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Corresponding Author:	Lay Teng Ang, Ph.D. Stanford University Stanford, UNITED STATES
Corresponding Author's Institution:	Stanford University
Corresponding Author E-Mail:	layteng@stanford.edu
Order of Authors:	Kyle M. Loh
	Amrita Palaria
	Lay Teng Ang
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TITLE:

Efficient Differentiation of Human Pluripotent Stem Cells into Liver Cells

AUTHORS & AFFILIATIONS:

Kyle M. Loh[1,2], Amrita Palaria[1], Lay Teng Ang[1]

[1]Institute for Stem Cell Biology & Regenerative Medicine, Stanford-UC Berkeley Siebel Stem Cell Institute, Stanford University School of Medicine, Stanford, CA
[2]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA

Kyle M. Loh: kyleloh@stanford.edu
Amrita Palaria: apalaria@stanford.edu
Lay Teng Ang: layteng@stanford.edu

Corresponding Author:
Lay Teng Ang
layteng@stanford.edu

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SUMMARY:

This protocol details a monolayer, serum-free method to efficiently generate hepatocyte-like cells from human pluripotent stem cells (hPSCs) in 18 days. This entails six steps as hPSCs sequentially differentiate into intermediate cell-types such as the primitive streak, definitive endoderm, posterior foregut and liver bud progenitors before forming hepatocyte-like cells.

ABSTRACT:

The liver detoxifies harmful substances, secretes vital proteins, and executes key metabolic activities, thus sustaining life. Consequently, liver failure—which can be caused by chronic alcohol intake, hepatitis, acute poisoning, or other insults—is a severe condition that can culminate in bleeding, jaundice, coma, and eventually death. However, approaches to treat liver failure, as well as studies of liver function and disease, have been stymied in part by the lack of a plentiful supply of human liver cells. To this end, this protocol details the efficient differentiation of human pluripotent stem cells (hPSCs) into hepatocyte-like cells, guided by a developmental roadmap that describes how liver fate is specified across six consecutive differentiation steps. By manipulating developmental signaling pathways to promote liver differentiation and to explicitly suppress the formation of unwanted cell fates, this method efficiently generates populations of human liver bud progenitors and hepatocyte-like cells by days 6 and 18 of PSC differentiation, respectively. This is achieved through the temporally-precise control of developmental signaling pathways, exerted by small molecules and growth factors in a serum-free culture medium. Differentiation in this system occurs in monolayers and yields hepatocyte-like cells that express characteristic hepatocyte enzymes and have the ability to engraft a mouse model of chronic liver failure. The ability to efficiently generate large

numbers of human liver cells in vitro has ramifications for treatment of liver failure, for drug screening, and for mechanistic studies of liver disease.

INTRODUCTION:

The purpose of this protocol is to efficiently differentiate human pluripotent stem cells (hPSCs) into enriched populations of liver bud progenitors and hepatocyte-like cells[2]. Access to a ready supply of human liver progenitors and hepatocyte-like cells will accelerate efforts to investigate liver function and disease and could enable new cellular transplantation therapies for liver failure[3-5]. This has proven challenging in the past since hPSCs (which include embryonic and induced pluripotent stem cells) can differentiate into all the cell-types of the human body; consequently, it has been difficult to exclusively differentiate them into a pure population of a single cell-type, such as liver cells[6].

To precisely differentiate hPSCs into liver cells, first it is critical to understand not only how liver cells are specified but also how non-liver cell-types develop. Knowledge of how non-liver cells develop is important to logically suppress the formation of non-liver lineages during differentiation, thereby exclusively guiding hPSCs towards a liver fate[2]. Second, it is essential to delineate the multiple developmental steps through which hPSCs differentiate towards a liver fate. It is known that hPSCs sequentially differentiate into multiple cell-types known as the primitive streak (APS), definitive endoderm (DE), posterior foregut (PFG) and liver bud progenitors (LB) before forming hepatocyte-like cells (HEP). Earlier work revealed the signals specifying liver fate and the signals that suppressed the formation of alternate non-liver cell-types (including bone, pancreatic, and intestinal progenitors) at each developmental lineage choice[2,7,8].

Collectively, these insights have given rise to a serum-free, monolayer method to differentiate hPSCs towards primitive streak, definitive endoderm, posterior foregut, liver bud progenitors and finally, hepatocyte-like cells[2]. Overall the method involves the seeding of hPSCs in a monolayer at an appropriate density, preparing six cocktails of differentiation media (containing growth factors and small molecules that regulate various developmental signaling pathways), and sequentially adding these media to induce differentiation over the course of 18 days (d). During the process, no passaging of cells is needed. Of note, because this method explicitly includes signals that suppress the formation of non-liver cell-types, this differentiation approach[1] more efficiently generates liver progenitors and hepatocyte-like cells by comparison to extant differentiation methods[2,9-12]. Furthermore, the protocol described in this text enables the much faster generation of hepatocytes (~2-3 times faster) that ultimately express higher levels of hepatic transcription factors and enzymes than those produced by other protocols[9-12].

The protocol described here has certain advantages over current differentiation protocols. First, it entails monolayer differentiation of hPSCs, which is technically simpler compared to three-dimensional differentiation methods, such as those that rely on embryoid bodies[13]. Second, this is a feeder-free and serum-free differentiation system, which enables more consistent and reproducible results compared to methods that use undefined media[10-12]. Third, this method exploits a recent advance whereby definitive endoderm cells (an early precursor to liver cells)

can be efficiently and rapidly generated within 2 days of hPSC differentiation[2, 7], thus enabling the subsequent production of hepatocytes with increased purity. Fourth, in side-by-side comparisons, the hepatocyte-like cells produced by this method[2] produce more albumin and express higher levels of hepatic transcription factors and enzymes compared to hepatocytes produced in other methods[10-12].

PROTOCOL:

1. Preparation of differentiation media

NOTE: Refer to the **Table of Materials** for manufacturer information regarding the materials and reagents used.

1.1. Preparation of base chemically defined media (CDM)

NOTE: CDM2, CDM3, CDM4 and CDM5 are chemically defined media that are used as base media for differentiating hPSCs to liver cells at various stages.

1.1.1. To make CDM2 or CDM3, prepare a stock solution containing polyvinyl alcohol (PVA). Dissolve 0.5 g of PVA powder in 50 mL of Iscove's Modified Dulbecco's Medium (IMDM) to generate a 10 mg/mL PVA stock.

NOTE: Since PVA does not dissolve easily, prepare the PVA/IMDM mixture in a conical flask with continuous stirring at 50 °C on a heating pad; stirring can be easily achieved using a magnetic stirrer.

1.1.2. After a homogenous solution of PVA is dissolved in IMDM, remove the PVA solution from the heating pad and allow it to cool down to room temperature.

1.1.3. Use a sterile, 0.2 µm filter to filter the PVA solution.

1.1.4. Prepare the CDM2 and CDM3 by combining the filtered PVA from step 1.1.1 and various commercially bought components as outlined in **Table 1** and the **Table of Materials**. Use sterile, 0.2 µm filter units to filter all media.

NOTE: Base medium can be stored at 4 °C but for no longer than 2 months.

1.1.5. Prepare the remaining base media CDM4 and CDM5 following **Table 1**.

1.2. Preparation of differentiation media

1.2.1. To prepare stock solutions for the small molecules and growth factors, reconstitute them as per the manufacturers' recommendations. For storage, aliquot into sterile tubes to reduce freeze-thaw cycles and keep at -20 °C or as recommended.

NOTE: Composition of differentiation medium to be used at various differentiation stages is described in **Table 2** and in the following steps.

1.2.2. To prepare the differentiation medium, first thaw the frozen small molecules and/or growth factors at room temperature. Next, aliquot out the required amount of base medium. Last, prepare the final differentiation media by adding the specified small molecules and growth factors to the base medium at the appropriate concentrations (**Table 2**).

NOTE: Freshly-prepared differentiation medium should ideally be used on the same day; otherwise it can be stored at 4 °C and used within three days.

1.2.3. Using a pipette tip, mix the differentiation medium several times to ensure supplements are homogeneously distributed before adding the medium to cells (e.g., add 1 mL of differentiation medium to each well of a 12-well plate.)

2. Seed hPSCs onto plates at defined densities for differentiation

2.1. Coat cell culture plastics that will be used for seeding with hPSCs.

2.1.1. Thaw the matrix (e.g., Geltrex) at 4 °C overnight before the day of use.

2.1.2. The next day, dilute the matrix 1:100 by adding 500 µL of the matrix into 50 mL of cold Dulbecco's Modified Eagle's Medium (DMEM)/F12. Since the matrix is a hydrogel that irreversibly polymerizes upon exposure to room temperature, when working with the matrix, always keep the matrix tubes and media on ice.

NOTE: The matrix dissolved 1:100 in DMEM/F12 can be stored at 4 °C but should be used within 2 months.

2.1.3. To coat a culture plate with the matrix, pipette the diluted matrix into the required number of wells using just enough volume of matrix solution to cover the surface of the well (e.g., add 0.5 mL of matrix to one well of a 12-well plate or 1 mL to one well of a 6-well plate). If needed, shake the plate gently to make sure that the matrix solution has fully covered the bottom of the well.

2.1.4. Leave the matrix-coated plate in a 37 °C incubator for at least 60 min. At this temperature, the matrix polymerizes to form a thin film at the bottom of the well.

NOTE: Matrix-coated plates can be kept in the 37 °C incubator and used within 3 days as long as the matrix has not dried up.

2.1.5. Aspirate the remaining matrix solution from the coated wells immediately prior to seeding the wells with hPSCs.

2.2. Passaging and seeding the coated plates with hPSCs

2.2.1. To seed matrix-coated culture plates with hPSCs prior to differentiation, grow undifferentiated hPSCs to >70% confluency in commercially-available mTeSR1 medium (**Table of Materials**) according to the manufacturer's protocol. It is critical to passage hPSCs before they become fully confluent, as hPSCs may spontaneously differentiate at high confluence.

2.2.2. To seed hPSCs for differentiation, aspirate mTeSR1 from largely-confluent hPSC cultures plate and add commercially bought dissociation agent (**Table of Materials**) to dissociate the hPSCs, using just enough dissociation agent to cover the surface of the well or dish on which the cells are growing (e.g., add 0.5 mL of the dissociation agent per well of a 12-well plate, 1 mL per well of a 6-well plate or 3 mL per 10 cm dish).

2.2.3. Incubate hPSCs in the dissociation agent at 37 °C for 5 min or until some colonies begin detaching. Gently tap the bottom of the well/plate several times; after several minutes of dissociation, most hPSC colonies should freely come into suspension.

NOTE: The dissociation agent contains enzymes that dissociate cells and it is important to leave hPSCs in the dissociation agent for as short of a timespan as possible.

2.2.4. To dislodge dissociated hPSCs from the plate, add 2 mL/well of DMEM/F12 if working with a 6-well plate (or 1 mL/well if working with a 12-well plate) to dilute the dissociation agent. Use a 5 mL serological pipette to gently pipette up and down multiple times to wash off all cells from the surface of the well; collect resuspended single cells in a 50 mL conical tube. Wash the plate a second time with the same volume of DMEM/F12 to ensure recovery of all hPSCs.

2.2.5. To the 50 mL conical tube containing the cells, add DMEM/F12 to dilute the original volume of the dissociation agent by 1:5-1:10 (e.g., if the original volume of the dissociation agent was 1 mL, adjust the total volume of cell suspension to 10 mL with DMEM/F12 to dilute the dissociation agent at 1:10)

2.2.6. Centrifuge the collected hPSCs in a 50 mL conical tube at 350 x *g* for 3 min at 4 °C to pellet cells.

2.2.7. While waiting for the cells to pellet, aspirate matrix from the plate in which the hPSCS will be seeded. Next, add sufficient amounts of mTesR1 media to recipient wells to cover them (e.g., add 0.5 mL mTeSR1 per well of a 12-well plate or 1 mL of mTeSR1 per well of a 6-well plate).

2.2.8. After centrifuging hPSCs, carefully aspirate the supernatant, leaving the pelleted hPSCs at the bottom of the conical tube. The supernatant contains dissociation agent which will inhibit subsequent adhesion of hPSCs and thus, it is important to aspirate the large majority of

the supernatant before proceeding.

2.2.9. Resuspend the cell pellet in mTeSR1 supplemented with 1 µM of commercially-obtained thiazovivin (a pharmacological ROCK inhibitor). With a p1000 pipette, gently triturate 2-3 times to evenly resuspend the cell pellet into a single cell suspension. Do not over-triturate the cell pellet as excessive mechanical force will damage hPSCs and lead to poor cell survival.

NOTE: Thiazovivin at a low concentration is included at this step to enhance single cell survival and hence the subsequent seeding density of hPSCs.

2.2.10. After resuspension of the hPSCs, immediately pipette 10 µL of the suspension into a hemocytometer and count the number of cells. It is important to pipette cells for counting as quickly as possible, as gravity will lead to hPSCs naturally settling into the bottom of the 50 mL tube, which will confound accurate cell counting.

2.2.11. Adjust the volume of the resuspended hPSCs with thiozavivin supplemented mTeSR1 to achieve the desired cell concentration for plating. For example, seed 150,000-225,000 cells into each well of a 12-well plate, use 0.5 mL of the cell suspension thus achieving a total of 1 mL in each well (0.5 mL of media was added to the well in step 2.2.7). If larger wells are being used, scale up accordingly.

NOTE: It is important to seed hPSCs at the indicated cell density, which will yield sparse hPSCs that are ready for subsequent differentiation. Overly-confluent hPSCs will not differentiate efificiently.

2.2.12. Shake the plate in a cross pattern (left, then right; forward, then backward) several times to make sure cells are evenly distributed across the plate/well. Do not swirl the plate in a circular motion, as the cells will settle in the center of the plate/well. Typically, hPSCs will begin adhering to the surface of the well within minutes or tens of minutes. Allow cells to grow for at least 24 hours before initiating differentiation.

3. Differentiation of hPSCs into endodermal cells and liver progenitors

3.1. After hPSCs have been plated for at least 24 hours, as described in step 2.2.12, before proceeding with differentiation, check the morphology of cells under a phase-contrast microscope, with specific emphasis on the diameter of plated hPSC colonies.

NOTE: Ideally, clumps will be small in size (less than or equal to 100 µm in diameter) and readily spaced throughout the well. Large colonies of hPSCs greater than 100 µm in diameter are not usable for differentiation. Only small- or mid-sized clumps are acceptable for differentiation; differentiation signals will not act evenly throughout large hPSC colonies, leading to inefficient differentiation.

3.2. If colony sizes are ideal in step 3.1, proceed to day 1 of differentiation, which entails differentiation of hPSCs into anterior primitive streak (APS).

3.3. Prepare day 1 APS differentiation medium by mixing all reagents outlined in **Table 2** using CDM 2 (**Table 1** and section 1.2) as the base medium. Pipet to mix several times to ensure even distribution of the components in the media.

3.4. Aspirate to remove the thiazovivin supplemented mTeSR1 from the plated hPSCs and briefly wash hPSCs with IMDM media. Do not wash with phosphate buffered saline (PBS) instead of IMDM, as PBS lacks calcium ions (Ca^{2+}) and is thus toxic to hPSCs; it will disrupt cell morphology.

3.5. After the brief IMDM wash, add day 1 medium to the hPSCs. Record the time and place cells back into the 37 °C incubator

3.6. Continue with the subsequent differentiation steps by preparing (**Table 2**) and adding the respective differentiation medium to the cells on the respective days (at 24 hour intervals) of differentiation around the same time of the day (**Figure 1**). Replace with fresh media daily, even if consecutive days of differentiation use the same differentiation media. Between media changes, wash cells once with IMDM media to remove dead cells and remnant of previous medium components.

3.7. As differentiation progresses beyond the liver bud stage, cell numbers increase and hence, add more media to each well of the plate to ensure that the amount of differentiation factors and nutrients is not limiting. For example, add 1 mL differentiation medium to each well of 12-well initially on days 1 to 6 of differentiation but add 1.5 mL-2 mL of subsequent differentiation media on later days of differentiation (days 7 to 18 of differentiation).

4. Characterization of endodermal cells and liver progenitors by immunostaining

4.1. Prepare blocking buffer: 10% donkey serum + 0.1% Triton X100 in deionized phosphate buffered saline (DPBS).

4.2. Prepare staining buffer: 1% donkey serum + 0.1% Triton X100 in DPBS.

4.3. Aspirate medium from cells in 12-well plate.

4.4. Add 4% paraformaldehyde (in DPBS) for 15 min at room temperature to fix cells and then wash the cells twice with DPBS.

4.5. Add blocking buffer for 1 h at room temperature to block and permeabilize the fixed cells.

4.6. Aspirate the blocking solution and add primary antibody diluted in staining buffer. (See **Table of Materials** for dilution ratios of antibodies.)

4.7. Stain the cells overnight at 4 °C.

4.8. Wash cells thrice with 0.1% Triton X100 in DPBS.

4.9. Add secondary antibody stain in the staining buffer for 1 h at room temperature. (See **Table of Materials** for dilutions of secondary antibodies.)

4.10. Remove the secondary antibody and add DAPI for 5 min at room temperature to conduct nuclear counterstain.

4.11. Wash twice with 0.1% Triton X100 in DPBS to remove excess antibody and DAPI.

4.12. Conduct fluorescence microscopy with a Zeiss Observer D1. Alternatively, store plate in 4 °C until imaging is performed. For expected results, see **Figure 3**.

5. Characterization of liver progenitors by Fluorescence Activated Cell-Sorting (FACS) analysis

NOTE: Use FACS to precisely quantify the percentage of AFP+ differentiated LB cells that emerge by day 6 of differentiation. Follow the same steps to quantify the percentage of ALB+ differentiated hepatocytes by day 18 of differentiation.

5.1. Conjugate an anti-Afp antibody.

5.2. Add R-phycoerythrin to anti-Afp antibody at a concentration of (0.55 µg/µL) using R-phycoerythrin conjugation kit (see **Table of Materials**).

NOTE: Ensure that the antibodies are purified and are reconstituted in buffers that do not contain amines. Ensure that amount of antibody used in a labeling reaction must be less than the amount of PE (i.e., 60 µg of antibody with 100 µg of PE). Poor conjugation of antibodies may lead to unreliable outcomes.

5.3. Add 1 µL of modifier reagent for 10 µL of antibody to be labeled. Mix gently.

5.4. Remove the R-PE mix (100 µL) and pipette the above mixture directly into the lyophilized R-PE material.

5.5. Place the cap back and leave the vial standing for 3 h in the dark at room temperature.

5.6. After incubating for 3 h or more, add 1 µL of quencher reagent for 10 µL of antibody used. The conjugate can be used after 30 minutes.

5.7. Store conjugated antibody at 4 °C.

5.8. Wash differentiated or undifferentiated hPSCs in 6-well format with DMEM/F12.

5.9. Briefly treat with dissociation agent (1 mL/well in a 6-well plate) for 5 min at room temperature until cells detach. Harvest and stain both undifferentiated hPSC and differentiated liver progenitor cells identically and analyze in parallel in the same experiment to ensure specificity of antibody staining.

5.10. Gently tap Petri dish to detach cells. Use a p1000 pipette to detach cells off the plate and collect cells in a 50 mL conical tube. Ensure that cells have mostly detached before proceeding with the next step. Subsequently, wash wells twice more with 1xDPBS buffer to collect residual cells.

5.11. In the 50mL conical tube, dilute dissociation agent in ~5 volumes of 1xDPBS buffer. Triturate rigorously 3-4 times with a p1000 pipette to ensure all cells are dissociated into single cells.

NOTE: It is imperative to generate single cells **prior** to centrifugation or clumps subsequently cannot be dissociated with ease. However, do not over-triturate as this may damage cell integrity. Count the number of cells and use the recommended proportion of cells to antibody ratio, which has been previously optimized for minimal background and maximal signal detection.

5.12. Centrifuge conical tube containing the cell suspension at 300 x *g* for 5 min at 4 °C.

5.13. Aspirate the supernatant with care not to disturb the cell pellet.

5.14. Resuspend cell pellet thoroughly in fixation/perm buffer to generate a single-cell suspension and fix on ice at 4 °C for 20 min. Note to transfer to a 2 mL microcentrifuge tube. Furthermore, usage of a 2 mL microcentrifuge tube at this step enables the cell pellet to deposit at the V-shaped-corner of the tube, minimizing cell loss during washes.

5.15. Wash each pellet with 1.8 mL of perm/wash buffer twice. Resuspend cell pellet thoroughly in perm/wash buffer by pipette mixing 6 times with a P1000 pipette. Then, centrifuge, remove supernatant and repeat the wash process with 1.8 mL of perm/wash buffer.

5.16. Resuspend cell pellet in perm/wash buffer so that there will be 100 µL/individual stain and subsequently transfer the cell suspension into a 2 mL microcentrifuge tube.

NOTE: It is imperative to generate a single-cell suspension at this step prior to antibody staining. Aggregates of cells will not be stained and thus will confound FACS analysis. Furthermore, usage of a 2 mL microcentrifuge tube at this step enables the cell pellet to deposit

at the V-shaped-corner of the tube, minimizing cell loss during washes.

5.17. After resuspending cells in FACS buffer, aliquot them into individual 2 mL tubes (for both unstained control and antibody-stained samples).

5.18. Stain with anti-AFP-PE 0.33 µL per 150,000 cells for 30 min at room temperature in the dark. For example, for a 100 µL individual stain, stain as follows: 0.33 µL of α-AFP PE and 100 µL of perm/wash buffer. Resuspend cells with p200 pipette well to ensure even staining.

NOTE: Only pipette-mix and do not vortex as this may reduce protein stability.

5.19. Wash, resuspend the cells twice in 1-2 mL of perm/wash buffer (1.9 mL/individual stain) and centrifuge at 800 x *g* for 5 min at 4 °C. Wash cells with no less than 1-2 mL of perm/wash buffer to ensure sufficient washing.

NOTE: Only pipette mix and do not vortex as this may reduce protein stability. After centrifuging, aspirate supernatant carefully to prevent cells from dislodging and remove as much supernatant as possible to minimize antibody carry-over and ensure a more complete wash.

5.20. Resuspend each washed pellet in 300 µL of perm/wash buffer and strain it through a 100 µm filter into a FACS tube to strain out large clumps of cells prior to FACS analysis.

5.21. Analyze cells on a FACS Aria flow cytometry on the PE channel. Analyze a minimum of 10,000 events for each individual stain, and parse events by virtue of FSC-A/SSC-A analysis, select cell singlets by gating on FSC-W/FSC-H followed by SSC-H/SSC-W.

REPRESENTATIVE RESULTS:

After 24 hours of APS differentiation, colonies will generally adopt a different morphology than undifferentiated colonies concomitant with a loss of the bright border that typically circumscribes hPSC colonies. Morphologically, primitive streak cells generally have ragged borders and are more spread and less compact than hPSCs—this is evocative of an epithelial-to-mesenchymal transition as pluripotent epiblast cells differentiate and ingress into the primitive streak in vivo. If the colony size of the hPSCs prior to differentiation was too large, there will be an overtly-raised center that comprises undifferentiated cells. Cultures containing such large clumps should be discarded, as these undifferentiated colony centers will confound subsequent differentiation. If clumps of the correct size were plated, APS differentiation is highly reproducible and uniform, generating a 99.3±0.1% *MIXL1-Gfp*+ primitive streak population (MIXL1 is a primitive streak marker)[7]. Note that some cell death is observed after 24 h of APS differentiation.

By day 2 of differentiation, primitive streak cells have differentiated into day 2 definitive endoderm cells, the vast majority of which express SOX17 (**Figure 2**) and FOXA2. In dozens of independent experiments with 14 hESC and hiPSC lines (including H1, H7, H9, HES2, HES3, BJC1,

BJC3, HUF1C4 and HUF58C4), this approach scalably, consistently and efficiently generated pure definitive endoderm populations (94.0% ± 3.1%)[7]. This method to generate definitive endoderm from hPSCs yields higher percentages of $CXCR4^{+}PDGFRA^{-}$ endodermal populations compared to other endoderm-forming approaches[2,14]. While some cell death is sometimes observed during this stage, most cells will remain adhered to the plate surface by end of day 2 of differentiation.

By day 3 of differentiation, endoderm has differentiated into foregut progenitors that appear polygonal in shape (**Figure 1**). Later, by 6 days of differentiation, the foregut progenitors differentiate into liver bud progenitors expressing AFP, TBX3, and HNF4A (**Figure 3**). Throughout 3 hPSC lines, this yielded an average 89±3.1% AFP+ LB population by FACS. Across three hESC lines (H1, H7, H9 hESCs), this method generates day 6 AFP^{+} liver progenitors form at an efficiency of 89.0±3.1% (**Figure 2**). Finally, after 18 days of liver differentiation from hPSCs, $ALBUMIN^{+}$ hepatocyte-like cells appear. Morphologically, they appear epithelial, forming bright borders reminiscent of bile canaliculi (**Figure 1**). At this stage, the cytoplasm of the day 18 hPSC-derived hepatocytes appears darker than the nucleus (**Figure 1**).

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of differentiation strategy and morphology of undifferentiated hPSCs, differentiating endoderm and hepatocyte-like cells. Differentiation process and timeline were depicted. Abbreviations: d = day, hPSC = human pluripotent stem cells, APS = anterior primitive streak, DE = definitive endoderm, PFG = posterior foregut, LB = liver bud progenitors, HP = hepatocyte progenitors, HEP = hepatocyte like cells. Scale bar = 400 μm.

Figure 2. Percentage of day 1 MIXL1+ primitive streak, day 2 SOX17+ definitive (def) endoderm, day 6 AFP+ liver bud progenitors and ALB+ hepatocyte populations as shown by FACS.

Figure 3. Immunostaining analysis of day 6 liver progenitors. hPSC were first differentiated into liver bud progenitors, which were immunostained for liver bud transcription factors HNF4A (red), TBX3 (green) as well as cytoplasmic liver bud marker AFP (red). Nuclei were counterstained with DAPI (blue) to assess total cell number. Scale bar = 50 μm.

Figure 4. Cell seeding density of hPSCs. Low (left) and appropriate (right) density of cells seeded. Scale bar = 1000 μm.

Table 1. Composition of chemically defined media CDM2, CDM3, CDM4, and CDM5

Table 2. Composition of differentiation media

Table 3. Potential problems and their possible causes and solutions

DISCUSSION:

This method enables the generation of enriched populations of liver bud progenitors, and subsequently hepatocyte-like cells, from hPSCs. The ability to generate enriched populations of human liver cells is important for the practical utilization of such cells. Previous methods to generate hepatocytes from hPSCs yielded impure cell populations containing both liver and non-liver cells that, upon transplantation into rodents, yielded bone and cartilage in addition to liver tissue[15]. Hence the explicit suppression of non-liver differentiation is critical to generate enriched liver populations that might be suitable for a variety of applications.

Notably, controlled plating of hPSCs at the very first step is essential for efficient downstream differentiation. It is important to accurately plate hPSCs at a certain density for differentiation, and to evenly distribute these hPSCs across the well or plate during the process of plating. For example, for each well of a 12-well plate, seed 150,000 to 225,000 hPSCs per well; overall, it is imperative to titrate the cell seeding density and to ultimately test the cell density that is appropriate for each cell line (**Figure 4**). If too many hPSCs are seeded per well, they will form large colonies, which will adversely affect differentiation efficiencies, as cells in the middle of large colonies are less accessible to differentiation signals compared to cells near the periphery; colonies of heterogeneous sizes also will present similar issues. If cell densities are too low, then there will not be sufficient material for differentiation and extensive cell death may be observed. The passaging and seeding method described above consistently generates hPSC clumps of suitable size for downstream differentiation.

One limitation of this method is that the hepatocyte-like cells generated are not identical to adult hepatocytes, as the hPSC-derived cells still express immature liver marker AFP. Moreover, CYP3A4 enzymatic activity is approximately 55 times lower in these hPSC-derived hepatocyte-like cells compared to primary adult human hepatocytes. A coming challenge will be to mature these hPSC-derived hepatocyte-like cells into fully-fledged, adult-like cells. A second limitation is that efficient differentiation is extremely dependent on the starting density of cells and therefore, it is very important to seed at the recommended density and to evenly disperse them across the plate (**Table 3**).

Overall, this protocol produces liver bud progenitors and hepatocyte-like cells at 89$\pm$3.1% purity in at least 3 hPSC lines. Second, hepatocyte-like cells expressed liver enzymes, secreted human ALBUMIN and expressed higher levels of liver genes than cells generated using extant differentiation approaches. Finally, the resultant hepatocyte-like cells not only exhibit certain hepatocyte functions in vitro, but most importantly they can function to some extent in vivo, as they can engraft a mouse model of chronic liver injury and improve short-term survival[2].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

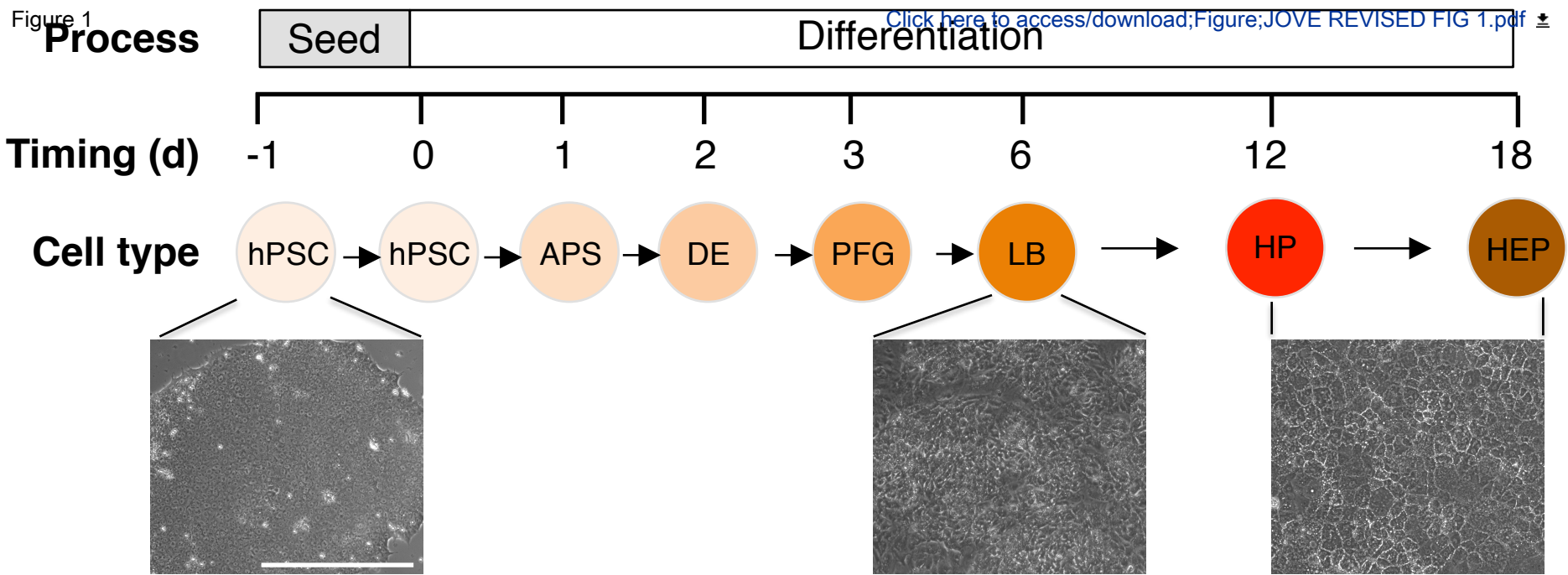


Figure 2

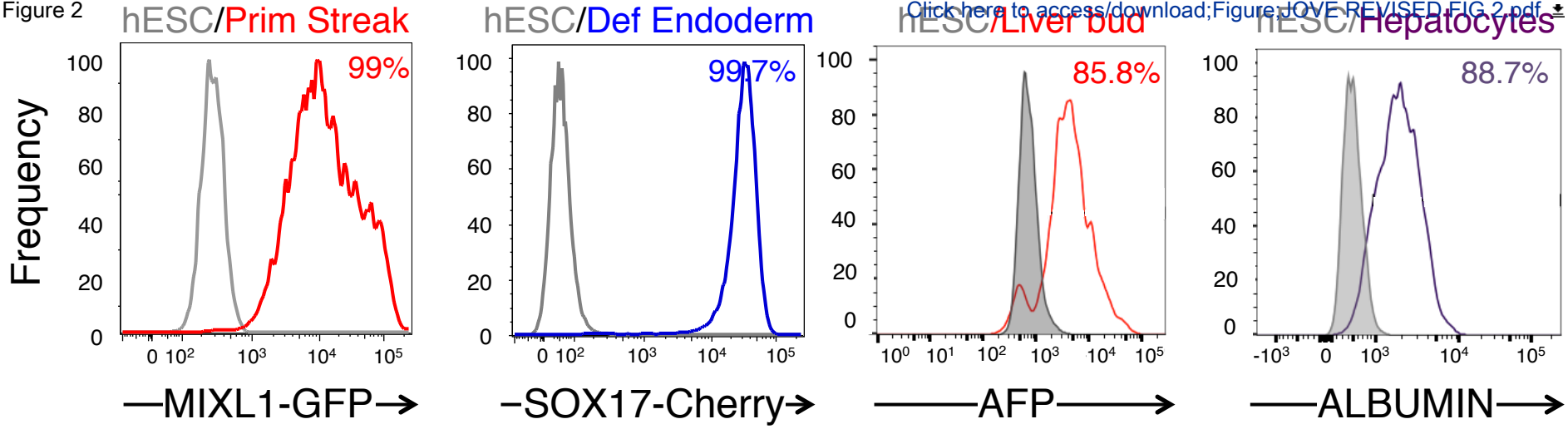


Figure 3

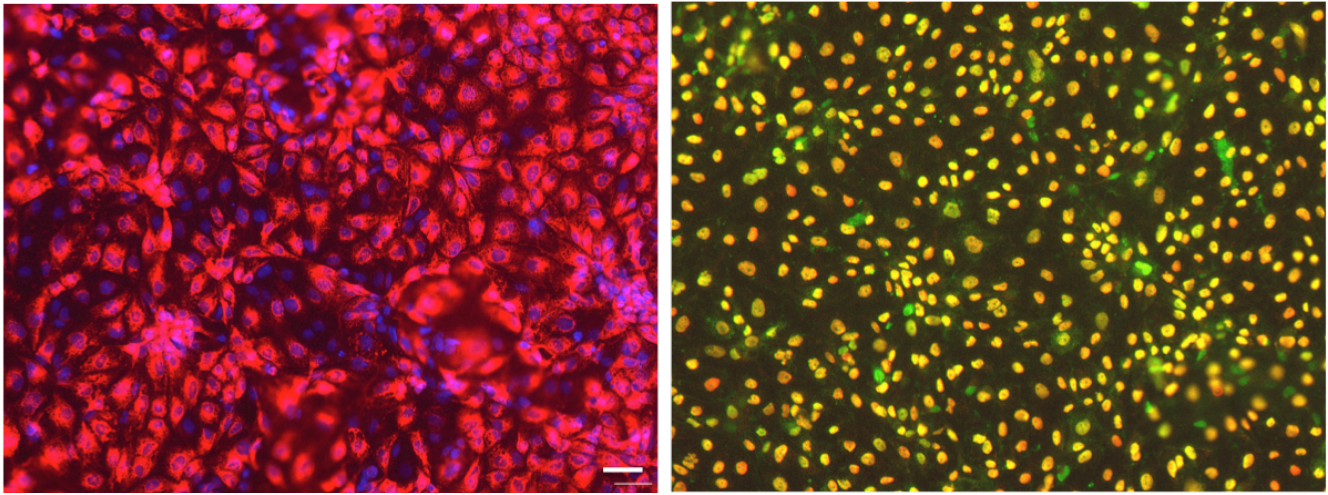
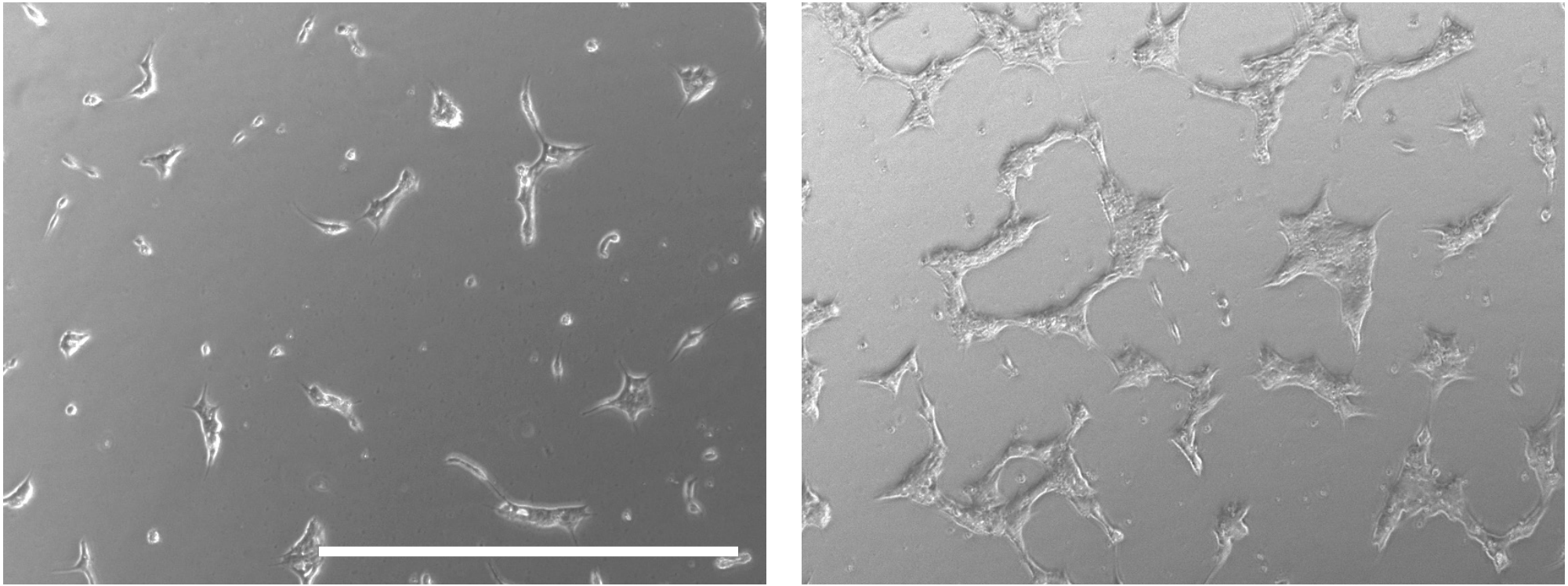


Figure 4



Base medium	Composition of base medium
CDM2	1:1 IMDM/F12, 0.1% m/v PVA, 1% concentrated lipids, 0.7 µg/mL human recombinant Insulin, 15 µg/mL Transferrin, 18 nM 1-thioglycerol
CDM3	1:1 IMDM/F12, 0.1% m/v PVA, 1% concentrated lipids, 10% KOSR
CDM4	1:1 IMDM/F12, 1% concentrated lipids, 15 µg/mL Transferrin
CDM5	CMRL, 10% KOSR, 1% Glutamax

Table 2

Differentiation Stages	Duration	Factors	Doses	Base medium
day 1 Primitive Streak	1 day	Activin CHIR99201 PI103 FGF2	100 ng/mL 3 µM 50 nM 10 ng/mL	CDM2
day 2 Definitive Endoderm	1 day	Activin DM3189 PI103	100 ng/mL 250 nM 50 nM	CDM2
day 3 Posterior Foregut	1 day	A83-01 TTNPB BMP4 FGF2	1 µM 75 nM 30 ng/mL 10 ng/mL	CDM3
day 6 Liver Bud progenitors	2 days	Activin C59 BMP4 Forskolin	10 ng/mL 1 µM 30 ng/mL 1 µM	CDM3
	1 day	Activin CHIR99201 BMP4 Forskolin	10 ng/mL 30 ng/mL 1 µM 1 µM	CDM3
day 12 Hepatic progenitors	6 days	BMP4 OSM Dexamethasone Forskolin Ro4929097 AA2P Insulin	10 µg/mL 10 ng/mL 10 µM 10 µM 2 µM 200 µg/mL 10 µg/mL	CDM4
day 18 Hepatocytes	6 days	Dexamethasone Forskolin Ro4929097 AA2P Insulin	10 µM 10 µM 2 µM 200 µg/mL 10 µg/mL	CDM4 or CDM5

Table 3 Click here to access/download;Table;JOVE TABLE 3.xlsx

Problem	Possible Reason	Proffered Solution
Cells in center of colony do not differentiate	i) Colony size was excessively large, cells in middle of large colonies were not accessible to differentiation signals	i) Check cell counting technqiue.
	ii) Uneven distribution of clumps which merged together in the center of the well (or its periphery), forming very large colonies	ii) Shake the plate in a cross fashion to evenly distribute clumps during plating, and check under a microscope before placing it into the incubator
	iii) Cells received insufficient differentiation signals	iii) Add adequate volumes of differentiation media: add 1 mL of medium per well in a 12-well plate and 3 mL per well in a 6-well plate
Poor efficiency of differentiation	i) Starting cell culture partially differentiated	i) Use a new, high-quality batch of undifferentiated hPSCs
	ii) Colonies were too densely seeded, forming a confluent sheet of cells	ii) Seed cells and count cells accurately for differentiation, and shake evenly to distribute them
	iii) Residual media and unwanted signals were not washed off due to inadequate washing	iii) Residual mTeSR1 or induction media from the previous stage of differentiation will block differentiation; wash cells with IMDM prior to adding differentiation medium
	iv) Washing too harsh; inappropriate washing conditions will severely disrupt cell morphology	iv) Prior to adding either differentiation medium, wash *briefly* with IMDM. Use of DPBS (or media with different osmolarity or cold media) or extended washing, will compromise cell morphology and viability
	v) Prolonged or shortened period of differentiation stages, longer or shorter than recommended.	v) Adhere to the recommended timings for each stage of differentiation.

Name of Material/ Equipment	Company
0.2 µm pore membrane filter	Millipore
1- 2 mM thiazovivin	Santa Cruz Biotechnology
1:1 DMEM/F12	Gibco
A83-01	Tocris
AA2P	Cayman chemicals
Accutase	Gibco or Millipore
C59	Tocris
Chemically Defined Lipid Concentrate	Thermofisher Scientific
CHIR99201	Tocris
Dexamethasone	Tocris
DM3189	Tocris
Forskolin	Tocris
Geltrex	Thermofisher Scientific
Ham's F-12 Nutrient Mix, GlutaMAX Supplement	Thermofisher Scientific
Human Activin	R&D
Human BMP4	R&D
human FGF2	R&D
human recombinant Insulin	Sigma-Aldrich
IMDM, GlutaMAX Supplement	Thermofisher Scientific
KOSR, Knockout serum replacement	Thermofisher Scientific
mTeSR1	Stem Cell Technologies
Oncostatin M	R&D
PI103	Tocris
Poly(vinyl alcohol)	Sigma-Aldrich
Ro4929097	Selleck Chem
Transferrin	Sigma-Aldrich
TTNPB	Tocris

Catalog Number	**Comments/Description**
GTTP02500	
sc-361380	
11320033	
2939/10	
16457	
Gibco A11105, Millipore SCR005	
5148	
11905031	
4423	
1126	
6053/10	
1099/10	
A1569601	
31765035	
338-AC	
314-BP	
233-FB	
11061-68-0	
31980030	
10828028	
5850	
295-OM	
2930/1	
P8136	
S1575	
10652202001	
0761/10	

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Author(s):	Nora Ou, Kyle M. Loh & Lay Teng Ang

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We thank the reviewers for their comments. Our responses are in **red**.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Thank you, corrected.

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4. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.
Thank you for the comment. First, we have now stated advantages of our approaches over alternative techniques. Second, we added references to previous studies that we compared our approach with. These references can be found in line 78.

5. Please spell out each abbreviation the first time it is used.
Thank you, corrected.

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Thank you, corrected. Please refer to line 190.

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Thank you, corrected.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Thank you, corrected.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
There are no personal pronouns in the protocol text now.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
We have made these changes.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
Line 84: Please specify the pore size of the filter.
Line 85: Listing an approximate volume to prepare would be helpful.
Line 91: This step is unclear. Please specify the small molecules and growth factors used in this step.
Line 94: Please specify centrifugation parameters.
Line 99: How to mix the medium? Vortex?
Line 138: Instead of writing “see above”, please state the specific step.
Line 145: Please specify the volume of mTeSR1 used.
Thank you, corrected.

12. Please consider describing FACS in the protocol because FACS data are presented in the Representative Results section.
The FACS protocol for intracellular FACS has been added in Section 5.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.
Thank you, corrected.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Yes, we have highlighted sections detailing essential steps for the video.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
This has been done.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
This has been done.

17. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. Please define all abbreviations in the figure legend.
This has been done.

18. Tables 1 and 2: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
This has been done.

19. Please provide a table of the essential supplies, reagents, and equipment. It should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
This has been done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The paper of Ou et al. describes the differentiation of pluripotent stem cells into hepatocyte-like cells by using a multistep protocol. The paper is well designed and written and it is really interesting for its possible application for regenerative medicine purposes and also for drug discovery and screening.

Minor Concerns:
1. It would be really useful to have the manufacturers and references of the products used as supplementary material in order researcher can exactly reproduce the protocol.
This has been done.

2. Since the authors have a wide experience in differentiating and characterizing the cells, they should include more detailed characterization (i.e. immunofluorescence, mRNA, enzymatic activities) in the figures and show them in the video.
This has been done.

3. ALthough the authors have included important references at the end of the introduction, maybe it could be useful to document some of the statements with a specific reference rather than all the references at the end.
We are not sure what the reviewer is asking here. All references have been listed at the end and they have been referred to in the text at the appropriate locations.

Reviewer #2:

Manuscript Summary:
In their manuscript, Ou et al, described an "Efficient differentiation of human pluripotent stem cells into liver cells". As it is explained in the introduction of the manuscript, it is important to develop alternatives to primary hepatocytes for the treatment of liver failure or for drug development studies. Methods published until now have described differentiation of hPSCs with various outcomes for the cells generated. This manuscript complements a previously published manuscript from the same group entitled "A Roadmap for Human Liver Differentiation from Pluripotent Stem Cells" and described in more

details the method of differentiation. Overall the manuscript is well written and provides sufficient information for any reader who wants to differentiate hPSCs into hepatocytes-like cells.

Major Concerns:
This manuscript guides well the author through the differentiation but supplementary information should be provided to help better the readers to generate hepatocytes-like cells from hPSCs and to be able to track their progress.

1. Together with the manuscript, the authors should provide phase-contrast images that will guide better the readers during the process. Like it is mentioned several times in the manuscript, cell density plays an important role in the success of the differentiation, therefore some more details should be given. The authors provide a wide range of plating ratio (40.000-80.000 cells/well of a 12 well plate). This information is important but as cell proliferation vary between cell lines, it would also be valuable to give the optimal number of cells on the 1st day of APS treatment.
This is a good point. Images will be incorporated.

2. Related to the density and to better illustrate, it is necessary that the authors provide pictures of lower magnification to show the optimal density at the beginning of each stage; it would also be informative to show pictures of suboptimal cells density at each stage that would lead to less efficient differentiation. Such information would provide valuable help to readers who want to reproduce the method described in the manuscript.
Images showing suboptimal and optimal densities at the start of the differentiation protocol have been added (Figure 4). The first few steps are critical as there is quite a bit of cell death. A lower magnification image at day 3 (Figure 4) has been provided to check oneself for optimal cell densities. Death at other steps is very less and is variable in different hands, hence, it is difficult to put an exact quantification to it. However, lower magnification images have been provided at critical steps as an example of optimal densities (Figure 4).

Lower magnification images have been provided.

3. Together with the FACS in figure 2, the authors should add immunostaining for each stage using 1 or 2 typical markers that would define best each stage.
This has been done.

4. The authors should provide the brand and catalogue number of each

reagents (medium, small molecules…) used in the described method. Different activities can be observed between different suppliers, and for a better start, these details should be provided.
This has been done.

5. The pictures in Figure1 are not providing enough information on cell morphology. These pictures should be replaced.
Additional lower magnification images have been added (Figure 1).

Minor Concerns:
In the chapter: "Passaging and seeding of hPSCs for liver differentiation", the 4th bullet point should clarify the type of pipette such as serological (5 or 10 ml) or 1ml pipette tip, used to detach the cells as it is also described in the 9th bullet.
In the same chapter, in the 9th bullet point, it is said that cells are dissociated into single cells and in the 12th point, cells are forming clumps, unless otherwise the authors should correct.
This has been corrected.

Reviewer #3:

Manuscript Summary:
The manuscript "Efficient differentiation of human pluripotent stem cells into liver cells" by Ou et al is an invited submission which describes in detail the generation of PSC derived hepatocyte-like cells. The investigators provide a detailed protocol to describe and delineate a platform that supports rapid differentiation into hepatocyte-like cells. Overall the submission is well crafted and detailed. Overall the manuscript should be accepted with minor revision to fill in a few details which would be helpful in reproducing the author's protocol and differentiation methodology.

Major Concerns:
1. The authors describe the culture of human induced pluripotent stem cells GelTrex and mTeSR. As many labs use a variety of culture matrixes (e.g. Matrigel (similar) or Laminin 511 as the backbone) of their mTESR iPSC cultures, do these other iPSC culture models support the homogeneity required for the rest of the differentiation platform? Has that been tested? If so that should be offered as an alternative for pluripotent stem culture. In addition have the authors examined whether the use of E8 or more traditional media conditions using SRKO produce PSC cells of enough quality and/or homogeneity to support the use of this differentiation protocol.

We thank the reviewer for the questions. We have maintained hPSCs using E8 and tested their differentiation efficiencies up to the endoderm stage. We could obtain highly pure populations of SOX17+ endoderm cells. However, we have not tested SRKO.

As for the matrices used, we have largely used growth factor reduced Geltrex, which is also derived from sarcoma, like Matrigel. Thus, we speculate that cells may differentiate fine on Matrigel. Apart from Geltrex, we have also successfully maintained hPSCs on vitronectin.

Minor Concerns:

1. The authors should include a library of antibodies for both IF and FACS characterization to determine the efficiency of differentiation at each step of the bifurcations of their differentiation that they describe in their manuscript. They show figures of them but do not include this information. Are there certain steps that the readers who are trying to reproduce this protocol should stop and characterize the efficiency and if below a certain threshold that they should first focus on those steps (i.e. Endoderm or liver bud). What specific tools (IF, FACS, qRTPCR) should be used at each step of the protocol for characterization and the authors should include that information with specific details to evaluate (with protocols). Which specific antibodies or PCR combinations (company/number/concentration and protocols of use) do the authors recommend?

This will be added as a reference to our previous publication.

2. The authors similarly give the media information and describe the preparation of the base media but include abbreviations that are nor defined (e.g. PVA, KOSR) and do include which company and the catalog number of the reagents they use for all media combinations. This should be included and be clearly spelled out.

3. The authors in instruction step 11 recommended a desired cell concentration and recommend a cell number for use in a 12-well plate. They should include at this step the surface area of the plate as there is a small variability in surface area based on the manufacture (i.e. X cells/cm2). To make this easier for the reader additional info for different well sizes (that the authors have been successful using their protocol) should be included. In addition does this protocol work similarly well across well-sizes as most stem cell differentiation protocols have peak efficiency for the 12-well-6-well size. Does this protocol have similar efficiency levels down to 96 or 384 well sizes?

The differentiation protocol ahs been tested for 12 well and 6 well plates and works well on them. We have not tested the efficiencies in 96 or 384 well

plates but we surmise that as we scale down the cell numbers for differentiation, the efficiencies are comparable.

4. The authors use thiazovivin (ROCK inhibitor) to prevent PSC loss during replating as single cells. Have the authors tested other ROCK inhibitors such as Y-27632 (more commonly used) to see if this is compatible with their platform. Each ROCK inhibitor likely leads to different levels of cell loss and subsequent cell density. How many hours after initial plating do you wait before differentiation initiation (as overnight as you describe is pretty variable in timing) and may impact ultimately the final concentration of PSC at the initiation of differentiation. Pictures of the plate at this stage would be very helpful in giving readers an idea of the ideal final density and spacing between PSC and PSC islands given the known importance of cell density in differentiation outcomes (as described as well as by the authors in their discussion). Moreover pictures of homogeneity versus heterogeneity would also add value for the readers given the importance of this step.

5. For step 2 (line 173) please define APS before using the first time. Similarly for figure 1 the authors should define the abbreviations for all steps.
This has been done.

6. When media is being transitioned from cocktail X to cocktail Y besides aspirating the media do you wash the well with anything to remove dead cells as well as lingering cytokines which may differ from the new combination. If so how many times?
Yes, cells are washed once with DMEM/F12. This has been added to the protocol in Step 3.2.

7. At the end of the differentiation process what protocols or methodologies do the authors use to validate robust hepatocyte-like cell differentiation (e.g. IF, functional characterization, or qRT-PCR) to ensure during setup that uniformity of differentiation is obtained. As an example, the authors denote albumin and CYP3A4 marker expression. Which specific antibodies (company/number) do the authors recommend and consideration for addition of the protocol will make the protocol more complete. Moreover the addition of functional assays such as examination of protein production (via ELISA) or other assays would strengthen the protocol as well as help readers who use this protocol validate that they are truly producing bona fide hepatocyte-like cells in vitro.
We use IF and qRT-PCR assays to validate the formation of HLCs. We have also used these cells in survival studies of mice. Please refer to our publication (Ang et al. 2018; Cell Reports). We have provided IF and qRT-

PCR data with our manuscript with a reference to the publication it was taken from.

Reviewer #4:

Manuscript Summary:
Based on their recently published studies, Ou et al. described in this manuscript a highly efficient, convenient and reliable method to achieve induced differentiation of human pluripotent stem cells (PSCs), i.e, ES cells and iPS cells, into hepatocyte-like cells. Below, a list of several concerns and comments are provided that the authors will hopefully consider to improve the clarity of their protocol.

Major Concerns:
- For the readers' reference, the list of specific names and/or clones of hPSC lines with which the present protocol exactly worked should be provided, including those described in line 203 [14 hESC and hiPSC lines], line 211 [3 hESC lines] and line 258 [3 hPSC lines].

Examples of these lines have been provided in the text.

- Table 2. The names for each of the differentiation media should be specified here and used consistently throughout the manuscript. The "Time point" shown in this table seems confusing and a bit misleading, as those described here (i.e., D1, D2, …) indicate the timing when the differentiation is completed, but not started by applying the corresponding medium: for instance, the "day 1 (D1) medium" is applied on day 0 in the time course shown in Figure 1.

The table has been edited.

- Line 192. "Primitive streak cells should generally have ragged borders and will become more spread and less compact". It should be better to include in Figure 1 a picture corresponding to this notion.

We have a representative picture for this but it does not have a scale bar.

- Line 213. Can the authors provide any evidence showing that bile canaliculi are indeed formed, for instance, by immuno-staining for canalicular markers (e.g., P-glycoprotein, MRP2) or dye efflux assays?

We have not performed these assays except for a preliminary study showing MRP2 protein expression by immunostaining.

Minor Concerns:
- Abbreviations used in the manuscript should be defined, such as CDM, PVA, and APS. The same applies to those used in Figure 1 (e.g., DE, PFG, LB, …)
This has been corrected (Line 445)

- Line 131. It would be better to clarify whether the cells should be resuspended as complete "single cells" at this step, or dissociation into clumps is also acceptable. In the latter case, typical and/or maximal size of the clumps amenable for the following differentiation steps should also be described.
This has been corrected to say ‘single cells’ at all places.

- Line 148. Description on thiazovivin should go to line 145.

We placed the description on Thiazovivin right after the paragraph it was mentioned.

- Line 176. What is "APS differentiation medium" ? (Maybe the same as the "day 1 medium" written in the following sentence)
This has been described line 259

- Line 180. Isn't is necessary to replace media with fresh ones everyday or at any time during each of the differentiation steps (i.e., on day 4 and 5, days 7-11, or days 13-17)?
Yes, this has been added in the protocol Step 3.6

- Line 220. "MIXL" should be "MIXL1+".
The errors are corrected (see lines 273 and 299).

- Table 3, Cells in center of colony do not differentiate, iii), Proffered Solution. Description here should be revised by taking what is written in lines 184-186 into account.

This has been corrected