Directed Differentiation of Human iPSCs to Hepatic Stellate Cells

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EDITORIAL SUMMARY. Human iPSCs are differentiated into hepatic stellate cells by culture with growth factors. They respond to fibrogenic stimuli, arising as a new source of HSC-like cells for *in vitro* modelling. Subsequent co-culture with hepatocytes facilitates self-assembly into 3D hepatic spheroids.

TWEET. Differentiation of human iPSCs to hepatic stellate cell-like cells for *in vitro* liver modelling.

COVER TEASER. Human hepatic stellate cell generation from iPSCs.

RELATED LINKS

Key reference(s) using this protocol

Coll, M et al. [Cell Stem Cell](#). 23, 101-113.e7 (2018)
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Abstract

Hepatic stellate cells (HSCs) are non-parenchymal liver cells responsible for extracellular matrix homeostasis and are the main cells involved in the development of liver fibrosis following injury. The lack of reliable sources of HSCs has hence limited the development of complex *in vitro* systems to model liver diseases and toxicity. Here we describe a protocol to differentiate human induced pluripotent stem cells (iPSCs) into hepatic stellate cells (iPSC-HSCs). The protocol is based on the addition of several growth factors important for liver development sequentially over 12 days. iPSC-HSCs present phenotypic and functional characteristics of primary HSCs and can be expanded or frozen and used to perform high-throughput *in vitro* studies. We also describe how to co-culture iPSC-HSCs with hepatocytes, which self-assemble into 3D hepatic spheroids. This protocol allows generating HSC-like cells for *in vitro* modelling and drug screening studies.

Introduction

The liver is a complex organ containing not only hepatocytes, the parenchymal cell population which are the main cell type of the organ, but also non-parenchymal cells including hepatic stellate cells (HSCs), Kupffer cells (liver macrophages) and liver sinusoidal endothelial cells (LSECs). Although hepatocytes exert the main metabolic and synthetic functions of the liver, non-parenchymal cells are essential for maintaining the structure, homeostasis and function of the organ, as well as enabling adequate response to tissue injury and inflammation¹.

HSCs are located in the space of Disse among hepatocytes and LSECs. In healthy liver, HSCs are in a quiescent state, which is characterized by low proliferative and metabolic activity. In the quiescent state they participate in the homeostasis of the extracellular matrix (ECM) and store retinyl esters in their cytoplasmic lipid droplets². When the liver is injured HSCs become activated, acquire a myofibroblast phenotype and participate in the wound-healing response of the tissue^{1,3}. If damage is acute or limited, the phenotype of HSCs can revert to a quiescent state, or they die by apoptosis or by natural killer cells activity⁴. However when damage persists, activated HSCs become proliferative and contractile, secrete pro-inflammatory and pro-fibrogenic cytokines and increase the production of ECM components, thus becoming the main cell type responsible for the development of liver fibrosis³.

The need for non-parenchymal cells in biomedical and biotechnological applications is increasing with the development of new complex *in vitro* systems to study liver toxicity and disease modelling, as well as the generation of liver devices containing parenchymal and non-parenchymal cells^{5,6}. Currently, the most reliable source of human HSCs is primary HSCs, which can be obtained from human liver resections. Shortcomings, however, are the complexity of the isolation process, the shortage of healthy livers and the high variability among donors. Moreover, the use of primary HSCs is hampered by their limited proliferative capacity and rapid activation when seeded in culture plates, far beyond the activation state of HSCs in fibrotic livers^{7,8}. As an alternative to primary cultures, cell lines such as LX-2 have been used to study liver fibrosis *in vitro*, but they have an intrinsic activated phenotype and their genetic profiles differ from primary HSCs^{9,10}.

Recently several papers have described the differentiation of induced pluripotent stem cells (iPSCs) to hepatocyte- and cholangiocyte- like cells^{11,12,13}, however these did not produce non-parenchymal cells. We recently generated HSC-like cells from human iPSCs (iPSC-HSCs) and used this method to produce 2D and 3D culture models of fibrosis and drug toxicity¹⁴. In this protocol we provide additional details of how to differentiate human iPSC to HSC-like cells and co-culture these cells with hepatocytes to generate hepatic spheroids.

DEVELOPMENT OF THE PROTOCOL

While little is known about the embryonic development of HSCs in humans, studies in animal models have provided important information regarding the origin of fetal HSCs. On day E9.5 of mouse embryogenesis, mesenchymal cells derived from mesoderm, which are situated in the *septum transversum mesenchyme*, differentiate to mesothelial cells. At day E11.5, these cells migrate to the liver surface generating fetal HSCs and perivascular mesenchymal cells^{15,16}. However, the cytokines and factors implicated in the differentiation of HSCs are not well

defined. We developed a differentiation protocol based on the addition of several growth factors that have been described to play an important role during embryonic and fetal liver development. Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor-beta (TGF β) family, induce differentiation of embryonic stem cells to a mesodermal state¹⁷. In the early stages of mouse embryogenesis (day E9.5), a member of the family, BMP4, is highly expressed in the *septum transversum mesenchyme*. Evseenko *et al.* (2010) demonstrated that BMP4 is required for the generation of a multipotent mesoderm progenitor population from human ESCs¹⁸. We therefore stimulated iPSCs with BMP4 to start our differentiation protocol, to induce the development of a mesodermal precursor state.

Other factors, such as fibroblast growth factor (FGF) family members, are involved in mesoderm induction. The presence of FGFs induces differentiation of iPSCs to hepatic progenitor cells and they have been shown to be involved in the proliferation and expansion of mesenchymal stem cells^{19,20}. In mouse models, FGF1 plays an important role during gastrulation, showing that homozygous mutant embryos for the FGF1 receptor develop an aberrant mesodermal pattern during gastrulation²¹. Others authors demonstrated that FGF3 is also necessary during the first stages of early mesoderm induction^{22,23}. Therefore, we added FGF1 and FGF3 at day 4 of culture to further enhance mesoderm formation.

As a final stage we cultured cells with palmitic acid and retinol to promote HSC differentiation, maturation and retinyl ester accumulation, an important characteristic of primary HSCs^{24,25}. As described by Asahina *et al.* (2009), ALCAM positive cells were able to differentiate to HSCs when treated with retinol and palmitic acid¹⁷. The important role of retinol for HSC development was also described by Ijpenberg *et al.* (2007), who demonstrated that depletion of Raldh2, a factor related to retinol metabolism, impaired the differentiation of HSCs in mice²⁶.

COMPARISON WITH OTHER METHODS

The main sources of HSCs that are currently used are primary cells obtained from human liver resections and commercial cell lines (LX-2). However, they present some limitations, such as the difficulty of isolation and expansion of primary HSCs, and for cell lines, an over-activated phenotype^{7,8,9,10}. The use of HSCs derived from iPSCs has several advantages over them. The main advantage is that iPSCs can be expanded extensively, allowing the generation of large numbers of progeny for high-throughput *in vitro* assays. Moreover, obtaining HSCs from multiple different patient-specific iPSCs can enable their use in personalized medicine strategies²⁷.

Alternative approaches have also been reported to generate HSC-like cells from iPSCs. Kouji *et al.* (2017) described the generation of ALCAM⁺ progenitor HSCs from human iPSCs. These progenitor HSCs were able to sustain hepatic progenitor cell maturation in a co-culture system²⁸. Moreover, ALCAM⁺ progenitor HSC-like cells could be expanded and further differentiated to mature HSC-like cells following inhibition of the Rho signaling pathway. Miyoshi *et al.* (2019) obtained HSC-like cells that stored vitamin A following 6 days of differentiation. When exposed to fibrogenic stimuli, the resulting HSC-like cells developed an activated phenotype. The maturation of the HSC progeny could be enhanced by co-culturing with iPSC-derived hepatic progenitor cells²⁹. Cells obtained following these strategies also promoted hepatocyte

maturation in co-culture studies, and thus alternative approaches can be used to model liver physiology and diseases^{31, 32}.

Following a different experimental approach, Ouchi *et al.* (2019) generated HSC-like cells by co-differentiating parenchymal and non-parenchymal cells from PSCs. These complex organoids were composed of hepatocytes, HSCs and Kupffer cells co-derived from human PSCs. The differentiation protocol recapitulates liver bud formation, and 31% of the resulting cells express HSC markers. This approach allows direct generation of liver organoids without the need to optimize the co-culture and result in the production of a multicellular liver model fully derived from PSCs³⁰.

As in the first two studies^{28, 29}, our approach¹⁴ is based on a directed differentiation protocol to obtain a HSC-like cell population. Although the other studies used similar factors such as BMP4 or FGFs to induce differentiation, timing and culture conditions are different and the extent of characterization of the resulting cells varies between them. In our protocol, the possibility to passage and cryopreserve iPSC-HSCs adds flexibility to downstream experimental designs. Thus, direct comparison of the methodologies is challenging and it cannot yet be determined whether a particular methodology is better. Direct side-by-side comparison and better characterization of the phenotype and function of the final population of cells are required to determine the best procedure to obtain HSC-like cells from iPSCs.

APPLICATIONS OF HSCs DERIVED FROM iPSCs

iPSC-HSCs have the potential to be used in multiple applications. They represent a new source of HSC-like cells that can be used to evaluate the role of HSCs in wound-healing, and in the fibrogenic and inflammatory responses. The differentiated cells can be aggregated with hepatocyte cell lines, and thus, cell-cell interactions can be modelled which might facilitate understanding of liver physiology and enable the development of *in vitro* models of chronic liver diseases. In addition, iPSC-HSCs can be used for toxicity assays, drug screening and discovery in both 2D and 3D systems. Additionally, this differentiation protocol could be adapted to facilitate the generation of pericytes from other organs. Finally, the differentiation part of the protocol may facilitate the study of human embryonic development of HSCs. Since iPSCs can be genetically modified, iPSC-HSCs can also be used to study the role of specific genes in human liver development and disease^{31,32}.

LIMITATIONS OF OUR PROTOCOL

Although mesenchymal and mesothelial markers are progressively expressed during the differentiation of iPSCs¹⁴, greater knowledge of the process might enable the protocol to be further optimized and better determine whether the differentiation is similar to the development of human HSCs that occurs in the embryo. Despite the fact that iPSC-HSCs present a similar phenotype and functionality to primary HSCs, at the end of the protocol we obtain a mix of cells with different degrees of PDGFR β expression.

Although differentiated cells are transcriptionally less activated than cultured primary HSCs, they show higher expression of activation markers than quiescent primary HSCs, indicating that iPSC-HSCs have to some extent an activated phenotype¹⁴. Moreover, passaging or thawing iPSC-

HSCs enhances their activation phenotype. For these reasons, the iPSC-HSCs generated via this protocol are not suitable as a model of completely quiescent HSCs. Therefore, further studies that modify or provide an alternative to the procedure are required if less activated iPSC-HSCs are required, particularly if passage and cryopreservation of cells is required..

EXPERIMENTAL DESIGN

We have optimized the concentration of cytokines and reagents, as well as the timing of the procedure to obtain HSC-like cells derived from human iPSCs. The procedure begins by describing the thaw and maintenance of iPSCs (steps 1-8). The preparation of iPSCs for induction of differentiation is described in steps 9-17. Induction of iPSCs differentiation towards HSC-like cells, which takes 12 days, is described in steps 18-25. At the end of the differentiation, iPSC-HSCs can be expanded, cryopreserved, stimulated or co-cultured with hepatocytes to form spheroids (described from step 26 onwards).

iPSCs are seeded at 15,000 cell/cm² in a 12-well plate coated with growth factor reduced matrigel (Matrigel) and are maintained in Essential 8™ Medium (E8 Medium). Once iPSCs achieve 70% confluence, the Liver differentiation medium (LdM) together with the different sequential growth factors are added to induce differentiation, according to Table 1.

Mesodermal induction is initiated by adding BMP4 until day 4 of differentiation. This is associated with a decreased expression of pluripotency markers such as octamer-binding transcription factor 4 (*OCT4*)¹⁴. On day 4 of differentiation, FGF1 and FGF3 are added together with BMP4. On day 6, BMP4 is removed and cells are cultured in retinol and palmitic acid medium, also containing FGF1 and FGF3, to promote a mesenchymal and mesothelial phenotype, which is characterized by the expression of platelet derived growth factor receptor alpha (*PDGFRα*), ecto-5'-nucleotidase (*CD73*) and neurotrophin receptor p75 (*NTR*) (*P75NTR*)^{14,33,34}. On day 8, FGF1 and FGF3 are removed and the culture is maintained in medium containing only retinol and palmitic acid until day 12 to promote expression of HSCs markers, such as platelet derived growth factor receptor beta (*PDGFRβ*), protocadherin 7 (*PCHD7*) and collagen type 1, alpha 1 (*COL1α1*) among others¹⁴. A schematic representation of the differentiation protocol and representative images of cell confluence and morphology changes are shown in Figure 1A. As shown by Coll *et al.* (2018), transcriptomic, phenotypic and functional characterization of iPSC-HSCs demonstrated that HSCs derived from iPSCs mimic the primary cell phenotype and properties¹⁴.

We recommend monitoring differentiation efficiency by flow cytometry (FACS) analysis of *PDGFRβ* expression and vitamin A incorporation (described in Box 1) at various time points during differentiation (Figure 2A). We suggest evaluating the expression of the main HSC markers by quantitative polymerase chain reaction (qPCR) and immunofluorescence as shown in Figure 1B and Figure 2C. Appropriate markers to compare derived cells with hepatocytes, quiescent and fibrogenic HSCs are detailed in Table 2. Details of the methods, primers and antibodies we used can be found in Tables 4 and 5 For further technical details, see the manufacturer's instructions.

At the end of the differentiation, 500,000 - 600,000 iPSC-HSCs are usually present in each well of a 12-well plate. Differentiated cells can be expanded, cryopreserved, stimulated or co-

cultured with hepatocytes to model liver fibrosis *in vitro* following the schemes represented in Figure 3A. Additional assays such as inflammatory stimulation and toxicity studies that have been successfully undertaken using the cells generated are described in Coll et al. (2018)¹⁴. Two different human iPSCs lines, BJ1 (made in house and used in this study) and from Sigma, have been successfully differentiated in two different laboratories, and both showed similar gene expression patterns³⁶. This differentiation protocol can also be used in embryonic cell lines such as H9, demonstrating the robustness of the protocol^{14, 36}. It is also low cost and has a short differentiation time (only 12 days) which is shorter than the differentiation protocols of other hepatic cell types such as hepatocytes or cholangiocytes^{11,12,13}.

EXPERTISE NEEDED TO IMPLEMENT THIS PROTOCOL

Any student or postdoctoral fellow with iPSCs culture experience can use this protocol. All equipment is standard to most cell culture facilities and reagents can be purchased from standard scientific companies.

Materials

REAGENTS

Biological Reagents

- **iPSC:** We have successfully used the human BJ1 iPSC line that was generated in the laboratory of Catherine Verfaillie, from human fibroblast (ATCC® CRL-2522™28). [RRID: CVCL_3653; https://scicrunch.org/resolver/RRID:CVCL_3653]. The results shown here are from that line³⁷
- **Differentiated HepaRG cells:** We use HPR116215-TA08 purchased from Biopredic (RRID: CVCL_9720; https://scicrunch.org/resolver/RRID:CVCL_9720).

! CAUTION. The cell lines used should be regularly checked to ensure they are authentic and are not infected with mycoplasma. We authenticate iPSC by evaluating pluripotency markers by gene expression, FACS and immunofluorescence; formation of embryoid bodies and SNP profiling.

! CAUTION. iPSCs research should always be conducted in accordance with all relevant governmental and institutional guidelines and regulations depending on each country.

Coating Reagents

- Vitronectin (VTN-N) Recombinant Human Protein, Truncated (ThermoFisher, Cat#A14700)
- Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-Free (Cultek, Cat#356230).

Cell Culture Medium

- Essential 8™ Medium (E8 Medium) (ThermoFisher, Cat#A1517001)
- DMEM, Low Glucose, Pyruvate (Thermo Fisher, Cat#31885)
- DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Thermo Fisher, Cat#31966)
- MCDB 201 medium (Sigma, Cat#M66770)
- L-Ascorbic Acid 2-phosphate sesquimagnesium salt hydrate (L-ascorbic Acid) (Sigma, Cat#A8960)
- Insulin-Transferrin-Selenium (100X) (ITS-G) (Invitrogen, Cat#41400045)
- Linoleic Acid-Albumin from bovine serum albumin (LA-BSA) (Sigma, Cat#L9530)
- 2-Mercaptoethanol (50mM) (Invitrogen, Cat#31350)
- Dexamethasone (Sigma, Cat#D1756)
- Penicillin-streptomycin Mixture (P/S) (LONZA, Cat#17-603E)
- HepaRG thawing medium supplement (HTM) (Biopredic, Cat#ADD670049)
- HepaRG serum-free induction medium supplement (HFM) (Biopredic, Cat#ADD650036)
- Gibco™ Fetal Bovine Serum, qualified, One Shot™ format (FBS) (Life Technologies, Cat#A3160802).

Cytokines

- Bone Morphogenic Protein (BMP4) (R&D, Cat#314-BP-010)
- Fibroblast Growth Factor 1 (FGF1) (R&D, Cat#232-FA-025)
- Fibroblast Growth Factor 3 (FGF3) (R&D, Cat#1206-F3-025)
- Retinol (Sigma, Cat#R7632-100MG)
- Palmitic Acid (Sigma, Cat#P0500).

Other Reagents

- UltraPure 0.5M EDTA (EDTA) (Life Technologies, Cat#15575-020)
- Dulbecco's Phosphate Buffered Saline DPBS without Calcium/Magnesium; DPBS (-/-) (LONZA, #BE17-515F)
- Trypsin-EDTA (0.25%), phenol red (Trypsin) (Thermo Fisher, Cat# 25200056)
- TGF-beta 1 human recombinant (TGFβ) (Peprotech, Cat#AF-100-21C)
- Dimethyl Sulfoxide (DMSO) (Sigma, Cat# 5879-100mL)
- 10X PBS buffer 4L OmniPur® (Calbiochem-Merck, Cat#6505-OP)
- Absolute ethanol for Molecular Biology (Panreac Quimica, Cat#A8075.1000)
- 2-Propanol (Panreac Química, Cat#8710901212)
- Chloridric acid 35% (AGROVIN, Cat#141019)
- Bovine Serum Albumin (BSA) (Sigma, Cat#A8806)
- Citric Acid 99% (Sigma, Cat#C0759).

EQUIPMENT

- Orbital cell shaker suited for use in cell culture incubator (Infors Celltron benchtop shaker for CO₂ incubator Ø 25 mm, Cat#: INF69222)
- Integra Viaflo Assist Base Unit (Integra, Cat# 4500)
- Corning® LSE™ Vortex Mixer with Standard Tube Head, 230V, EU Plug (Corning, Cat#6776)
- Milli-Q water purification system (Millipore, Cat#7003/7005)
- Pipetboy Acu Classic (Cultek, Cat# 39155000)
- Serologic tips pipettes of 5mL, 10mL and 25mL (Cultek, Cat#4487, Cat#4488 and Cat#4489)
- Tips pipettes of 10µl, 100µl, 200µl and 1000µl (Eppendorf, Cat# 0030078500, Cat#0030078543, Cat#0030078837, Cat#0030077571)
- 10µl Graduated Filter Tip (Sterile) (StarLab, Cat#S1121-3810)
- CLEARLine® Filter tips racked sterile 100µl (DDBiolab, Cat#036060CL)
- 200µl and 1000µl Filter Tips (Labclinics, Cat#LAB200ULFNL, Cat#LAB1000ULFNL)
- 8-channel INTEGRA Viaflo electronic pipettes 10-300µL and 50-1250µL (IntegraCat# 4623, Cat# 4624)
- Tissue culture test plates 6 wells (TPP_Reactiva, Cat# 92006)
- Tissue culture test plates 12 wells (TPP_Reactiva, Cat# 92012)
- Microplate 96 well, ps, clear, sterile, u-bottom, cell-repellent surface (Greiner-Bio, Cat# 650970)
- Stericup® and Steritop™ Vacuum Driven Sterile Filters (Merck, Cat#SCGVU05RE)
- Falcon 15mL and 5mL tubes (Corning, Cat# 15430791 and Cat#10604551)

- Eppendorf Tubes 1,5 ml PYROGEN FREE, RNase Free (STARLAB, Cat#S1615-5510)
- Acrodisc Syringe Filters with Supor Membrane 0.2µm (Pall life sciences, Cat#PN4612)
- BD Plastipak Syringe Luer 20mL 300613 (BD Plastipack, Cat#300613)
- Mr. Frosty™ Freezing Container (Thermo Scientific, Cat# 5100-0001)
- Cryogenic vials (Greiner CRYOS.S, Cat#121263).

Software equipment

- ZEN Lite from ZEISS Microscopy (ZEISS, <http://www.zeiss.com/microscopy/bint/products/microscope-software/zen-lite.html/>)
- Graphpad Pism 5 (GraphPad Software, <http://www.graphpad.com/>).

REAGENTS SETUP

- **Vitronectin-coated 6-well plates:** To coat a 6-well plate, thaw a 60µL aliquot and dilute it in 6mL of sterile DPBS without calcium and magnesium DBPS (-/-) to give a final concentration of Vitronectin of 1% volume over volume (v/v). Resuspend, add 1mL to each well, move the plate gently to spread the solution evenly across the well and incubate the plate at room temperature (RT) for 1h or at 37°C for 20-30min before seeding the cells. Coated plates can alternatively be stored wrapped in parafilm at 4°C for a maximum of 1 week. Before use, aspirate and discard excess Vitronectin solution and wash the wells once with DPBS (-/-).
- **Matrigel-coated 12-well plates:** To coat a 12-well plate, resuspend a 120µL Matrigel aliquot in 6mL ice-cold DMEM, low glucose, pyruvate (DMEM) to give a final concentration of Matrigel of 2% (v/v). Add 500µL of the solution to each well, move the plate gently to spread the solution evenly across the well and incubate the plate at RT for 1h or at 37°C for 20-30min before seeding the cells. Alternatively, coated plates can be stored wrapped in parafilm at 4°C for a maximum of 1 week. Before use, aspirate and discard excess Matrigel solution and wash the wells once with DPBS (-/-).

Δ **CRITICAL.** Matrigel should be kept under cold conditions during the whole process in order to avoid spontaneous polymerization.

- **Essential 8™ Medium (E8 Medium):** To prepare 500mL of complete Essential 8™ Medium, add 10mL of Essential 8™ Supplement (50X) to 490mL of Essential 8™ Medium. E8 Medium supplemented medium can be stored at 2-8°C for up to 2 weeks, according to the manufacturer's instructions.
- **0.5mM EDTA:** Dissolve 50µL of UltraPure™ 0.5M EDTA pH 8 in 50mL of DPBS (-/-) and filter with a 0.22µm filter. EDTA should be stored at RT. Diluted EDTA can be stored at 2-8°C for up to 6 month.
- **MCDB:** Dissolve 8.85g of MCDB in 500mL of MilliQ water. Stir with magnetic bar for 10-20min and adjust the pH to 7.2. Filter the solution with a 0.22µm filter. Store the dry powder medium at 2-8°C. Once the solution is made, store it at 2-8°C for up to 2 weeks.
- **Dexamethasone:** Dissolve 5mg in 1mL of pure ethanol to obtain a 5,000ng/µL stock solution. Dilute the stock dexamethasone solution to a final working concentration of 98ng/µL in MilliQ water. Filter the solution with a 0.22µm filter. Stock solution should

be stored at -20°C. The working solution is stable at 4°C for up to 1 month, according to the manufacturer's instructions.

- **BMP4:** Reconstitute the vial (10µg) in 1mL of 4mM HCl containing 0.1% weight over volume (w/v) BSA to obtain a final concentration of 10ng/µL. Filter the solution with a 0.22µm filter. Powder cytokine can be stored for up to 12 months at -20 to -70°C. After reconstitution, it can be stored for 1 month at 2-8°C and for longer storage (3 months) at -70°C, according to the manufacturer's instructions.
- **FGF1:** Reconstitute the vial (25µg) in 2.5mL of DPBS (-/-) containing 0.1% w/v BSA to obtain a final concentration of 10ng/µL. Filter the solution with a 0.22µm filter. Powder cytokine can be stored for up to 12 months at -20 to -70°C. After reconstitution, it can be stored for 1 month at 2-8°C and for longer storage (3 months) at -20°C, according to the manufacturer's instructions.
- **FGF3:** Reconstitute the vial (25µg) in 2.5mL of DPBS (-/-) containing 0.1% w/v BSA to obtain a final concentration of 10ng/µL. Filter the solution with a 0.22µm filter. Powder cytokine can be stored for up to 12 months at -20 to -70°C. After reconstitution, it can be stored for 1 month at 2-8°C and for longer storage (3 months) at -70°C, according to the manufacturer's instructions.
- **Retinol:** Reconstitute the vial (100mg) in 0.348mL of pure ethanol to obtain a stock solution of 1M under dark conditions. Filter the solution with a 0.22µm filter. Dilute it in pure ethanol to a final working concentration of 5mM. Retinol is stable for up to 1 year at -20°C. Once diluted should be stored in dark conditions at -70°C, according to the manufacturer's instructions.
- **Palmitic Acid:** Dissolve 25.643mg of palmitic acid in 1mL of pure ethanol to obtain a final concentration of 0.1M. Filter the solution with a 0.22µm filter. Vortex the solution vigorously in order to dissolve it. Powder palmitic acid should be stored at RT. Working solution should be stored at -20°C to -70°C for up to 1 month.
- **TGFβ:** Resuspend the vial (2µg) in 1mL of 10mM citric acid at pH 3, filter the solution using a 0.22µm filter and aliquot it to obtain a final concentration of 2,000ng/mL. Store TGFβ at -20°C and after reconstitution at -70°C for 1 month.
- **L-Ascorbic Acid:** Dissolve 1.45g of L-ascorbic acid in 500mL PBS 1x to obtain a 10mM solution. Stir with magnetic bar for 10-20min in dark conditions and filter with a 0.22µm filter. L-Ascorbic Acid should be stored at -20°C for up to 2 years, according to the manufacturer's instructions.
- **Liver differentiation medium (LdM):** To prepare a final volume of 500mL, add 285mL DMEM Low Glucose (can be stored at 2-8°C for up to 12 months from date of manufacture), 200mL of MCDB, 5mL of P/S (store at -10 to -20°C up to the expiry date according to the manufacturer's instructions), 5mL of L-Ascorbic Acid, 1.25mL of ITS-G (store at 2-8°C for up to 2 years), 1.25mL of LA-BSA (can be stored at 2-8°C for up to 2 years), 0.5mL of 2-Mercaptoethanol (can be stored at 2-8°C for up to the expiring date according to the manufacturer's instructions) and 2mL of Dexamethasone (working solution). Filter the solution with a 0.22µm filter. This prepared medium can be maintained at 4°C for 1 month.

- **Freezing Medium:** 80% FBS (can be stored at 2-8°C up to the expiry date) and 20% DMSO (store at RT). Filter the solution with a 0.22µm filter.
- **Expansion Medium:** DMEM Glutamax (can be stored at 2-8°C for up to 12 months from date of manufacture), 10% FBS and 1% P/S. Filter the solution with a 0.22µm filter.
- **Low FBS Medium:** DMEM Glutamax, 1% FBS and 1% P/S. Filter the solution with a 0.22µm filter.

Thawing Differentiated HepaRG® cells • Timing 20min

- Δ **CRITICAL.** Pre-warm the HepaRG thawing medium supplement (HTM) in a 37°C water bath.
1. Pipette 9mL of pre-warmed HTM into a sterile tube.
 2. Under the laminar flow hood, briefly twist the cap of the cryovial a quarter turn (do not open it completely) to release the internal pressure and then close it again.
 3. Quickly transfer the cryovial to a water bath set at 37°C. Do not submerge it completely and be careful not to allow water to penetrate the cap.
 4. Aseptically transfer the “semi”-thawed HepaRG® cell suspension into the tube containing 9mL of the pre-warmed HTM.
 5. Rinse out the cryovial once with approximately 1mL of HTM and return the resulting suspension to the tube.
 6. Centrifuge the differentiated HepaRG® cell suspension for 3min at 500g at RT.
 7. Aspirate the supernatant and resuspend the differentiated HepaRG® cell pellet gently with HTM in 5mL.
- Δ **CRITICAL STEP.** Do not try to dissociate any large cluster of cells.

Procedure

Maintenance of human iPSCs • Timing 3-4 days (15min for plating cells)

Δ **CRITICAL.** E8 Medium should be warmed at RT prior to thawing the cells. Keep the Vitronectin-coated plate inside the incubator for a minimum of 20min before seeding the cells. Ensure that the water bath is at 37°C before starting the procedure.

1. Thaw the cells at 37°C by swirling the cryovial in a water bath. When only a small ice crystal remains, which is usually after 1-2min, take the cryovial out of the water bath.
2. Spray the cryovial with ethanol and place it in the laminar flow hood.
3. Add 0.5mL of warmed E8 Medium in the cryovial and transfer the cells into a 15mL conical tube containing 9.5mL of E8 Medium.
4. Centrifuge cell suspension at 300g for 5min at RT.
5. Meanwhile, wash the Vitronectin-coated 6-well plate with DPBS (-/-).
6. Remove the supernatant and resuspend cells in 2mL of E8 Medium. Pipette carefully as it is important to maintain cell clusters. Transfer the cell suspension to one well.

? TROUBLESHOOTING

7. Incubate cells at 37°C and replace medium daily with fresh E8 Medium.
8. When cells reach 70% confluence move to next step to disaggregate the colonies.

? TROUBLESHOOTING

Preparation of human iPSCs for HSCs differentiation • Timing 1-3 days (15min for plating cells)

Δ **CRITICAL.** One well of undifferentiated iPSCs must be expanded into a full 6-well plate in order to have enough cells to start a 12-well plate differentiation. To expand iPSCs, cells have to be detached following steps 9-12.

Δ **CRITICAL.** Coat 12-well plate with Matrigel before starting iPSC differentiation. Incubate the plates at 37°C for at least 20min before seeding the cells. Also, warm E8 Medium and EDTA solution at RT.

9. Remove E8 Medium from cells and rinse the wells with DPBS (-/-) to wash them.
10. Remove DPBS (-/-) and add 1mL of EDTA 0.5mM to each well. Wait 1-2min at 37°C or 2-3min at RT.
11. Check the cell colonies under the microscope. Colonies should start to detach.
12. Aspirate and discard EDTA. Add 1mL of E8 Medium to each well and disrupt the cell colonies by gently pipetting the medium several times using a 1mL pipette. Collect the cell suspension in a 15mL tube.
13. Wash the Matrigel coated plate with DPBS (-/-).
14. Count the cells collected in the 15mL tube. Plate cells at a density of 15,000 cells/cm² in E8 Medium. ? TROUBLESHOOTING

Δ **CRITICAL.** 720,000 iPSCs are usually obtained from 1.5 wells of a 6-well plate at 70% confluence but may differ between iPSC cell lines.

15. Move the plate to distribute the cells homogenously.
16. Keep the cells in the incubator.
17. Change the medium daily to fresh E8 Medium until the culture reaches 70% confluency (this usually takes 1-2 days). Then go on to the next step to start the differentiation protocol. ? **TROUBLESHOOTING**

iPSCs differentiation to HSCs • Timing 12 days for complete the differentiation (10min for medium change)

Δ **CRITICAL STEP.** Prepare the corresponding media to the day of differentiation before starting culture work and warm it up at RT or 37°C. Medium changes should be performed every 48h as detailed in Table 1.

18. Remove and discard E8 Medium and wash cells once with DPBS (-/-).

Δ **CRITICAL STEP.** Washing is important to remove any remains of the E8 Medium.

19. Add LdM supplemented with 2μL per mL of medium of BMP4 stock solution (10ng/μL). This is considered Day 0 of differentiation. To characterize the cells at the start of differentiation, we recommend you follow the analyses described in Box 1 for flow cytometry analysis and Table 4 for qPCR. Use one well for qPCR analysis, two wells for flow cytometry analysis of PDGFRβ and one well for flow cytometry analysis of vitamin A.
20. On day 2, remove and discard medium, wash cells with DPBS (-/-) and add LdM supplemented with 2μL of BMP4 stock solution (10ng/μL) per mL of LdM.
21. On day 4, remove and discard medium, wash the cells with DPBS (-/-) and add LdM supplemented with 2μL of BMP4, 2μL of FGF1 and 2μL of FGF3 stock solutions (10ng/μL) per mL of LdM. We recommend you use one well of cells for qPCR analysis (Table 4).

Δ **CRITICAL.** On day 4, the wells should be completely confluent.

22. On day 6, remove and discard medium, wash cells with DPBS (-/-) and add LdM containing 2μL of FGF1 and 2μL of FGF3 stock solutions (10ng/μL), 1μL of retinol working solution (5mM) and 1μL of palmitic acid stock solution (0.1M) per mL of LdM.

Δ **CRITICAL.** Palmitic acid is difficult to dilute, and therefore, the stock solution may precipitate. Vortex the solution before use.

23. On day 8, remove and discard the medium, wash cells with DPBS (-/-) and add LdM supplemented with 1μL of retinol working solution (5mM) and 1μL of palmitic acid stock solution (0.1M) per mL of LdM. We recommend you use one well for qPCR analysis

(Table 4) and another three wells for FACS analysis to check the expression of PDGFR β and presence of vitamin A as described in Box 1.

Δ CRITICAL STEP. Around 40% and 90% of cells should express PDGFR β and present vitamin A, respectively. If the expression of PDGFR β is less than 25%, do not continue with the differentiation.

24. On day 10, remove and discard the medium, wash cells with DPBS (-/-) and add LdM supplemented with 1 μ L of retinol working solution (5mM) and 1 μ L of palmitic acid stock solution (0.1M) per mL of LdM. We recommend you process one well for qPCR analysis (Table 4).

Δ CRITICAL. The cell population should have a more homogenous morphology compared to previous days and lipid droplets should be visible by viewing the culture under the microscope using a 10x objective.

25. On day 12, iPSC-HSC should have been obtained and can be used in downstream assays, as described in the next step. We also recommend one well of cells be used for qPCR analysis (Table 4) and two for PDGFR β FACS analysis and one for vitamin A analysis as described in Box 1. At day 12, supernatant can be collected to perform ELISA analysis if required. **? TROUBLESHOOTING**

Δ CRITICAL STEP Around 60% of iPSC-HSCs should be positive for PDGFR β . Close to 100% of cells should present vitamin A.

Δ CRITICAL. At the end of the differentiation each well should contain 500,000 - 600,000 cells.

Δ CRITICAL. Gene expression assays and immunofluorescence procedures for further characterization of the differentiation are explained in Tables 4 and 5. For more information about technical details, see the manufacturer's instructions.

Δ CRITICAL. Be aware that iPSC-HSCs are not quiescent and show some degree of activation, and therefore their activation state could potentially limit downstream applications.

□ PAUSE POINT. iPSC-HSCs can be incubated in the same plate until day 14 without further changes. However, we recommend to use them on day 12. Cells maintained in the same plate more than 14 days start to detach and die.

Downstream assays that can be undertaken with iPSC-HSCs

26. The iPSC-HSCs can now be used in a variety of ways. Follow option A to subculture and expand iPSC-HSCs. Follow option B to freeze and subsequently thaw iPSC-HSCs.

Follow option C to stimulate iPSC-HSCs. Follow option D to co-culture with hepatocytes and form spheroids.

A) Subculture and expansion of iPSC-HSCs • Timing 2-3 days (10min for cell passage)

- Δ **CRITICAL.** A new 12-well plate coated with Matrigel should be used to expand iPSC-HSCs. Depending on the experiments to be performed, other plate types can be used. Incubate at 37°C for a minimum of 20min before seeding cells. Warm the Expansion Medium and Trypsin-EDTA 0.25% at 37°C or RT before use.
- i. Remove and discard medium, incubate iPSC-HSCs with DPBS (-/-) for 1min at RT, remove and add 0.5mL of Trypsin-EDTA 0.25% to each well. Incubate cells at 37°C for 2-3min to detach.
- Δ **CRITICAL STEP.** Differentiated iPSC-HSCs are strongly attached to the Matrigel-coated plate as they secrete ECM proteins, therefore dissociation with trypsin may take longer than is usual for iPSCs. If cells are detached before day 8 or 10 of differentiation, dissociation times tend to be shorter (1-2min at 37°C). If the percentage of PDGFRβ positive cells is less than 60%, dissociation may take longer.
- ii. Check the cells under a microscope and when cells start detaching, add 0.5mL per well of medium containing 10% FBS to stop the reaction. Collect the cells by gently pipetting the medium. ? **TROUBLESHOOTING**
- Δ **CRITICAL STEP.** When detaching cells, Matrigel can also be released, if so, carefully remove it.
- iii. Centrifuge at 300g for 5min at RT.
- iv. During the centrifugation, wash the Matrigel pre-coated 12-well plate with DPBS (-/-).
- v. After centrifugation, aspirate the supernatant and add an appropriate volume of Expansion Medium. Usually, a cell split ratio of 1:2 for the first passage in a 12-well plate enables seeding of 73,000 cells/cm². The split ratio should be adapted according to the plate type and the number of wells being used.
- vi. Seed the cell suspension in the Matrigel-coated plate, move the plate to distribute cells homogenously and place it in the incubator.
- vii. Discard and replace the medium the day after seeding and subsequently every other day with fresh Expansion Medium.
- viii. After 2-3 days the wells should be around 70% confluent. They can be used for *in vitro* studies or expanded and passaged for a total of 3 passages. ? **TROUBLESHOOTING**
- Δ **CRITICAL.** Passaging iPSC-HSCs promote cell activation, which could potentially limit downstream applications.
- Δ **CRITICAL STEP.** Passaged iPSC-HSCs can be seeded without a Matrigel coating for *in vitro* studies of HSC activation.

B) Freezing and thawing iPSC-HSCs • Timing 2-3 days to obtain a confluent culture post thaw (30min to freeze and 20min to thaw)

- Δ **CRITICAL.** Prepare the Freezing Medium and store it at 4°C. It should be cold when added to the cell suspension. Warm Trypsin-EDTA 0.25% and medium containing FBS at RT or 37 °C. Program the centrifuge at 4°C.
- i. **Freezing:** Remove and discard medium, incubate iPSC-HSCs with DPBS (-/-) for 1min at RT, remove and add 0.5mL of Trypsin-EDTA 0.25% to each well. Incubate cells at 37°C for 2-3min to detach.
- Δ **CRITICAL STEP.** Differentiated iPSC-HSCs are strongly attached to the Matrigel-coated plate as they secrete ECM proteins, therefore dissociation with trypsin may take longer than is usual for iPSC. If cells are detached before day 8 or 10 of differentiation, dissociation times tend to be shorter (1-2min at 37°C).
- ii. Check the cells under a microscope and when cells start detaching, add 0.5mL per well of medium containing 10% FBS to stop the reaction. Collect the cells by gently pipetting the medium. ? **TROUBLESHOOTING**
- Δ **CRITICAL STEP.** When detaching cells, Matrigel can also be released, if so carefully remove it.
- iii. Count the collected cells.
- Δ **CRITICAL STEP.** 2 wells of the 12-well plate should contain 1 million iPSC-HSCs, which is usually sufficient for one cryovial.
- iv. Centrifuge the cells at 300g for 5min at 4°C.
- v. Discard the supernatant, resuspend the cells with the corresponding volume of Freezing Medium to give a final concentration of 1 million iPSC-HSCs per mL of medium. Add 1mL cell suspension per cryovial.
- vi. Place the cryovials rapidly into a cryofreezing container and keep overnight at -80°C.
- vii. After 24h, transfer the cryovial to a liquid nitrogen tank for long-term storage.
- **PAUSE POINT.** Cells can be stored in liquid nitrogen.
- viii. **Thawing:** Incubate plate pre-coated with Matrigel at 37°C for at least 20min and also warm the Expansion Medium. Ensure the water bath is at 37°C before moving to the next step. Thaw a cryovial of cells in the 37°C water bath. When only an ice crystal remains, take the cryovial out of the bath and spray it with ethanol before placing it into the hood.

- ix. Add 0.5mL warmed Expansion Medium to the cryovial and transfer the cells to a 15mL tube containing 9.5mL of the same medium.

Δ **CRITICAL.** After thawing, cell viability should be approximately 70%.

- x. Centrifuge cells at 300g for 5min at RT.
- xi. Meanwhile, wash the Matrigel-coated plates with DPBS (-/-).
- xii. After centrifugation, aspirate the supernatant, resuspend cells with the corresponding volume of Expansion Medium to plate 145,000 cells/cm². (As one million cells were frozen per cryovial, approximately, 1 cryovial should be split across 2 wells of a 12-well plate). Move the plate gently in order to distribute cells homogenously.
- xiii. Incubate the cells at 37°C.
- xiv. After 24h, discard and replace with Expansion Medium. **? TROUBLESHOOTING**
- xv. Replace medium every 48h until cells reach 70-80% confluence (this usually occurs after 2-3 days). Cells are now ready to be used for further studies.

C) Stimulation of iPSC-HSCs • Timing 15min (24h or 7 days of stimulation)

Δ **CRITICAL** Passaged or Thawed iPSC-HSCs at 70% confluence can be used.

Δ **CRITICAL.** Low FBS Medium should be warmed before use.

- i. Remove medium and replace it with Low FBS Medium.
- ii. After 24h incubation in Low FBS Medium, aspirate and discard the medium and incubate with Low FBS Medium containing TGFβ at 2.5μL of the stock solution per mL (final concentration of 5ng/mL).

CRITICAL. Inflammatory assays can be performed by LPS stimulation instead of TGFβ stimuli. Incubate the cells 24h with a final concentration of LPS of 100ng/mL as described in Coll et al. (2018)..

- iii. After 24h or 7 days of stimulation, remove medium and harvest RNA from iPSC-HSCs for activation analysis (Table 4).

Δ **CRITICAL.** For chronic TGFβ stimulation, change medium every other day.

Δ **CRITICAL.** As a positive control for the activation of iPSC-HSCs, cells can be compared to primary HSCs. Also, Passaged cells can be used as a positive control for the activation.

D) Spheroid Formation • Timing 21 days for the complete formation and stabilization of the culture (1h for seeding cells and 10min for medium refreshment every 48h)

Δ **CRITICAL** Differentiated HepaRG cells are also required, thawed immediately prior to starting this section following the procedure described in the Materials section (Thawing differentiated HepaRG cells).

- i. **Spheroid formation:** Fill the outer wells of a cell repellent Microplate 96-well with 100µL of DPBS (-/-) supplemented with P/S, to prevent medium evaporation during culture.
- ii. Remove and discard medium, incubate iPSC-HSCs with DPBS (-/-) for 1min at RT, remove and add 0.5mL of Trypsin-EDTA 0.25% to each well. Incubate cells at 37°C for 2-3min to detach.

Δ CRITICAL STEP. Differentiated iPSC-HSCs are strongly attached to the Matrigel-coated plate as they secrete ECM proteins, therefore dissociation with the trypsin may take longer than is usual for iPSCs. If cells are detached before day 8 or 10 of differentiation, dissociation times tend to be shorter (1-2min at 37°C).
- iii. Check the cells under a microscope and when cells start detaching, add 0.5mL per well of medium containing 10% FBS to stop the reaction. Collect the cells by gently pipetting the medium.

Δ CRITICAL STEP. When detaching cells, Matrigel can also be released, if so, carefully remove it.
- iv. Centrifuge at 300g for 5min at RT.
- v. Mix HepaRG and iPSC-HSCs in a 1:2 ratio to give a volume of 2,000 cells total per 10µL medium. Also allow an extra ~10% cell mix to compensate for loss of cells and medium when using a multichannel pipette for seeding.
- vi. Carefully seed 10µL of cell suspension mix in the center of each well of the microplate. The hydrophobic properties of the plate ensure that drop integrity is maintained during seeding and after starting the shaker in step viii.

Δ CRITICAL. The use of an electronic multichannel pipette is recommended to obtain uniform spheroids.
- vii. Incubate plates for 15-30min in a cell culture incubator to allow cells to settle at the bottom of the wells.
- viii. Place the plates on an orbital shaker suited for use in a cell culture incubator at 80 rpm during 24h.
- ix. The day after, most cells should be aggregated forming spheroids. **? TROUBLESHOOTING**

Δ CRITICAL STEP. If a large volume of cells was seeded in step vi, more time will be needed for cells to form aggregates. Add 100µL of HepaRG serum-free induction medium supplement (HFM) culture medium and place the plates back to the orbital shaker inside the incubator.
- x. Replace 90% of the HFM medium after 24h. Subsequently replace 90% of the medium in the culture every 48h. Follow Box 2 if you wish to fix and stain the co-culture at any point during the culture. **? TROUBLESHOOTING**

- Δ **CRITICAL STEP.** Medium refreshing must be done with precision to avoid aspiration and loss of samples. Ideally, medium change is done using an electronic multichannel 1,000 pipette. The medium is removed by placing the pipette tip at the medium-air interface at low aspiration speed, and fresh medium is added at the lowest dispense speed to the side of the well.
- Δ **CRITICAL.** After 7-14 days in culture, there should be increased hepatocyte activity due to the presence of the iPSC-HSCs¹⁴. From this time point onward fibrosis or toxicity studies can be started as shown in Coll et al. (2018). Cultures can be maintained for at least 21 days with both cell types present and functional. For longer culture periods replenish the DPBS (-/-) with P/S in the outer wells to minimize evaporation of the medium in outer wells containing spheroids.
- Δ **CRITICAL.** Guidance on how to carry out analysis of gene expression or immunofluorescence of liver spheroids can be found in Coll *et al.* (2018)¹⁴, Leite *et al.* (2016)³⁵, Table 2, Table 4, Figure 3B and Box 2.

Box 1: Flow cytometry analysis of PDGFR β and vitamin A across differentiation • Timing 1 hour and a half

Additional materials required for Flow Cytometry

Antibodies and Reagents

- PE Mouse Anti-Human CD140b Clone 28D4 (BD PharMingen, Cat#558821 RRID: AB_397132; https://scicrunch.org/resolver/RRID:AB_397132)
 - PE Mouse IgG2a, κ Isotype Control Clone MOPC-173 (Biolegend, Cat#400214 RRID:AB_326460; https://scicrunch.org/resolver/RRID:AB_326460)
 - FACs Tubes (BD biosciences, Cat#352052)
 - Sodium azide Natriumazid (Sigma, Cat#S2002)
 - MACS SmartStrainer (100 μ m) (Milteny Biotec, Cat#130-098-463)
- FACS Buffer: 2% FBS, 1% Sodium Azide and DPBS (-/-). Filter the solution with a 0.22 μ m filter. Once prepared, keep it at 4°C up to 2 months.

Equipment

- FACSCanto II Cytometer and GACSDiva Software (BD Biosciences)
- BD FACS DIVA SOFTWARE (BD PharMingen, <http://www.bdbiosciences.com/us/home>)

Flow Cytometry Analysis of PDGFR β

- Δ **CRITICAL.** Program the centrifuge at 4°C. Warm Trypsin-EDTA 0.25% and medium containing 10% FBS before starting.

1. Detach cells from two wells as described in steps i-iii of Step 26 Option A (Subculture and Expansion of iPSC-HSCs).
2. Resuspend cells in 3mL of cold DMEM Glutamax and place 1mL in each of 3 FACS tubes for analysis of Autofluorescence, Isotype and PDGFR β .
3. Add DPBS (-/-) to the top of each tube to wash the cells.
4. Centrifuge at 300g for 5min at 4°C.
5. Discard the supernatant and add 100 μ L of cold FACS Buffer to each tube. Resuspend cells and in dark conditions, add 1 μ L of each antibody to Isotype and PDGFR β tubes.

Δ CRITICAL STEP. The final volume of antibodies will depend on the cell concentration. For this analysis we optimized two wells of a 12-well plate at the end of differentiation (approximately 340,000 cells) and found the best dilution to be 1:100.

6. Incubate for 30min at RT under photo-protected conditions. At the end of the incubation, add DPBS (-/-) to the top of the tube to wash the cells.
7. Centrifuge at 300g for 5min at 4°C.
8. Discard the supernatant and resuspend cells in 200 μ L of FACS Buffer and perform FACS analysis. Follow the gate strategy described in Figure 2A in order to analyze the percentage of PDGFR β positive cell population.

Δ CRITICAL STEP. When detaching cells, Matrigel can also be released. Filter the final solution with a cell strainer of 100 μ m to avoid Matrigel obstructing the cytometer.

Flow Cytometry Analysis of Vitamin A

Δ CRITICAL. Warm Trypsin-EDTA 0.25% and medium containing 10% FBS before starting.

1. Detach one well of cells as described in steps i-iii of Step 26 Option A (Subculture and Expansion of iPSC-HSCs).
2. After centrifugation at 300g for 5min at 4°C, remove and discard the supernatant.
3. Add DPBS (-/-) to the top of the tube to wash cells.
4. Centrifuge at 300g for 5min at 4°C
5. Discard the supernatant and resuspend cells in 200 μ L of FACS Buffer. Transfer cell suspension to a FACS tube.
6. As for PDGFR β FACS analysis, select the singlet population by SSC-A and FSC-A. Detect vitamin A storage positive cells by assessing cells that are autofluorescent at 350-450/50-A, thus autofluorescent at this wavelength is an indication that cells contain vitamin A. Figure 2B shows the percentage of vitamin A storage positive cells typically obtained on days 0, 8 and 12 of the differentiation. iPSCs cells can be run as a negative control for autofluorescence.

Box 2: Immunostaining of the liver spheroids • Timing 2 hours

Fixing spheroids

1. Remove medium (keep for ELISA analysis if desired) from the wells, wash spheroids twice with DPBS (-/-).
2. Add 100μL of 4% Formaldehyde solution (formalin) to fix spheroids and incubate 8min at RT.
3. Dispose of formalin waste according to institutional disposal instructions and wash spheroids 3 times with PBS 1x.

Δ **CRITICAL STEP.** Fixed spheroids can be stored in PBS 1x at 4°C for several weeks. Spheroids can be used for whole spheroid staining (conditions need to be optimized for each antibody) or can be further processed for paraffin embedding and sectioning as described in the next step. ? **TROUBLESHOOTING**

Embed spheroids in Paraffin

4. Transfer 3-6 fixed spheroids per condition to a microscope slide, placing them close together. Remove as much PBS 1x as possible.
5. Carefully add a drop of approximately 20μL of 2% low melting point agarose on top of the spheroids and leave to solidify. This should not take more than 1 minute.

Δ **CRITICAL.** Aim at keeping the spheroids in 1 plane. This will allow staining, imaging and quantification of the spheroids at the same time.

6. Use a razor blade to remove the excess of agarose and cut into a square shape. Gently slide the agarose cube with spheroids into a paraffin embedding cassette.
7. Use a bench top tissue processor to embed the spheroids in paraffin. Cut paraffin sections of 3-7μm with a microtome according to Figure 3B.

TROUBLESHOOTING

See Table 6 for troubleshooting guidance. Figure 1A shows examples of the morphology of cells on day 12 following successful and poor differentiations.

Anticipated Results

Differentiation can be started when iPSCs plated on Matrigel-coated 12-well plate reach 70% of confluence. After approximately 4 days of differentiation, cells should be 100% confluent (Figure 1A).

At day 12, the percentage of PDGFRβ should be around 60-70%, while in undifferentiated cells (day 0) less than 1% of cells are PDGFRβ and 47% of cells are PDGFRβ positive at day 8 (Figure 2A). The majority (98%) of the day 12 progeny, contain vitamin A, detected by autofluorescence measurement, again consistent with the acquisition of an HSC-like cells phenotype (Figure 2B). Presence of vitamin A can also be detected in more than 85% of cells at day 8, whereas at day 0 autofluorescence is not seen. Lipid droplets should be visible under a 10x magnification microscope. In addition, the gene expression of HSC markers such as *COL1α1*, *PCDH7*, *PDGFRβ*

and actin alpha 2 smooth muscle (*ACTA2*) increase during differentiation, whereas *lecithin retinol acyltransferase* (*LRAT*) expression is maintained, indicative of acquisition of an HSC phenotype (Figure 1B and Table 3). Consistent with the gene expression, day 12 iPSC-HSCs stain positive for PDGFR β , PCDH7 and Vimentin, detected by immunofluorescence using the antibodies listed in Table 4 (Figure 2C).

iPSC-HSCs can be split and cultured with Expansion Medium, seeding 73,000 cells/cm². In addition, iPSC-HSCs progeny can be cryopreserved by freezing 1 million and thawing afterwards in Expansion Medium, seeding 145,000 cells/cm². Once cells are 70% confluent they can then be used for further assays. Although there are differences in the expression of some activation markers, Passaged iPSC-HSCs and Thawed iPSC-HSCs maintain an HSC-like morphology, and the expression of constitutive markers such as PCDH7 (Figure 4A and 4B). Be aware that passaged and thawed cells differ from the final population of the differentiation and are more activated. It is important to take into account the activation state of iPSC-HSCs when designing the studies, particularly those aiming at evaluating quiescent HSCs function.

As shown in Figure 4C and 4D, Passaged and Thawed iPSC-HSCs stimulated with TGF β for 24h become activated, with significantly increased expression of activation markers, such as *COL1 α 1*, *ACTA2*, *Lysyl Oxidase (LOX)* and non-significantly *SMAD family member 7 (SMAD7)*. Although modest, the induction of activation and fibrogenic genes is comparable to the differentiated cells at day 12 and primary HSCs¹⁴. However, when they are exposed to TGF β for a longer period of time (7 days), expression of HSC activation markers further increases indicating their potential use for chronic injury modelling. In addition, differentiated cells express pro-inflammatory genes in response to mediators such as lipopolysaccharide (LPS)¹⁴. For qPCR analysis, reference Table 3.

Differentiated cells can also be co-cultured at a ratio of 2:1 (iPSC-HSCs:HepaRG) with hepatocyte cell lines to generate multicellular spheroids. As shown in Figure 3A, complete cell aggregation occurs on day 2-3 and the spheroids can be maintained 21 days for long-term *in vitro* studies. Under this condition, iPSC-HSCs present a reduced activation phenotype and hepatocytes maintain their metabolic profile during, at least, 21 days of culture. Further data can be found in Coll *et al.* (2018)¹⁴, which describes how treatment of iPSC-HSCs-HepaRG spheroids with TGF β , TAA (Thioacetamide) and APAP (Acetaminophen) induced expression of HSC activation markers and secretion of pro-collagen type I.

Altogether, these results indicate, that the protocol presented here is efficient, as a large fraction of iPSC-HSCs express PDGFR β , contain vitamin A and express the main markers of HSCs, quite closely mimicking cultured primary HSCs. Moreover, they represent a source of iPSCs-HSCs to perform *in vitro* fibrogenic, inflammation and toxicity assays, under acute and chronic insults. In addition, the possibility of culturing iPSC-HSCs in 3D co-cultures, allows the generation of complex *in vitro* systems and the study of cell-cell interactions in liver spheroids.

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Author contributions

J.V. and R.A.M equally contributed to the optimization of expansion and freezing processes, characterization of the cells and drafting the manuscript. I.M. and A.S. performed the 3D spheroid experiments, and S.V. and. A.S. carried out parallel iPSC-differentiations using a different iPSC line (not included in the manuscript). M.C. participated in the differentiation protocol design and reviewed the manuscript. S.A., T.R-T., B.A-B., C.M-S., D.B., participated in the differentiation protocol process. C.M.V. contributed to the design of the IPSCs differentiation protocol and reviewed the manuscript. L.A.v.G. guided the iPSC and spheroid studies in Brussels and critically reviewed the manuscript. P.S-B. designed and supervised the

study and critically reviewed the manuscript.

Competing interests

M.C., C.M.V., and P.S.-B. have a patent application PCT/EP2016/079464 and L.A.v.G. has a patent application PCT/EP2015/062551.

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the paper.

FIGURES LEGENDS

Figure 1. Time course of the 12 day differentiation protocol. (A) Schematic representation indicating which growth factors and cytokines (BMP4, FGF1, FGF3, retinol and palmitic acid) are included at different timepoints. Microscopy images show the changes in cell morphology observed during differentiation (Scale bars 100µm). Homogeneous and heterogeneous final populations are shown as representative images of good and poor differentiation. (B) Gene expression of *COL1α1*, *PCDH7*, *LRAT*, *PDGFRβ* and *ACTA2* assayed by qPCR in samples taken on days 0, 4, 8, 10 and 12 of the differentiation protocol. qPCR was undertaken following the manufacturer's instructions and using the materials and reagents detailed in Table 4. Experiment performed in 5 independent differentiations. *p< 0.05 compared with day 0 by t-test.

Figure 2. Characterization of iPSCs differentiation to HSC-like cells. (A) Representative flow cytometry plots of total cell population and PDGFRβ positive cells on days 0, 8 and 12 of the differentiation. PE Mouse Anti-Human CD140b Clone 28D4 (PDGFRβ antibody) and PE Mouse IgG2a, κ Isotype Control Clone MOPC-173 (Isotype antibody) were used in a 1:100 dilution as indicated in Box 1. (B) Representative histogram of flow cytometry analysis of the expression of vitamin A at days 0, 8 and 12 as indicated in Box 1. (C) Representative immunofluorescence confocal images of PDGFRβ, PCDH7 and Vimentin in iPSC-HSCs at day 12. Details of the antibodies used are given in Table 5. PDGFRβ antibody was used at a 1:50 dilution, PCDH7 antibody was used at a 1:100 dilution, Vimentin antibody was used at a 1:100 dilution and Hoechst (blue) was used at a 1:2000 dilution. Secondary antibodies (Goat Anti-Mouse and Donkey Anti-Rabbit) were used at a final dilution of 1:500 (Scale bars 100µm).

Figure 3. Scheme of the downstream assays with iPSC-HSCs. (A) Schematic representations of the different downstream assays that can be undertaken at the end of the differentiation (step 26). Option A) Subculture and expansion of iPSC-HSCs. Option B) Freezing and thawing iPSC-HSCs. Option C) Stimulation of iPSC-HSCs. Option D) Spheroid Formation. (B) Schematic process of spheroid embedding used for immunofluorescence analysis of spheroids for PDGFRβ antibody used in a 1:50 dilution, CYP3A4 antibody used in a 1:100 dilution and DAPI (mounted DAPI containing medium) staining. For more technical information, check Box 2 (Scale bar 100µm).

Figure 4. Passage and Thawed iPSC-HSCs characterization and stimulation. (A) Gene expression of *PCHD7*, *LRAT*, *P75NTR*, *PDGFRβ*, *COL1α1*, *ACTA2* and *LOX* in iPSC-HSCs Passage 1 and Thawed iPSC-HSCs compared to cells at day 12. qPCR was undertaken following the manufacturer's instructions and using the materials and reagents detailed in Table 4. Experiments were performed using 3 independent differentiations. *p< 0.05 compared with iPSC-HSCs at day 12 by t-test. (B) Bright field microscopy images of iPSC-HSCs morphology at day 12, Passage 1 and Thawed cells (Scale bar 100µm). (C) Gene expression analysis of *COL1α1*, *ACTA2*, *LOX* and *SMAD7* in Passaged iPSC-HSCs after 24h or 7 days TGFβ stimuli (5ng/mL), as detailed in step ii option C. qPCR was undertaken following the manufacturer's instructions and using the materials and reagents detailed in Table 4. Experiments were performed using 5 and 4 independent differentiations* p< 0.05 compared to vehicle by t-test. (D) Gene expression analysis of *COL1α1*, *ACTA2*, *LOX* and *SMAD7* in Thawed iPSC-HSCs after 24h or 7 days TGFβ

stimuli (5ng/mL). qPCR was undertaken following the manufacturer's instructions and using the materials and reagents detailed in Table 4. Experiment were performed using 4 independent differentiations. *p< 0.05 compared to vehicle by t-test.

Table 1: Scheme of the differentiation protocol indicating all the medium changes. At each medium change cells should first be washed with DPBS (-/-)

Day	Treatment	Analysis
0	LdM + 2µL of BMP4 per mL of LdM	Collect one well for qPCR analysis and two for PDGFRβ FACS analysis and one well for vitamin A FACS analysis
2	LdM + 2µL of BMP4 per mL of LdM	N/A
4	LdM + 2µL of BMP4 + 2µL of FGF1 + 2µL of FGF3 per mL of LdM	Collect one well for qPCR analysis
6	LdM + 2µL of FGF1 + 2µL of FGF3 + 1µL retinol + 1µL palmitic acid per mL of LdM	N/A
8	LdM + 1µL retinol + 1µL palmitic acid per mL of LdM	Collect one well for qPCR analysis and two for PDGFRβ FACS analysis and one well for vitamin A FACS analysis
10	LdM + 1µL retinol + 1µL palmitic acid per mL of LdM	Collect one well for qPCR
12	LdM + 1µL retinol + 1µL palmitic acid per mL of LdM	Collect one well for qPCR analysis and two for PDGFRβ FACS analysis and one well for vitamin A FACS analysis

Table 2: Main hepatocytes, quiescent and fibrogenic HSC markers used for gene expression analysis.

Hepatocytes	Quiescent HSCs	Fibrogenic HSCs
<i>Albumin</i>	<i>LRAT</i>	<i>LOXL2</i>
<i>CYP3A4</i>	<i>PPARγ</i>	<i>COL1α1</i>

<i>GSTAT1</i>	<i>SPARCL</i>	<i>COL3α1</i>
<i>SLCO1B1</i>		<i>ACTA2</i>
	<i>PDGFRβ</i>	
	<i>PCDH7</i>	

Table 3: Additional materials we use for Quantitative Real Time PCR (qPCR) analysis

Oligonucleotides	Reference	Company
<i>GAPDH</i>	Hs99999905_m1	Thermo Fisher Scientific
<i>PCDH7</i>	Hs00941345_m1	Thermo Fisher Scientific
<i>LRAT</i>	Hs00428109_m1	Thermo Fisher Scientific
<i>SMAD7</i>	Hs00178696_m1	Thermo Fisher Scientific
<i>LOX</i>	Hs00184700_m1	Thermo Fisher Scientific
<i>ACTA2</i>	Hs00909449_m1	Thermo Fisher Scientific
<i>COL1α1</i>	Hs00164004_m1	Thermo Fisher Scientific
<i>PDGFRβ</i>	Hs00182163_m1	Thermo Fisher Scientific
<i>P75NTR</i>	Hs00609976_m1	Thermo Fisher Scientific
Other reagents	Reference	Company
RNeasy Micro Kit	Cat#74004	QIAGEN
High capacity cDNA reverse Transcription kit	Cat#4368813	Applied Biosystems
TaqMan™ Fast Advanced Master Mix	Cat#4444556	Thermo Fisher Scientific
TaqMan™ Gene Expression Assay	Cat#4351372	Thermo Fisher Scientific
Equipment	Reference	Company
PTC-100™ Programmable	Cat#78299-1	MJ Research, INC

Thermal Controller		
Applied Biosystems 7900HT Fast Real-Time PCR System	Cat#4329001	Applied Biosystems

Table 4: Additional materials we use for Immunofluorescence analysis

Antibodies	Reference	Company
Recombinant Anti-PDGFR beta antibody [Y92] - C- terminal	Cat#ab32570 (RRID:AB_777165; https://scicrunch.org/resolver/RRID: AB_777165)	Abcam
Anti-PCDH7 antibody [OTI2G6]	Cat#ab139274 (RRID:AB_2868608; https://scicrunch.org/resolver/RRID: AB_2868608)	Abcam
Novocastra™ Liquid Mouse Monoclonal Antibody Vimentin	Cat#NCL-VIM-V9 (RRID:AB_442141; https://scicrunch.org/resolver/RRID: AB_442141)	Leica Biosystems
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Cat#A-11001 (RRID:AB_2534088; https://scicrunch.org/resolver/RRID: AB_2534088)	Thermo Fisher Scientific
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Cat#711-545-152 (RRID:AB_2313584; https://scicrunch.org/resolver/RRID: AB_2313584)	Jackson Immuno Research
Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL	Cat#H3570 (RRID:AB_2868610; https://scicrunch.org/resolver/RRID: AB_2868610)	Thermo Fisher Scientific
Other reagents	Reference	Company

Formaldehyde solution 4%, buffered, pH 6.9	Cat# 100496	Millipore Corporation
Equipment	Reference	Company
Laser Scanning Confocal spectral High Speed Multiphoton Microscope	TCS SP8 MP	Leica
Fiji ImageJ	https://imagej.net/Using_Fiji	Fiji

Table 5: Troubleshooting

Step	Problem	Possible reasons	Solutions
6	Cells stay as single cells or colonies do not grow	The growth rate of different cell lines may vary, as may the growth rate following cryopreservation.	Incubate iPSCs for an additional day and/or adjust initial seeding density. Ensure that cell pipetting is done carefully so cell clusters are maintained. Be sure to thaw cells correctly and do not overheat the cryovial.
7	iPSCs die	Cell contamination might be impacting iPSC culture. Also, the number of cells plated or the number of colonies that have been plated may affect cell growth.	Check the incubator and the reagents used. E8 Medium can be supplemented with antibiotics such as P/S at a 1:1000 dilution without affecting cell growth.
8	Colonies become elongated instead of rounded	The cells might be starting to differentiate and lose pluripotent characteristics.	A new cryovial of iPSCs should be thawed. Note that this can happen due to the addition of cold E8 Medium, which can induce cell stress.
14	Cells do not attach to the Matrigel-coated plate	Matrigel concentration might not be correct. Matrigel might not have been prepared in cold conditions. Plate was prepared more than 5 days ago.	Check the Matrigel concentration and expiry date, and prepare the coating in cold conditions. It is also critical to ensure uniform Matrigel distribution. Ensure the correct

				number of iPSCs are plated and that they are viable.
17	Cells do not grow sufficiently at 1-2 days of culture		Low concentration of plated cells or quality of the iPSCs. Sometimes this occurs because the Matrigel coating was not prepared correctly.	Check plating cell number, quality of iPSCs and the concentration, lot and expiration of reagents. Ensure that the Matrigel-coated plate is correctly prepared and Matrigel evenly distributed across the plate.
25	Suboptimal differentiation	HSCs	The confluence of the cells was not around 60-70% at the start of differentiation. The Matrigel coating was not prepared correctly.	Check the quality of iPSCs and the confluence as well as the distribution of the cells when starting the differentiation. It is important to move the plate after cell seeding in order to avoid cell accumulation in the center of the well. Growth factors and cytokines should not be stored at 4°C for more than one week.
26A)ii	Differentiated cells do not detach from the culture plate		The differentiated cells might produce more ECM proteins which can impact detaching efficiency.	Check the quality of the trypsin used. Increase the time of exposure if there is a lot of ECM in the well and be sure to pipette the medium several times across the entire well to collect the maximum number of cells. It is important to wash the cells with DPBS (-/-) for 1min before the addition of trypsin.
26A)viii	Cells do not attach or do not grow		PDGFR β expression is lower than 60%. Matrigel coating was not prepared correctly. Cells were incubated for more than 5 min with trypsin.	Ensure medium is warmed before plating cells. Check the number of cells plated and check cells are expressing PDGFR β .
26B)xiv	Thawed cells do not grow well		The quality of the differentiation may affect the thawing process, thus only iPSC-HSCs with more than	Before freezing cells, check the quality of the differentiation by assessing the percentage of PDGFR β positive cells. Check cell

		60% cells that are positive for PDGFR β should be used. Low viability before freezing the cells may affect the thawing efficiency. Also, exposure to DMSO at RT for too long may affect cell viability.	viability when freezing and thawing cells. Count the number of frozen cells. Transfer the cryovials from the -80°C freezer to a liquid nitrogen tank 24h after freezing. After freezing, ensure a sufficient number of cells is plated. We recommend plating 1 vial in 2 wells of a 12-well plate.
26D)ix	Less than 90% of cells are integrated in a round spheroid after 24h culture	The quality of the differentiation may affect the capacity to form spheroids. Also, check the viability before seeding cells.	If the cells do not form spheroids after 24h in culture the culture should be abandoned.
26D)x	Absence of spheroids	Small spheroids can be aspirated when changing medium.	Carefully perform 50% medium changes and check the pipette when aspirating. Add fresh medium carefully to the wall of the wells.
Box 2	Spheroid staining is heterogenous	Staining intensity depends on the depth of the spheroid and can vary among spheroids.	Multiple spheroids need to be analyzed per condition and, preferentially, multiple sections per condition need to be stained.

FIGURE 1

A.

B.

FIGURE 2

FIGURE 3

A.

OPTION A:
PASSAGE

OPTION B:
FREEZE/THAW

OPTION C:
STIMULATION

OPTION D:
SPHEROID FORMATION

B.

FIGURE 4

A.

B.

C.

D.