qPCR was performed on a MasterCycler RealPlex-2 (Eppendorf, Hamburg, Germany) using pre-designed probe-primer gene expression assays (GE Dharmaco, Lafayette, CO). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression levels were calculated using the delta-delta CT method relative to the level in freshly processed iHeps.

Global gene expression profiling was performed using Affymetrix Human Genome (HG) U133 Plus 2.0 Microarrays by the Genomics Shared Resource of the University of Colorado Denver Cancer Center (P30-CA046934) using published protocols.1,5 Raw intensity values on the microarrays were normalized, modeled, and compared using dChip (DNA Chip Analyzer,
The invariant set normalization method was used to compare arrays and the PM-MM model-based expression index analysis was used for computing expression values for gene comparison. Gene expression values from iHeps were compared against two donors of freshly isolated PHHs and PHH from the same donors cultured in PHH-MPCCs. The PHH data was obtained from a previous publication. CellNet, a network biology platform, was used to analyze the microarray samples to provide tissue-specific classification scores and to verify the establishment of liver-specific gene regulatory networks (GRNs). All Affymetrix arrays utilized in this study have been submitted to Gene Expression Omnibus (GEO accession: GSE62962).
REFERENCES