Serum-Free Production of Three-Dimensional Hepatospheres from Pluripotent Stem Cells

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Abstract

Developing renewable human liver tissue from stem cells has been pursued as a potential source of biological material for pharmaceutical and clinical endeavours. At present, two-dimensional differentiation procedures deliver tissue lacking long-term phenotypic and functional stability. Efforts to overcome these limiting factors have led to the development of protocols to generate three-dimensional cellular aggregates. Here we describe a methodology to generate 3D hepatospheres from human pluripotent stem cells using defined and commercially available reagents.

Key words: Human Pluripotent stem cells, Hepatic differentiation, Parenchymal cells, 3D cell culture, Regenerative Medicine.

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1. Introduction

The liver is a vital organ and significant loss of its function has serious consequences for human health [1]. In the UK, liver disease is one of the leading cause of premature death. Orthotopic liver transplantation (OLT) has been considered as the most effective mode of treatment for acute or end-stage liver failure. However, the shortage of organ donors and complications associated with lifelong immunosuppression has driven the quest to develop alternative therapies.

While donor hepatocytes have been used to successfully to treat metabolic liver

disease [1], the scarcity of human hepatocytes and their transient phenotype following isolation has remained a major bottleneck for widespread clinical application. Unlimited self-renewal capacity and ability to differentiate into all cell types in the body has made the human pluripotent stem cells (hPSCs) a credible alternative with which to generate a stable source of quality assured human liver tissue for applied medicine. To this end, several protocols have been developed to efficiently generate hepatocyte-like cells (HLCs) under conventional two-dimensional (2D) differentiation systems [2-6]. Despite recent improvements [7-10], 2D-derived HLCs exhibit foetal features and unstable phenotype in vitro, limiting their clinical application.

To improve hepatocyte stability, three-dimensional (3D) approaches have been explored to generate human liver tissue [11, 12]. Most developed 3D protocols have employed extracellular matrix such as Matrigel® with or without inclusion of other cell types [13-17] or synthetic niche scaffold [18]. These models have been important to further our understanding of human development and physiology. However, they were manufactured using animal-derived components and suffer from batch variation which limits widespread application of the technology.

Herein, a scalable and defined 3D differentiation process is described to generate 3D human liver tissue (Figure 1). Using this platform, hPSC-derived liver tissue can be generated that display modest liver function for an extended period in culture. During differentiation, cell organisation become evident (Figure 2 & 3), correlating with stable function, reduced proliferation and loss of foetal protein secretion (Figure 4) [19, 20]. The *in vivo* functionality of hPSC-derived 3D Heps was also demonstrated following transplantation into immune-competent or deficient recipients with compromised liver function [19].

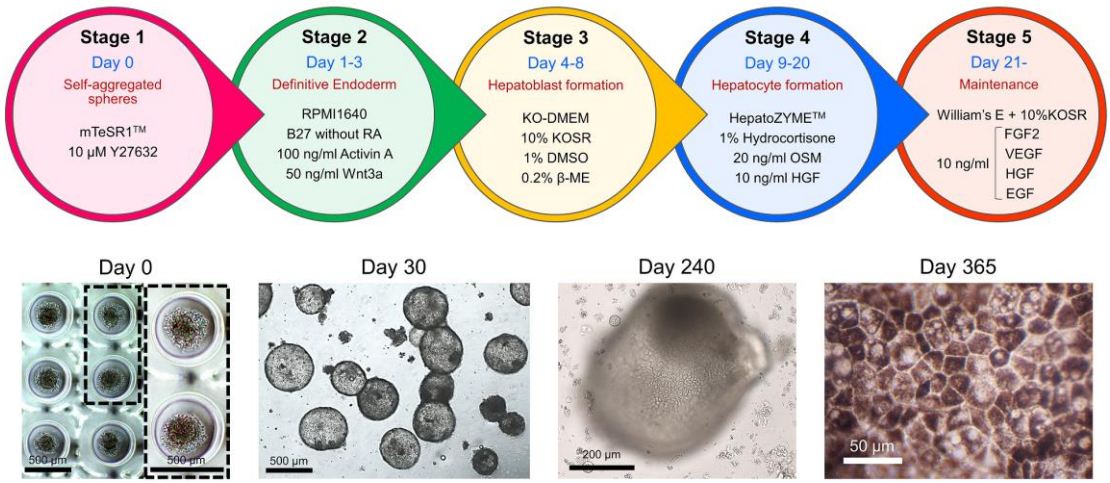

Figure 1: Schematic representation detailing of stepwise differentiation protocol to generate 3D hepatospheres (3D Heps) from hPSCs. The hPSCs are sequentially directed through different stages to yield 3D Heps which maintained their phenotype for an extended period in culture.

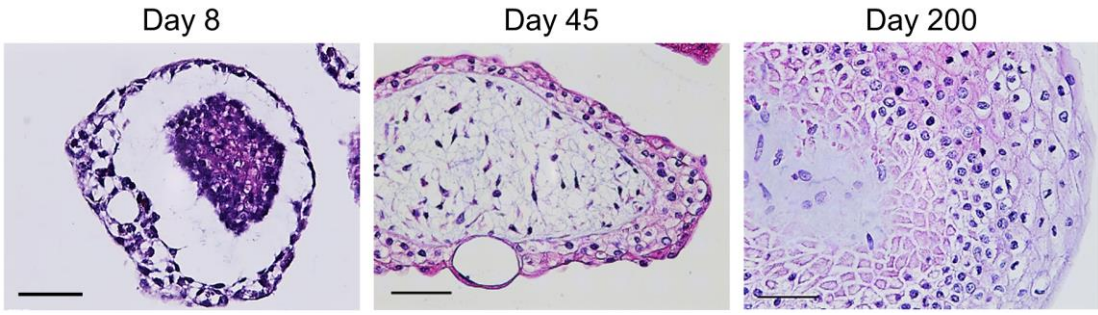

Figure 2: H&E staining of 3D Heps at various time points. 3D Heps form of two distinct population. A central mesenchymal core surrounded by several layers of hepatocytes. Scale bar, 50 µm.

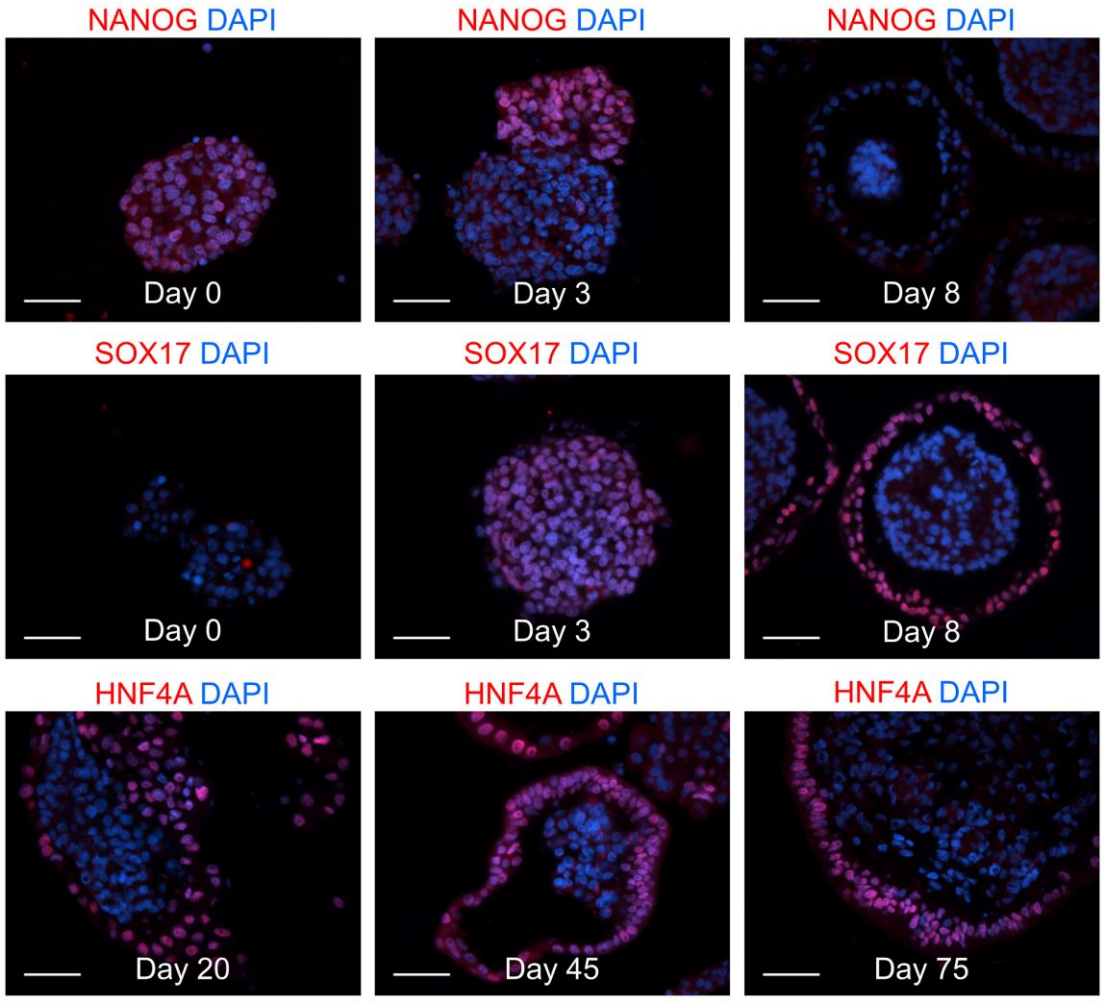

Figure 3: Detection of pluripotent (NANOG), definitive endoderm (SOX17) and hepatic (HNF4A) markers. Loss of NANOG and expression of SOX17 indicated successful differentiation into definitive endoderm at early stages of differentiation (Top two rows). Expression of HNF4A as an important transcription factor for hepatic function can be detected during stage 4 and 5 of differentiation (bottom row). Scale bar, 50 µm.

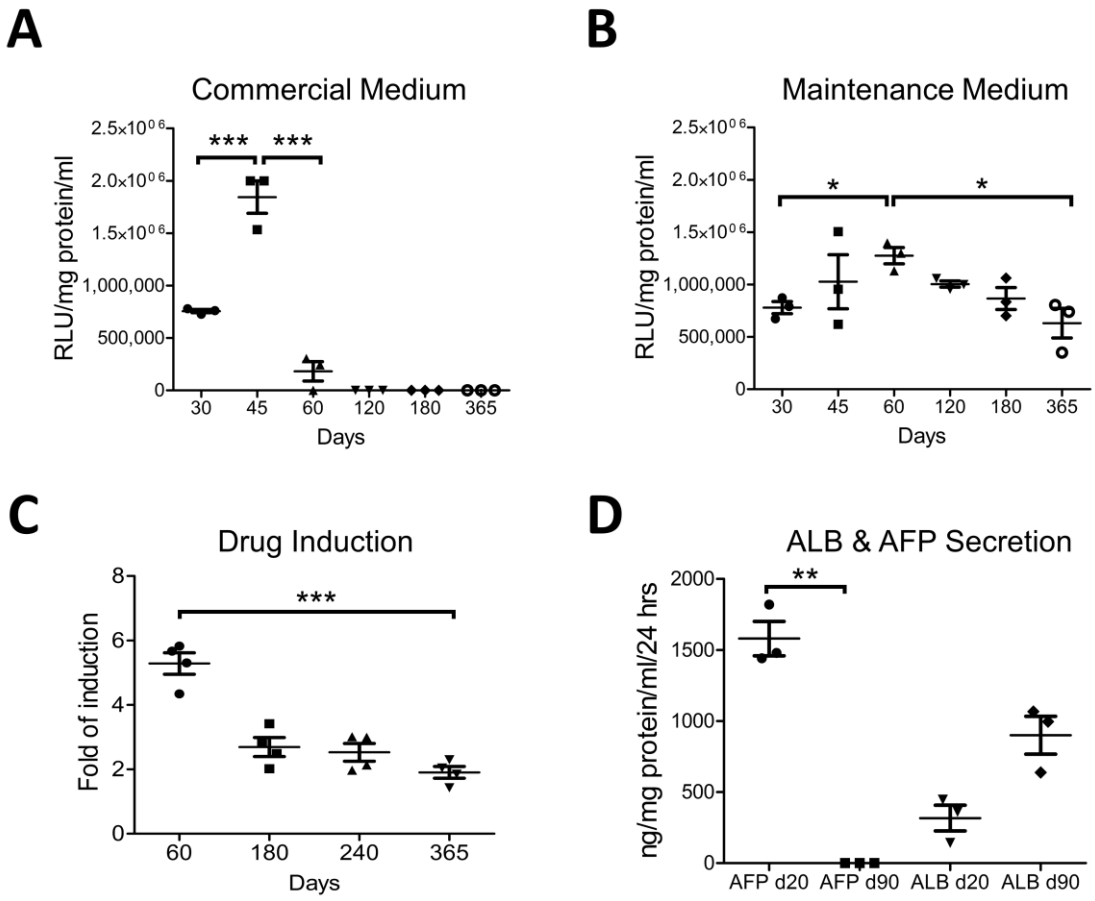

Figure 4: Cyp3A activity of differentiated 3D Heps maintained for short-term using a commercially available medium (A) and for long-term in maintenance medium (B). 3D Heps remain drug inducible for 365 days in vitro (C). Secretion of AFP and ALB in 3D Heps at days 20 and 90 of differentiation (D).

2. Materials

2.1 Human Pluripotent Stem Cell (hPSC) Culture

Product Name	Catalogue Number	Source
H9 hESC line	WA09	WiCell
mTeSR1 Feeder-free growth media	5850	STEMCELL Technologies
Y-27632 hydrochloride (Ri)	10005583	Cayman Chemical Company
PBS without $CaCl_2$ and $MgCl_2$	14190250	Thermo Fisher Scientific
PBS with $CaCl_2$ and $MgCl_2$	14040133	Thermo Fisher Scientific
Recombinant laminin 521	LN521	BioLamina

2.2 Generation of self-aggregated spheres (Stage 1)

Product Name	Catalogue Number	Source
MicroTissues® 3D Petri Dish®	Z764000-6EA	Sigma-Aldrich
Gentle Cell Dissociation Reagent	7174	STEMCELL Technologies
Agarose, low gelling temperature	A9045	Sigma-Aldrich

2.3 Differentiation of 3D aggregates into Definitive Endoderm (Stage 2)

Product Name	Catalogue Number	Source
RPMI1640	21875	Thermo Fisher Scientific
B27 supplement	12587-010	Thermo Fisher Scientific
Penicillin-Streptomycin	15140122	Thermo Fisher Scientific
Recombinant human Activin A	120-14E	Peprotech
Recombinant mouse Wnt3a	1324-WN-500/CF	bio-techne

- Prepare a 1,000x stock solution of human activin A by dissolving human activin A lyophilized protein in sterile 0.2% bovine serum albumin (BSA) in 1x Dulbecco's phosphate-buffered saline (DPBS) to a final concentration of 100 µg/mL. Store at -20 °C in small aliquots. Use at 1:1,000 (Note 1).
- Prepare a 1,000x stock solution of Wnt3a by dissolving mouse Wnt3a lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:200 (Note 1).

2.4 Differentiation of 3D aggregates into hepatoblast (Stage 3)

Product Name	Catalogue Number	Source
KnockOut™ DMEM	10829	Thermo Fisher Scientific
KnockOut™ Serum Replacement	10828	Thermo Fisher Scientific
GlutaMAX™	35050038	Thermo Fisher Scientific
100× MEM-NEAA	11140050	Thermo Fisher Scientific
DMSO	D5879	Sigma-Aldrich
Beta-mercaptoethanol	31350	Thermo Fisher Scientific
Penicillin-Streptomycin	15140122	Thermo Fisher Scientific

2.5 Differentiation 3D aggregates into hepatocyte (Stage 4)

Product Name	Catalogue Number	Source
HepatoZYME-SFM	17705021	Thermo Fisher Scientific
Hydrocortisone 21-hemisuccinate	H4881	Sigma-Aldrich
Human recombinant OSM	300-100	Peprotech
Human recombinant HGF	100-39	Peprotech
Penicillin-Streptomycin	15140122	Thermo Fisher Scientific

- Prepare a 1,000x stock solution of human hepatocyte growth factor (HGF) by dissolving human HGF lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1,000 (Note 1).
- Prepare a 1,000x stock solution of oncostatin M (OSM) by dissolving OSM in sterile 0.2% BSA/DPBS to a final concentration of 20 µg/mL. Store at -20 °C in

small aliquots. Use at 1:1,000 (Note 1).

2.6 Maintenance and long-term culture of 3D Heps (Stage 5)

Product Name	Catalogue Number	Source
William's E Medium	A1217601	Thermo Fisher Scientific
KnockOut™ Serum Replacement	10828	Thermo Fisher Scientific
GlutaMAX™	35050038	Thermo Fisher Scientific
Human recombinant EGF	236-EG	bio-techne
Human recombinant HGF	100-39	Peprotech
Human recombinant VEGF	293-VE	bio-techne
Human recombinant FGF2	100-18B	Peprotech
Penicillin-Streptomycin	15140122	Thermo Fisher Scientific

- Prepare a 1000x stock solution of epithelial growth factor (EGF) by dissolving the lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1,000 (Note 1).
- Prepare a 1,000x stock solution of basic fibroblast growth factor (bFGF) by dissolving the lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1,000 (Note 1).
- Prepare a 1,000x stock solution of vascular endothelial growth factor (VEGF) by dissolving the lyophilized protein in 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1,000 (Note 1).

3. Methods

All Media and buffered used in these experiments must be sterile and at room temperature (RT) for cell culture.

3.1. Preparation of Agarose Microplate Molds

1. Dissolve 2 g of low melting temperature agarose into 100 mL of distilled water and sterilized by autoclaving.

2. Carefully heat the sterilized 2% agarose in a microwave with interval shaking to dissolve completely.

3. Add 520 µL of melted agarose to a 256-well format mold and leave to solidify.

   - Transfer each agarose microplate into a single well of a 12-well plate.
   - Add 1.5 mL of DPBS to each well and remove the air bubbles from the microwells by gently pipetting up and down several times using a P1000 pipette tip. This is performed to ensure uniform cell seeding. Agarose microplates can be stored for up to 6 months at 4 °C in DPBS.

3.2. Preparation of polyHEMA-coated plates

Dissolve 2 g of poly-HEMA (Sigma-Aldrich) in 100 mL of 95% ethanol. Stir the solution overnight using a hot plate at 55 °C. Add 250 µL of poly-HEMA solution

per well of a 24-well plate and dry overnight at 60 °C using an oven.**3.3. Stage 1 – Formation of hPSCs aggregates in Agarose Microwell Plates**

1. Cultured hPSCs on LN-521-coated plated in mTeSR1 with the medium changed every 24 h and passaged regularly once the cells reach 75% to 85% of confluency.

2. Rinse the cells with 5 mL of RT 1x DPBS without $CaCl_2$/$MgCl_2$ and remove the buffer.

3. Add 5 mL of 1x cell dissociation reagent to the cells and allow cell dissociation by incubating cells at 37 °C for 6-8 min. Examine cell detachment under the microscope. The cells should be partially detached from the plate. Extend the incubation for an extra 1-2 min if longer time is required (Note 2).

4. To dissociate colonies into single cells, gently pipette the dissociation buffer up and down while washing the whole surface of the plate using a P1000 pipette tip.

- Centrifuge the suspension at 200 RCF for 5 minutes, remove supernatant and resuspend in mTeSR1 supplemented with 10 µM ROCK inhibitor Y27632.
- Count the viable cells using a hemocytometer and trypan blue staining exclusion. Following this, prepare the cell suspension at 2.0×10^6 viable cells per ml.
- Transfer 190 µl of cell suspension to agarose wells (3.84×10^5 cells per agarose microplate) to generate spheroids with 100-150 µm in diameter.
- After seeding, return the plates to the cell incubator at 37 °C and 5% CO2 for 2 h to allow the cells to settle.
- After 2 h, add 1 mL of fresh and warm mTeSR1 medium supplemented with 10 µM ROCK inhibitor Y27632 to each well.
- Return the plates to the cell incubator at 37 °C and 5% CO2 for 24 h and examine the formation of spheres the next day to allow the cells to attach.

3.4. Stage 2 – Differentiating self-aggregated spheres to 3D definitive endoderm

1. Make endoderm differentiation medium consisting of Roswell Park Memorial Institute 1640 (RPMI 1640) basal medium supplemented with 2% B27 supplement (50x, without insulin), and 1% penicillin/streptomycin (final concentrations: 100 IU/mL and 100 µg/mL, respectively).

2. Check 3D aggregate formation 24 h post seeding and initiate hepatocyte differentiation by carefully removing the mTeSR1 medium and replace it with 1 mL of fresh endoderm differentiation medium supplemented with 100 ng/mL activin A and 50 ng/mL Wnt3a (Note 3-4).

- Change supplemented endoderm priming medium every 24 h for 3 days.

3.5. Stage 3 – Generation of 3D hepatoblasts

1. Make hepatoblast differentiation medium consisting of knockout Dulbecco's modified Eagle medium (KO-DMEM) with 20% knockout serum replacement (KOSR) and supplemented with 0.5% GlutaMAX™, 1% non-essential amino acids (NEAA), 0.1 mM beta-mercaptoethanol, 1% DMSO, and 1% penicillin/streptomycin.

2. Following definitive endoderm induction, switch to hepatoblast differentiation medium for hepatoblast specification for 5 days.

3. Refresh the medium every 2 days and perform the last change on the last day of the hepatoblast specification.

3.6. Stage 4 – Maturation of 3D hepatoblasts to generate 3D Heps

1. Make hepatocyte maturation medium consisting of HepatoZYM-SFM medium supplemented with 1% of GlutaMAX™, 10 μM hydrocortisone 21-hemisuccinate sodium salt (HCC), 1% penicillin/streptomycin.

2. Aspirate KSR/DMSO medium and wash the cells once by hepatocyte maturation medium without supplements.

- Add 1 mL of hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM (Note 3-4).
- Using a P1000 pipette, lift up the 3D Heps from the agarose microplate by pipetting up and down the solution several times.
- Transfer the medium containing the 3D Heps to a poly-HEMA coated well.
- Wash the agarose microplate using 1 mL of hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM and transfer the medium to the poly-HEMA coated well.
- Change the medium every 48 h for 12 days.

3..7. Stage 5 – Long-term culture of 3D Heps

1. Make hepatocyte maintenance medium consisting of William's E medium supplemented with 10% knockout serum replacement, 1% of GlutaMAX™, and 1% penicillin/streptomycin.

2. At day 20, remove hepatocyte maturation medium and wash cells once with hepatocyte maintenance medium without the supplements.

- Add 1 mL of hepatocyte maintenance medium supplemented with 10 ng/mL of HGF, EGF, FGF and VEGF (Note 3-4).
- Refresh the medium for fresh hepatocyte maintenance medium every 48 h. For each medium change, supplement the required volume with HGF, EGF, bFGF and VEGF (final concentration of each growth factor at 10 ng/mL).
- 3D Heps will remain phenotypically and functionally stable for extended period of over a year in culture.

3.8. Measuring CYP activity of Long-term Cultured 3D Heps

1. Switch to hepatocyte maturation medium supplemented with 10 µM HCC, L-glutamine, 1% penicillin/streptomycin and 10 ng/mL HGF 48 h prior to performing functional analysis.

2. Analyze hepatocyte metabolic function using cytochrome (CYP) P450 assays.

- Replace medium with 1 mL of fresh hepatocyte maturation medium supplemented with 50 µM luciferin-6'-pentafluoro-benzyl ether (luciferin-PFBE) substrate to detect CYP3A basal activity or 100 µM luciferin-methyl ether (luciferin-ME) substrate to detect CYP1A2 basal activity (minimum number of replicates = 3). Incubate cells for 24 h at 37 ° C.
- Collect the supernatants using a P100 pipette tip and carry out the assay as per manufacturer's instructions.
- Use tissue culture media as a negative control.
- Measure the relative levels of basal activity and normalize to per mg protein as determined by the bicinchoninic acid assay (BCA).

3.9. Assessment of protein production using enzyme-linked immunosorbent assay (ELISA).

1. Replace medium with 1 mL of fresh hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM and incubate cells at 37 ° C (minimum number of replicates = 3).

- Collect the supernatant after 24 h using a P100 pipette tip and measure the relative levels of serum protein production as per manufacturer's instructions.
- Use tissue culture media as a negative control.
- Normalize to per mg protein as determined by the BCA Assay.

3.10. Immunocytochemistry

To prepare paraffin sections containing 3D Heps:

- Wash the 3D Heps three times with 1x DPBS.
- Fix the 3D Heps with ice-cold methanol for 30 min.
- Wash three times with 1x DPBS.
- Embed the 3D Heps in 300 µL of a tempered solution of 2% agarose dissolved in H2O using an empty well of a 24-well plate as a mold and leave it to solidify for 30 min.
- Embed the agarose containing 3D Heps in paraffin.
- Section the paraffin block containing fixed 3D Heps in 4 µm thick sections using a microtome.

To de-wax and rehydrate the sections:

- Place sections in a slide rack.
- Immerse slide rack containing sections in a staining trough containing 300 mL of xylene for 5 min.
- Repeat previous step.
- Immerse in absolute ethanol for 30 s.
- Immerse in 95% ethanol for 30 s.
- Immerse in 90% ethanol for 30 s.
- Immerse in 80% ethanol for 30 s.
- Immerse in 70% ethanol for 30 s.
- Rehydrate the sections with water for 5 min.

To perform antigen retrieval of paraffin sections containing 3D Heps prior to immunostaining:

- Heat de-waxed and rehydrated sections in 1x Tris-EDTA (TE) pH 9.0 buffer solution for 15 min in a microwave at 800 W.
- Cool down samples by immersing them in tap water for 5 min.

To perform immunostaining:

- Incubate slides with blocking solution made of 10%BSA dissolved in PBS and contained 0.5% Tween 20 for 1 h at RT.
- Replace blocking solution with the appropriate primary antibody diluted in 1% BSA with 0.5% Tween 20 and incubate at 4 °C with gentle agitation overnight.
- Wash cells with PBST for 5 min and repeat three times.
- Incubate with the appropriate secondary antibody diluted in 1% BSA with 0.5% Tween 20 and incubate in the dark at RT for 1 h with gentle agitation.
- Wash sections with PBS/T for 5 min and repeat three times.
- Add 4',6-diamidino-2-phenylindole (DAPI) to the cells according to manufacturer's instructions and place a glass coverslip gently to reduce air bubbles.

  Keep fixed cells at 4 °C in the dark. Observe staining under a microscope with appropriate filter and fluorescent lamp.

4. Notes

1. Prepare small aliquots of growth factors and avoid repeated freeze-thaw process.
2. To dissociate cells into single cell suspension, it is better to extend the

incubation with Gentle Cell Dissociation Reagent for 1-2 minutes than implying too much shear stress by pipetting cells up and down forcefully.

3. Always prepare freshly growth factor-supplemented media on the day and before changing the media.

4. Freshly thawed growth factors can be stored at 4 °C and can be used within a week.

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