Journal of Visualized Experiments

An efficient method for directed hepatocyte-like cell induction from human embryonic stem cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62654R1
Full Title:	An efficient method for directed hepatocyte-like cell induction from human embryonic stem cells
Corresponding Author:	Xiaoling Zhou Shantou University Medical College Shantou, CHINA
Corresponding Author's Institution:	Shantou University Medical College
Corresponding Author E-Mail:	xlzhou@stu.edu.cn
Order of Authors:	Qi Zhou
	Xiaoling Xie
	Zhiqian Zhong
	Pingnan Sun
	Xiaoling Zhou
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TITLE:

An Efficient Method for Directed Hepatocyte-like Cell Induction from Human Embryonic Stem Cells

AUTHORS AND AFFILIATIONS:

Qi Zhou*[1,2,3], Xiaoling Xie*[1,2,3], Zhiqian Zhong[1,2,3], Pingnan Sun[1,2,3], Xiaoling Zhou[1,2,3]

[1]Stem Cell Research Center, Shantou University Medical College, Shantou, China
[2]The Center for Reproductive Medicine, Shantou University Medical College, Shantou, China
[3]Guangdong Provincial Key Laboratory of Infectious Diseases and Molecular Immunopathology, Shantou University Medical College, Shantou, China

*These authors contributed equally.

1479288377@qq.com
xxling1995@163.com
969482235@qq.com
pnsun@stu.edu.cn
xlzhou@stu.edu.cn

Correspondence authors:
Pingnan Sun: pnsun@stu.edu.cn
Xiaoling Zhou: xlzhou@stu.edu.cn

KEYWORDS:

hepatocyte-like cell, human embryonic stem cells, cell differentiation, definitive endoderm, activin A, CHIR99021

SUMMARY:

This manuscript describes a detailed protocol for differentiation of human embryonic stem cells (hESCs) into functional hepatocyte-like cells (HLCs) by continuously supplementing activin A and CHIR99021 during hESC differentiation into definitive endoderm (DE).

ABSTRACT:

The potential functions of hepatocyte-like cells (HLCs) derived from human embryonic stem cells (hESCs) hold great promise for disease modeling and drug screening applications. Provided here is an efficient and reproducible method for differentiation of hESCs into functional HLCs. The establishment of an endoderm lineage is a key step in the differentiation to HLCs. By our method, we regulate the key signaling pathways by continuously supplementing activin A and CHIR99021 during hESC differentiation into definitive endoderm (DE), followed by generation of hepatic progenitor cells, and finally HLCs with typical hepatocyte morphology in a stagewise method with completely defined reagents. The hESC-derived HLCs produced by this method express stage-specific markers (including albumin, HNF4α nuclear receptor, and sodium taurocholate cotransporting polypeptide (NTCP)), and show special characteristics related to mature and

functional hepatocytes (including indocyanine green staining, glycogen storage, hematoxylin-eosin staining and CYP3 activity), and can provide a platform for the development of HLC-based applications in the study of liver diseases.

INTRODUCTION:

The liver is a highly metabolic organ that plays several roles, including deoxidation, storing glycogen, and secretion and synthesis of proteins[1]. Various pathogens, drugs and heredity can cause pathological changes in the liver and affect its functions[2,3]. Hepatocytes, as the main functional unit of the liver, play an important role in artificial liver support systems and drug toxicity elimination. However, the resource of primary human hepatocytes is limited in cell-based therapy, as well as in liver disease research. Therefore, developing new sources of functional human hepatocytes is an important research direction in the field of regenerative medicine. Since 1998 when hESCs have been established[4], hESCs have been widely considered by researchers because of their superior differentiation potential (they can differentiate into various tissues in a suitable environment) and high degree of self-renewability, and thus provide ideal source cells for bioartificial livers, hepatocyte transplantation and even liver tissue engineering[5].

Currently, the hepatic differentiation efficiency can be greatly increased by enriching the endoderm[6]. In the lineage differentiation of stem cells into endoderm, levels of the TGF-β signaling and WNT signaling pathways are the key factors in the node of the endoderm formation stage. Activation of a high-level of TGF-β and WNT signaling can promote the development of endoderm[7,8]. Activin A is a cytokine belonging to the transforming growth factor β (TGF-β) superfamily. Therefore, activin A is widely used in endoderm induction of human induced pluripotent stem cells (hiPSCs) and hESCs[9,10]. GSK3 is a serine-threonine protein kinase. Researchers have found that CHIR99021, a specific inhibitor of GSK3β, can stimulate typical WNT signals, and can promote stem cell differentiation under certain conditions, suggesting that CHIR99021 has potential for inducing stem cell differentiation into endoderm [11-13].

Here we report an efficient and reproducible method for effectively inducing the differentiation of hESCs into functional HLCs. The sequential addition of activin A and CHIR99021 produced about 89.7±0.8% SOX17 (definitive endoderm marker)-positive cells. After being further maturated *in vitro*, these cells expressed hepatic specific markers and exerted hepatocyte-like morphology (based on hematoxylin-eosin staining (H & E)) and functions, such as uptake of indocyanine green (ICG), glycogen storage and CYP3 activity. The results show that hESCs can be successfully differentiated into mature functional HLCs by this method and can provide a basis for liver disease-related research and *in vitro* drug screening.

PROTOCOL:

1. Stem cell maintenance

NOTE: The cell maintenance protocol described below applies to the hES03 cell line maintained in an adherent monolayer. For all the following protocols in this manuscript, cells should be handled under a biological safety cabinet.

1.1. Prepare 1x mTesR stem cell culture medium by diluting 5x supplementary medium to mTesR basic medium.

1.2. Prepare 30x hESC-qualified Matrigel medium by diluting 5 mL of hESC-qualified Matrigel with 5 mL of DMEM/F12 on the ice. Store at -20 °C.

1.3. Prepare 1x hESC-qualified Matrigel medium by diluting 33.3 µL of 30x hESC-qualified Matrigel with 1 mL of DMEM/F12.

1.4. Coat sterile 6 well, tissue culture-treated plates with 1 mL of 1x hESC-qualified Matrigel in each well in advance and store at 4 °C overnight. Leave at room temperature for at least 30 min before use.

1.5. Thaw the cryopreserved hESCs in a 37 °C water bath for 3 min without shaking. Then immediately transfer the cells by pipetting into a 15 mL centrifuge tube containing 4 mL prewarmed 37 °C mTesR medium, pipet up and down gently 2 times.

1.6. Centrifuge the hESCs at 200 x *g* for 2 min at room temperature (RT).

1.7. Aspirate the supernatant and gently resuspend the cells in 1 mL of mTesR medium.

1.8. Aspirate the DMEM/F12 medium from the plate and seed the cells into a well of 6-well plate at a density of 1 x 10^5 cells in 2 mL of mTesR.

1.9. Incubate in a 5% CO_2 incubator and maintain the cells by replacing with preheated mTesR medium daily. Passage the cells at roughly 70-80% confluence or when the cell colonies begin to make contact.

2. Stem cell passage and differentiation

2.1. For passaging cells, aspirate the medium and incubate the cells with 1 mL per well of enzyme solution for 5 min at 37 °C. Then transfer the cells by pipetting to a 15 mL centrifuge tube containing 4 mL of DMEM/F12 prewarmed to 37 °C.

2.2. Centrifuge the cells at 200 x *g* at RT for 2 min.

2.3. Aspirate the medium and gently resuspend the cell pellet in 1 mL of mTesR medium.

2.4. Prepare 24-well plates by coating with 250 µL of 1x hESC-qualified Matrigel medium in advance. Seed hESCs as described above at a density of 1 - 1.5 x 10^5 cells per well in 500 µL of mTesR medium.

2.5. Allow cells to incubate for 24 h at 37 °C in a CO_2 incubator.

3. Definitive endoderm formation

3.1. Prepare stock solutions of 100 µg/mL activin A with DPBS containing 0.2% BSA and 3 mM CHIR99021 with DMSO.

3.2. Prepare Stage I differentiation basic medium by supplementing RPMI medium with 1x B27.

3.3. Add activin A to a final concentration of 100 ng/mL and CHIR99021 to a final concentration of 3 µM in an appropriate volume of preheated Stage I differentiation basic medium.

3.4. Aspirate mTesR medium from the cells and replace with Stage I differentiation media containing added differentiation factors (e.g., 0.5 mL per well of a 24-well plate).

3.5. Allow cells to incubate for 3 days at 37 °C in a CO_2 incubator, replacing Stage I media daily with freshly added differentiation factors (Days 0-2).

NOTE: After 3 days of differentiation, differentiated cells should express markers of definitive endoderm cells, such as FOXA2, SOX17, GATA4, CRCR4, and FOXA1 (minimum 80% of SOX17 needed for proceeding).

4. Differentiation of hepatic progenitor cells

4.1. Prepare Stage II differentiation basic media by dissolving knockout serum replacement (KOSR) to a final concentration of 20% in Knock Out DMEM.

4.2. Prepare Stage II differentiation medium containing 1% DMSO, 1x GlutaMAX, 1x non-essential amino acid (NEAA) solution, 100 µM 2-mercaptoethanol and 1x penicillin/streptomycin (P/S) to the appropriate volume of KOSR/knockout DMEM.

4.3. On Day 3 of differentiation, aspirate the medium and replace with Stage II medium (e.g., 0.5 mL per well of a 24 well plate). Then incubate for 24 h.

4.4. On Day 4 of differentiation, aspirate the medium and replace with Stage II medium (e.g., 1 mL per well of a 24 well plate).

4.5. Change the medium every other day (replace medium on Day 6).

NOTE: After 8 days of differentiation, differentiated cells should express appropriate markers of hepatic progenitor cells, such as HNF4α, AFP, TBX3, TTR, ALB, NTCP, CEBPA (minimum 80% of HNF4α needed for proceeding).

5. Hepatocyte differentiation

5.1. Prepare 10 µg/mL hepatocyte growth factor (HGF), 20 µg/mL Oncostatin (OSM) stock solutions with DBPS (containing 0.2% BSA) and filter with a 0.22 µm filter membrane.

5.2. Prepare Stage III differentiation basic medium (HepatoZYME-SFM (HZM) medium supplemented with 10 µM hydrocortisone-21-hemisuccinate and 1x P/S).

5.3. Add HGF to a final concentration of 10 ng/mL and OSM to a final concentration of 20 ng/mL to the appropriate volume of preheated Stage III differentiation medium.

5.4. On Day 8 of differentiation, aspirate Stage II medium from cells and replace with Stage III medium (e.g., 1 mL per well of a 24 well plate).

5.5. Allow cells to incubate for 10 days at 37 °C in a CO_2 incubator, changing Stage III media every other day with freshly added differentiation factors (Days 8-18).

NOTE: After 18 days of differentiation, differentiated cells should express characteristic markers of hepatocytes, such as AAT, ALB, TTR, HNF4α, NTCP, ASGR1, CYP3A4. The percentage of Albumin-positive cells usually is more than 90%.

6. RNA isolation, cDNA synthesis and RT-PCR analysis

6.1. Aspirate medium from cells and add the proper amount of RNAiso Plus reagent, (e.g., 0.3 mL per well of a 24-well plate). Collect the supernatant in a 1.5 mL tube and let stand at RT for 5 min.

6.2. Add 60 µL of chloroform reagent and leave it at RT for 5 min. Then centrifuge at 12,000 x *g* for 15 min.

6.3. Collect the 125 µL supernatant and transfer it to another centrifuge tube. Add 160 µL of isopropanol, mix well, and stand at RT for 10 min.

6.4. Centrifuge at 12,000 x *g* for 10 min.

6.5. Remove the supernatant, add 75% ethanol to the precipitated, and centrifuge at 7,500 x *g* for 5 min.

6.6. After drying at RT, add 40 µL of DEPC-treated water to dissolve. For qPCR, reverse transcribe 2 µg of total RNA with a reverse transcription kit according to the manufacturer's protocol.

6.7. Perform Q-PCR using a commercial kit according to the manufacturer's protocol.

6.8. Normalize gene expression relative to non-induced stem cells and determine fold variation following normalization to GAPDH.

7. Immunofluorescence Validation of Differentiation

7.1. Prepare 1x PBST washing buffer by adding 0.1% Tween-20 to PBS.

7.2. Aspirate medium from adherent cells and wash briefly with PBS at RT on a shaker.

7.3. Aspirate PBS and incubate cells with 500 µL of ice-cold methanol per well of a 24-well plate to fix the cells at -20 °C overnight.

7.4. Remove the methanol and wash the cells 3 times with PBS on a shaker for 10 min each time at RT.

7.5. Block cells with 500 µL of 5% bovine serum albumin prepared in PBS for 1-2 h at RT on a shaker.

7.6. Dilute primary antibody at 1: 200 in the same solution used for blocking.

7.7. Incubate the fixed cells in 300 µL primary antibody solution overnight at 4 °C on a shaker.

7.8. After overnight incubation, wash cells 3 times with PBST for 10 min each time at RT on a shaker.

7.9. Prepare secondary antibody solution in blocking solution at 1:1000.

7.10. Incubate in 300 µL of secondary antibody solution protected from light for 1 h at RT on a shaker.

7.11. Aspirate secondary antibody solution and wash cells 3 times with PBST for 10 min each time at RT on a shaker.

7.12. Incubate cells with 300 µL of 1 µg/mL DAPI for 3-5 min, and then wash twice with PBST.

7.13. After aspirating PBST, add an appropriate amount of PBS for taking pictures under a fluorescence microscope.

8. Western blot analysis

8.1. Prepare 1x TBST washing buffer by adding 0.1% Tween-20 to Tris-buffered saline (TBS).

8.2. Prepare SDS-PAGE electrophoresis buffer and western transfer buffer by using a commercial kit according to the manufacturer's protocol.

8.3. Resolve total cell protein (40 μg) on a 10% sodium dodecyl sulfate polyacrylamide gel and run in SDS-PAGE electrophoresis buffer at 15 mA constant current for about 2 h.

8.4. Transfer the gel to a polyvinylidene difluoride (PVDF) membrane at 300 mA constant current for 2 h at low temperature.

NOTE: The running time and transfer time may vary depending on the equipment used and the type and percentage of the gel.

8.5. Block the membrane with 3% bovine serum albumin prepared in TBST for 1 h at RT on a shaker.

8.6. Incubate in primary antibody solution (1:1000 dilution) overnight at 4 °C on a shaker.

8.7. After overnight incubation, wash blotting membrane 3 times with TBST for 15 min per time at RT on a shaker.

8.8. Incubate in secondary antibody solution (1:2000 dilution) for 2 h at RT on a shaker.

8.9. Discard secondary antibody solution and wash the membrane 3 times with TBST, for 15 min each time, at RT on a shaker.

8.10. Visualize immunoreactive bands using a chemiluminescence reagent followed by autoradiography. Use β-actin as the loading control.

9. Indocyanine green uptake

9.1. Prepare 200 mg/mL indocyanine green stock solution in DMSO.

9.2. Prepare a working solution by supplementing Stage III differentiation medium with indocyanine green solution to a final concentration of 1 mg/mL.

9.3. Incubate in a 37 °C, 5% CO_2 incubator for 1-2 h.

9.4. Aspirate medium and wash the cells 3 times with PBS.

9.5. Photograph under a microscope.

10. Periodic Acid-Schiff (PAS) staining and Hematoxylin-Eosin (H&E) staining

10.1. Aspirate medium from adherent cells and briefly wash twice with PBS.

10.2. For cell fixation, aspirate PBS and incubate cells with ice-cold methanol at -20 °C

overnight.

10.3. Visualize glycogen storage by PAS staining and observe binucleated cells by H & E staining using a kit following the manufacturer's instructions.

10.4. Take images with a fluorescence microscope.

11. Assay for CYP Activity

11.1. Measure CYP450 activity using commercially available cell-based assays (P450-Glo Assays) according to the manufacturer's instructions.

11.2. Use three independent repeats for testing. Data are represented as the mean ± SD.

REPRESENTATIVE RESULTS:

The schematic diagram of HLC induction from hESCs and representative bright-field images of each differentiation stage are shown in **Figure 1**. In Stage I, activin A and CHIR99021 were added for 3 days to induce stem cells to form endoderm cells. In Stage II, the endoderm cells differentiated into hepatic progenitor cells after being treated with differentiation medium for 5 days. In Stage III, early hepatocytes had matured and differentiated into hepatocyte-like cells after 10 days in HGF and OSM (**Figure 1A**). In the final stage of differentiation, cells showed a typical hepatocyte phenotype (The cells are polygon and distributed evenly and regularly) (**Figure 1B**).

To further confirm hepatic differentiation, RT-PCR, immunofluorescence staining and western blotting were used to detect markers of endoderm cells, hepatic precursor cells, and mature hepatocytes (**Figure 2**). The differentiated cells showed high expression levels of differentiation-related genes and proteins in each stage, such as endoderm markers SOX17 (89.7± 0.8%) at Day 3, hepatic precursor marker HNF4α (81.3±2.9%) at Day 8, AFP (86.6±0.3%) at Day 14, and mature hepatocyte marker ALB (94.5±1.1%) at Day 18. These results indicate that hepatocyte-like cells are generated by this protocol.

In order to detect whether HLCs have hepatocyte functions, we detected ICG uptake, glycogen storage, and CYP activity of HLCs, as well as performed H&E staining. As can be seen the HLCs exhibited green staining (**Figure 3A**) and extensive cytoplasmic periodic acid-Schiff staining (pink to purple) was observed (**Figure 3B**), which is consistent with glycogen storage. In addition, we can see the representative morphology of a typical binucleated hepatocyte (**Figure 3C**). Finally, the enzymatic activity of CYP3A4, the most important metabolic CYP in the liver, was confirmed by enzymatic activity assay (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Table 1: Components of cell culture and differentiation base media.

Table 2: Primer sequences for q-PCR analysis.

Figure 1: Schematic diagram of protocol for the specification of definitive endoderm and hepatocyte-like cells. (**A**) Schematic presentation of the 3-stage differentiation strategy used in this study. (**B**) Morphology of the differentiated cells at Days 0, 3, 8, 14 and 18. Scale bar = 100 μm.

Figure 2: Stage-specific marker expression during hESC differentiation into HLCs. (**A**) mRNA expression of stage-specific genes. (**B-C**) Protein expression of stage-specific genes.

Figure 3: Functions of hESC-derived hepatocytes (**A**) HLCs treated for 1 h with 1 mg/mL indocyanine green demonstrate uptake as assessed by phase microscopy. (**B**) Representative PAS staining images indicating glycogen storage. (**C**) Representative HE staining images showing binucleates cells. (**D**) Basal level activity of major cytochrome P450 enzymes. All values are presented as mean ± SD. Scale bar = 100 μm.

DISCUSSION:

Here, we present a stepwise method that divides hepatocyte-like cell induction from human embryonic stem cells in three stages. In the first stage, activin A and CHIR99021 were used to differentiate hESCs into definitive endoderm. In the second stage, KO-DMEM and DMSO were used to differentiate definitive endoderm into hepatic progenitor cells. In the third stage, HepatoZYME-SFM (HZM) plus HGF, OSM and HC were used to continue to differentiate hepatic progenitor cells into hepatocyte-like cells.

The following critical steps need to be taken into consideration while using the protocol. The initial differentiation density of cells and the accurate timing of medium changes are important factors for successful differentiation. When we begin to differentiate, we must ensure that the initial seeding density is appropriate, at a confluence of about 30-40%, when cells are just beginning to come into contact with each other, and that the intercellular spaces are evenly arranged in a network under the field of vision. During differentiation, the corresponding differentiation medium must be changed precisely at the indicated time, especially in the first stage and the key moment of each replacement stage, to ensure that cells differentiate in the mode and stage imitating the process of embryonic development.

The first stage of differentiation of hESCs into DE is particularly important. There are usually a large number of cells that detach and float up during Stage I of differentiation, which is a critical stage for the cell fate, but at this time the cells still retain the ability to proliferate. Therefore, the confluence of cells can reach about 90-100% at the end of Stage I. In addition, it should be noted that at the beginning of Stage III of differentiation (i.e., Day 8), the cytoplasm of the cells begins to condense, resulting in the cells gradually acquiring a slender morphology. However at Day 10, the cytoplasm gradually recovers, and at Day 14, the cells begin to show the obvious polyhedral morphology of hepatocytes.

In this study, the HLCs obtained are actually closer to fetal hepatocytes as AFP is higher expressed than ALB at day 18. Although the generated HLCs exert hepatocyte characteristics and functions,

there is still a gap between HLCs and primary human hepatocytes, indicating our differentiated cells are still not functionally mature. Therefore, future work will need to focus on further optimizing the differentiation method.

At present, small molecular compounds such as activin A[9,10,14], Wnt3a[15], CHIR[16], Torin2[17] and IDE1[18,19] are commonly used to induce DE differentiation in various differentiation systems. The Song group used activin A to differentiate stem cells into DE, and used FGF4 (Fibroblast Growth Factor 4), BMP2(Bone morphogenetic protein 2), HGF, KGF (Keratinocyte growth factor), OSM and Dex for hepatic specification and HLCs maturation[20]. The Sullivan group induced DE by activin A and Wnt 3a, and induced HLCs by β-ME (2-mercaptoethanol), DMSO, Insulin, HGF, OSM[21]. The Siller team used CHIR99021 for DE differentiation, DMSO, Dihexa (Hepatocyte growth factor receptor agonist N-hexanoic-Tyr), and Dex for HLC differentiation to obtain HLC with liver function characteristics[16]. However, some of these methods use many recombinant growth factors to generate DE with high cost and some of them use only small molecules to generate DE with lower efficiency. Activin A is critical factor for DE generation. We have tried to replace Activin A with other small molecules but usually get a lower efficiency. Therefore, we adopt the method of adding activin A and CHIR99021 as inducing factors of DE, and detect differentiation-related genes in each stage by real-time quantitative PCR, immunofluorescence and western blotting.

In recent years, the establishment of an experimental system for directed HLC induction from hESCs is an important basis for the modeling of hepatocyte development and screening drugs for liver-related diseases. At the same time, it can also provide an effective source of cells for hepatocyte transplantation, liver tissue engineering, bioartificial livers and other research. It has been reported that HLCs derived from stem cells have been used to conduct various studies on viral infection as a drug screening model to predict hepatotoxic drug-induced responses and to study the innate immune pathway and signaling pathways of cells[10,22-25]. Generally, this method introduces in detail an efficient hepatocyte-like cell differentiation technique from hESCs that can successfully differentiate hESCs into mature functional hepatocytes. The results provide a platform for the development of HLC-based applications.

ACKNOWLEDGMENTS:

This work was supported by the National Natural Science Foundation of China (No. 81870432 and 81570567 to X.L.Z.), (No. 81571994 to P.N.S.); the Natural Science Foundation of Guangdong Province, China (No. 2020A1515010054 to P.N.S.), The Li Ka Shing Shantou University Foundation (No. L1111 2008 to P.N.S.). We would like to thank Prof. Stanley Lin from Shantou University Medical College for useful advice.

DISCLOSURES:

The authors have nothing to disclose.

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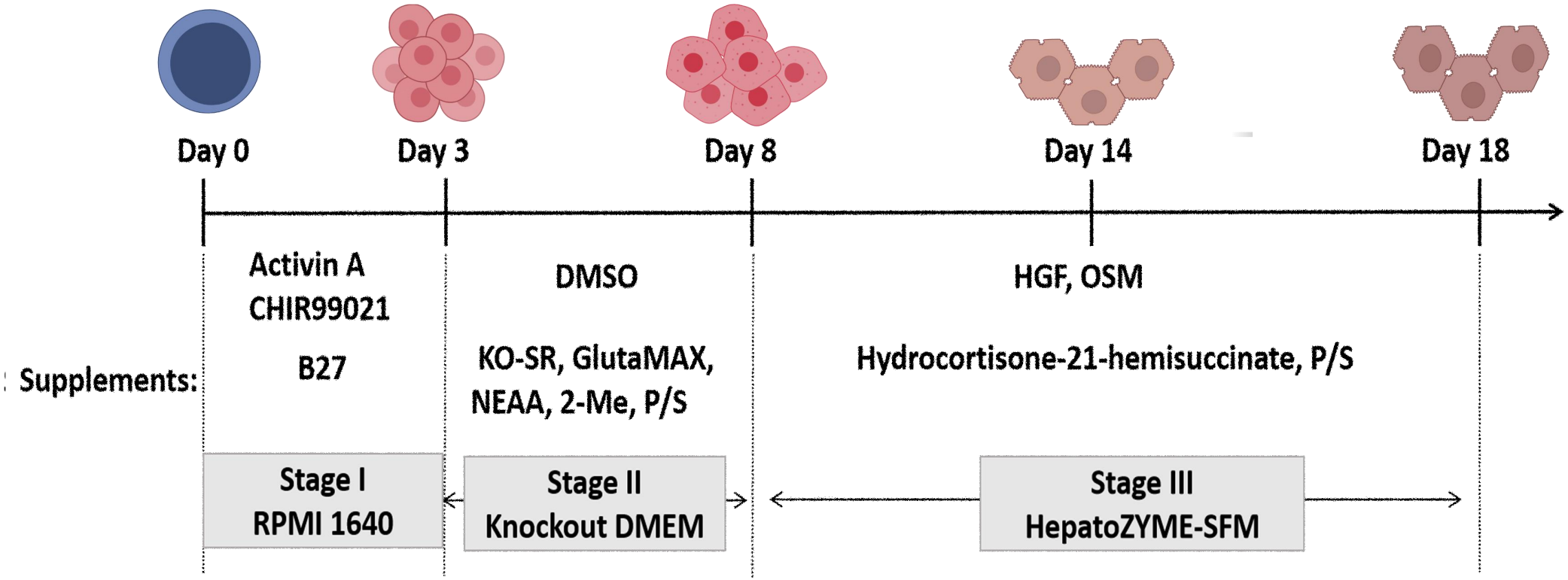
Figure 1

A



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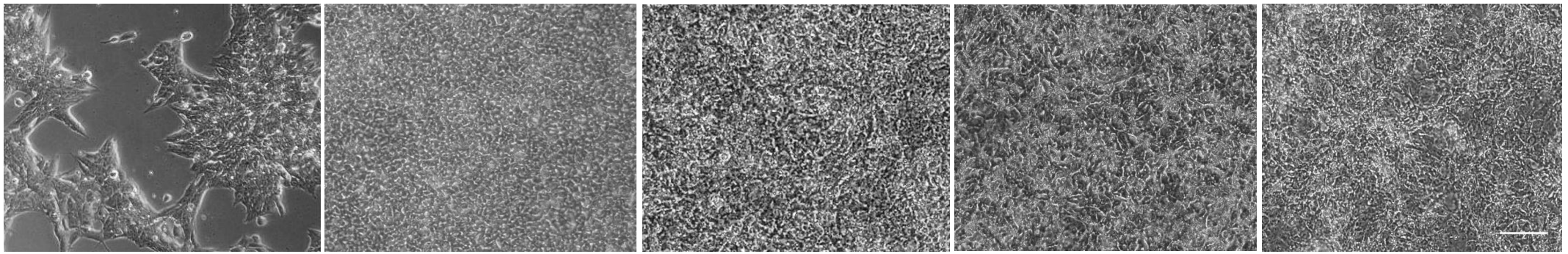
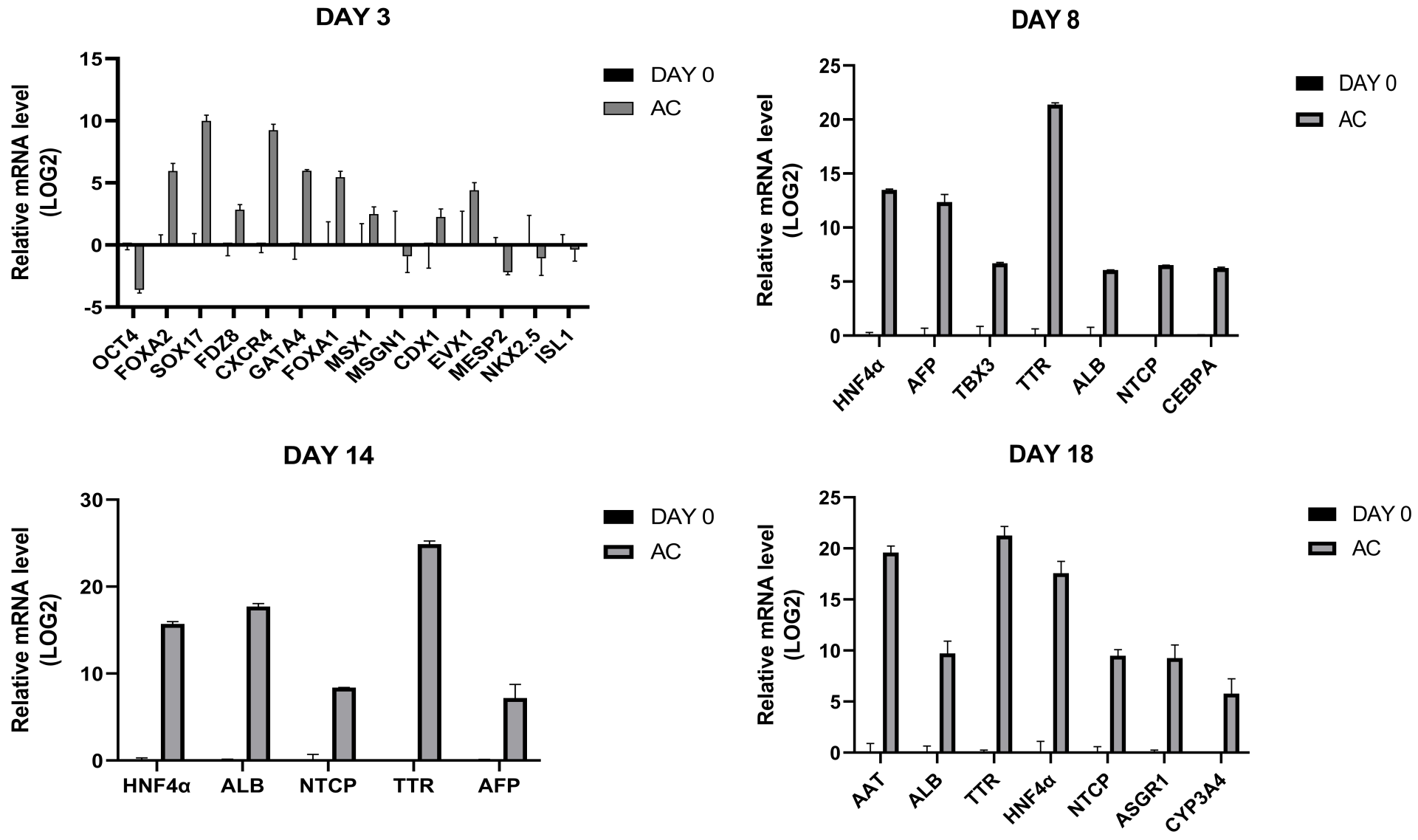
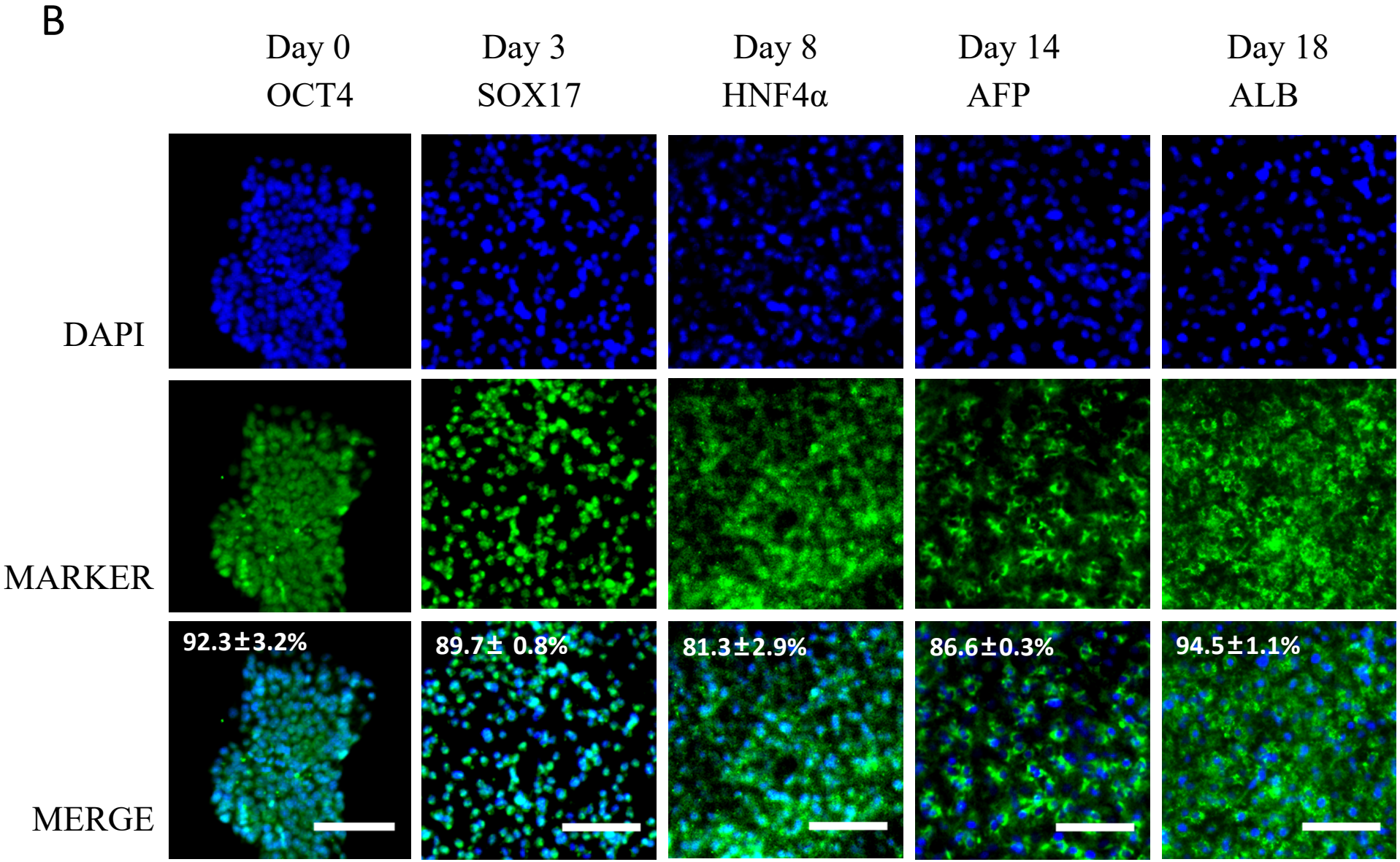


Figure 2

A




B
Day 0
OCT4
Day 3
SOX17
Day 8
HNF4α
Day 14
AFP
Day 18
ALB
DAPI
MARKER
MERGE
92.3±3.2%
89.7± 0.8%
81.3±2.9%
86.6±0.3%
94.5±1.1%

C

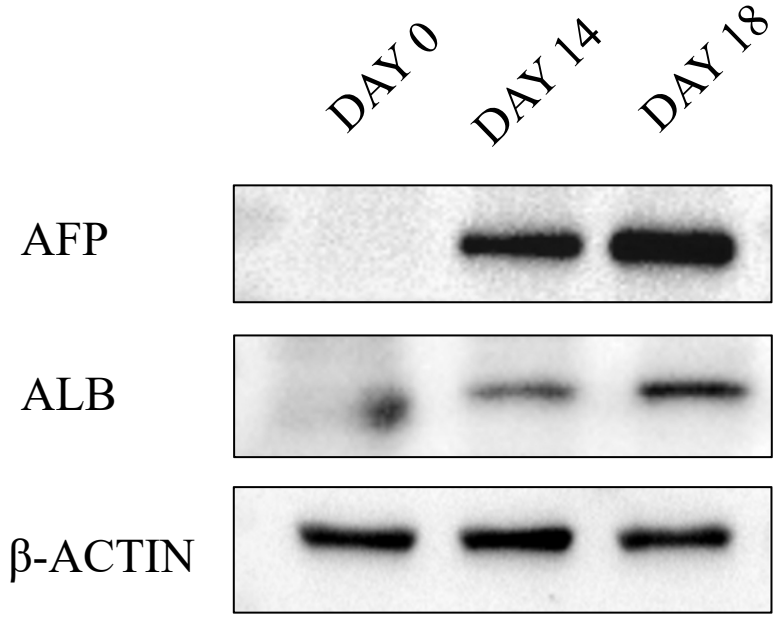


Figure 3

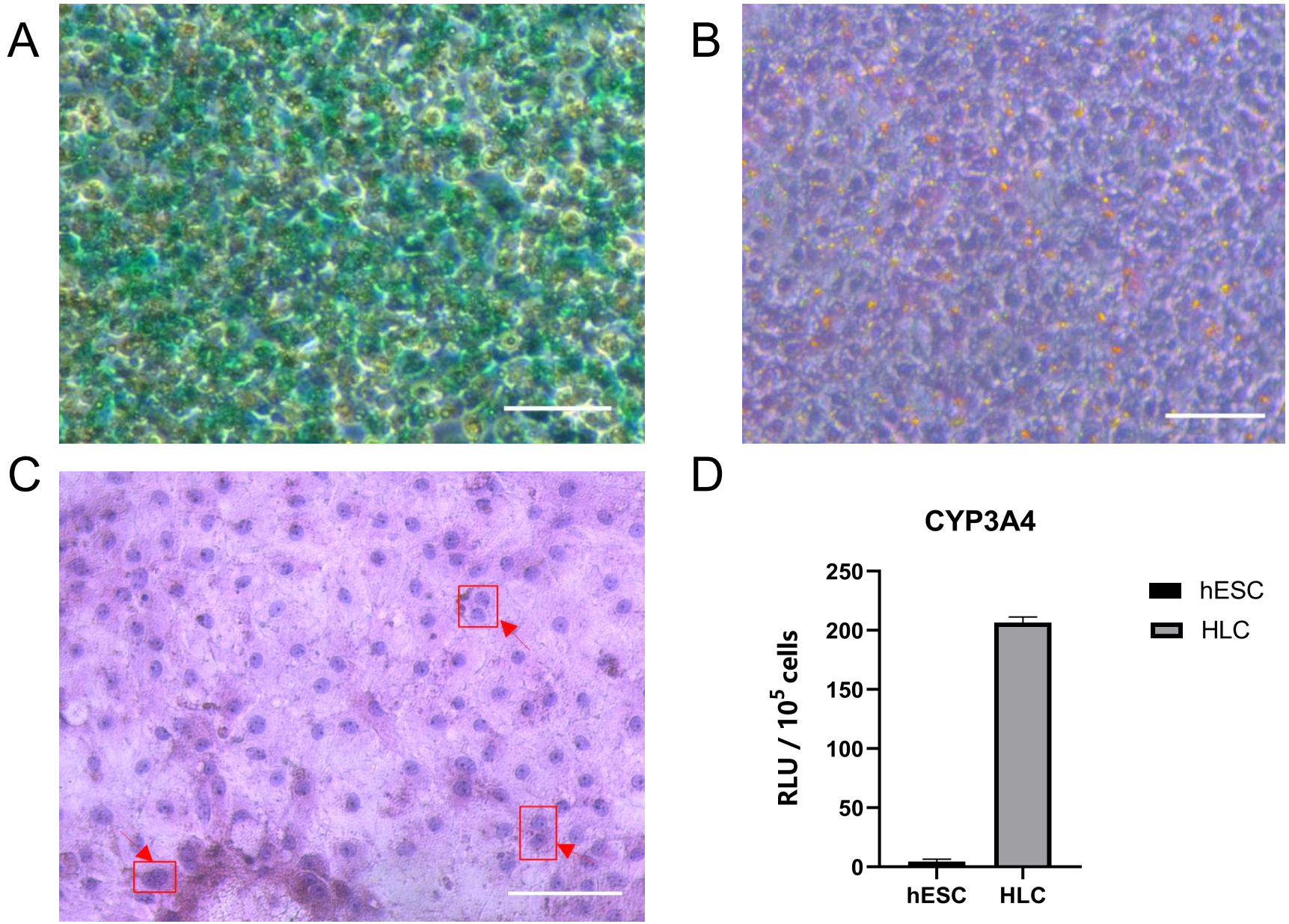


Table 1

Name of Reagent	Company	Catalog Number	Stock Concentration
2-Mercaptoethanol	Sigma	M7522	50 mM
488 labeled goat against mouse IgG	ZSGB-BIO	ZF-0512	-
488 labeled goat against Rabbit IgG	ZSGB-BIO	ZF-0516	-
Accutase	Stem Cell Technologie	7920	1 x
Activin A	peproTech	120-14E	100 µg/ml
Anti - Albumin (ALB)	Sigma-Aldrich	A6684	-
Anti - Human Oct4	Abcam	Ab19587	-
Anti - α-Fetoprotein (AFP)	Sigma-Aldrich	A8452	-
Anti -SOX17	Abcam	ab224637	-
Anti-Hepatocyte Nuclear Factor 4 alpha (HNF4α)	Sigma-Aldrich	SAB1412164	-
B-27 Supplement	Gibco	17504-044	50 x
BSA	Beyotime	ST023-200g	-
CHIR99021	Sigma-Aldrich	SML1046	3 mM
DAPI	Beyotime	C1006	-
DEPC-water	Beyotime	R0021	-
DM3189	MCE	HY-12071	500 µM
DMEM/F12	Gibco	11320-033	1 x
DMSO	Sigma-Aldrich	D5879	1 x
DPBS	Gibco	14190-144	1 x
GlutaMAX	Gibco	35050-061	100 x
H&E staining kit	Beyotime	C0105S	-
Hepatocyte growth factor (HGF)	peproTech	100-39	10 µg/ml
HepatoZYME-SFM (HZM)	Gibco	17705-021	-
Hydrocortisone-21-hemisuccinate	Sigma-Aldrich	H4881	1 mM
Indocyanine	Sangon Biotech	A606326	200 mg/mL
Knock Out DMEM	Gibco	10829-018	1 x
Knock Out SR Multi-Species	Gibco	A31815-02	-
Matrigel hESC-qualified	Corning	354277	60 x
MEM NeAA	Gibco	11140-050	100 x
mTesR 5X Supplement	Stem Cell Technologie	85852	5 x
mTesR Basal Medium	Stem Cell Technologie	85851	1 x

Oncostatin (OSM)	peproTech	300-10	20 μg/ml
P450 - CYP3A4（Luciferin - PFBE）	Promega	V8901	-
PAS staining kit	Solarbio	G1281	-
Pen/Strep	Gibco	15140-122	100 x
Peroxidase-Conjugated Goat anti-Mouse IgG	ZSGB-BIO	ZB-2305	-
Primary Antibody Dilution Buffer for Western Blot	Beyotime	P0256	-
ReverTraAce qPCR RT Kit	TOYOBO	FSQ-101	-
RNAiso Plus	TaKaRa	9109	-
RPMI 1640	Gibco	11875093	1 x
Skim milk	Sangon Biotech	A600669	-
SYBR Green Master Mix	Thermo Fisher Scienti	A25742	-
Torin2	MCE	HY-13002	15 μM
Tween	Sigma-Aldrich	WXBB7485V	-

Final Concentration
100 μM
IF: 1:1000
IF: 1:1000
1 x
100 ng/ml
IF: 1:200； WB:1:1000
IF: 1:200
IF: 1:200； WB:1:1000
IF: 1:200
IF: 1:200
1 x
5.00%
3 μM
-
-
250 nM
1 x
1 x
1 x
1 x
-
10 ng/ml
-
10 μM
1 mg/mL
1 x
20%
1 x
1 x
1 x
1 x

20 ng/ml
-
-
1 x
WB: 1:2000
-
-
-
1 x
5.00%
-
15 nM
0.10%

Table 2

Name of Gene	abbreviations	primers sequences (F)
POU Class 5 Homeobox 1	OCT4	AGCGAACCAGTATCGAGAAC
Forkhead Box A2	FOXA2	GCATTCCCAATCTTGACACGGTGA
SRY-Box Transcription Factor 17	SOX17	TATTTTGTCTGCCACTTGAACAGT
Frizzled Class Receptor 8	FZD8	ATCGGCTACAACTACACCTACA
C-X-C Motif Chemokine Receptor 4	CXCR4	CACCGCATCTGGAGAACCA
Forkhead Box A1	FOXA1	AAGGCATACGAACAGGCACTG
Msh Homeobox 1	MSX1	CGCCAAGGCAAAGAGACTAC
Mesogenin 1	MSGN1	GCTGGAATCCTATTCTTCTTCTCC
Caudal Type Homeobox 1	CDX1	AAGGAGTTTCATTACAGCCGTTAC
Even-Skipped Homeobox 1	EVX1	GCTGTCTCTCTGAACAAAATGCT
Mesoderm Posterior BHLH Transcription Factor 2	MESP2	AGCTTGGGTGCCTCCTTATT
NK2 Homeobox 5	NKX2.5	CAAGTGTGCGTCTGCCTTT
ISL LIM Homeobox 1	ISL1	AGATTATATCAGGTTGTACGGGATCA
Hepatocyte Nuclear Factor 4 Alpha	HNF4α	ACATGGACATGGCCGACTAC
Alpha Fetoprotein	AFP	ACTGAATCCAGAACACTGCA
T-Box Transcription Factor 3	TBX3	TTATGTCCCAGCGAGGGTGA
Transthyretin	TTR	AGAAAGGCTGCTGATGAC
Albumin	ALB	CCCCAAGTGTCAACTCCA
Sodium taurocholate cotransporting polypeptide	NTCP	CATAGGGATCGTCCTCAAATCCA
CCAAT Enhancer Binding Protein Alpha	CEBPA	AGGAGGATGAAGCCAAGCAGCT
Serpin Family A Member 1	AAT	AAATGAACTCACCCACGAT
Asialoglycoprotein Receptor 1	ASGR1	CAGACCCTGAGACCCTGAGCAA
Cytochrome P450 Family 3 Subfamily A Member 4	CYP3A4	TTGTAATCACTGTTGGCGTGGG

primers sequences (R)

TTACAGAACCACACTCGGAC

GCCCTTGCAGCCAGAATACACATT

TTGGGACACATTCAAAGCTAGTTA
GTACATGCTGCACAGGAAGAA
GCCCATTTCCTCGGTGTAGTT
TACACACCTTGGTAGTACGCC
GCCATCTTCAGCTTCTCCAG
TGGAAAGCTAACATATTGTAGTCC
AC
TGCTGTTTCTTCTTGTTCACTTTG
CATCTCTCACTCTCTCCTCCAAA
TGCTTCCCTGAAAGACATCA
CAGCTCTTTCTTTTCGGCTCTA
ACACAGCGGAAACACTCGAT
CGTTGAGGTTGGTGCCTTCT
TGCAGTCAATGCATCTTTCA
ACGTGGTGGTGGAGATCTTG
GTGCCTTCCAGTAAGATTTG
GTTCAGGACCACGGATAG
GCCACACTGCACAAGAGAATG
AGTGCGCGATCTGGAACTGCAG
ACCTTAGTGATGCCCAGT
TCCTGCAGCTGGGAGTCTTTTCT
AATGGGCAAAGTCACAGTGGA

Name of Material/ Equipment	Company	Catalog Number
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Activin A	peproTech	120-14E
Anti - Albumin (ALB)	Sigma-Aldrich	A6684
Anti - Human Oct4	Abcam	Ab19587
Anti - α-Fetoprotein (AFP)	Sigma-Aldrich	A8452
Anti -SOX17	Abcam	ab224637
Anti-Hepatocyte Nuclear Factor 4 alpha (HNF4α)	Sigma-Aldrich	SAB1412164
B-27 Supplement	Gibco	17504-044
BSA	Beyotime	ST023-200g
CHIR99021	Sigma-Aldrich	SML1046
DAPI	Beyotime	C1006
DEPC-water	Beyotime	R0021
DM3189	MCE	HY-12071
DMEM/F12	Gibco	11320-033
DMSO	Sigma-Aldrich	D5879
DPBS	Gibco	14190-144
GlutaMAX	Gibco	35050-061
H&E staining kit	Beyotime	C0105S
Hepatocyte growth factor (HGF)	peproTech	100-39
HepatoZYME-SFM (HZM)	Gibco	17705-021
Hydrocortisone-21-hemisuccinate	Sigma-Aldrich	H4881
Indocyanine	Sangon Biotech	A606326
Knock Out DMEM	Gibco	10829-018
Knock Out SR Multi-Species	Gibco	A31815-02
Matrigel hESC-qualified	Corning	354277
MEM NeAA	Gibco	11140-050
mTesR 5X Supplement	Stem Cell Technologie	85852
mTesR Basal Medium	Stem Cell Technologie	85851

Oncostatin (OSM)	peproTech	300-10
P450 - CYP3A4（Luciferin - PFBE）	Promega	V8901
PAS staining kit	Solarbio	G1281
Pen/Strep	Gibco	15140-122
Peroxidase-Conjugated Goat anti-Mouse IgG	ZSGB-BIO	ZB-2305
Primary Antibody Dilution Buffer for Western Blot	Beyotime	P0256
ReverTraAce qPCR RT Kit	TOYOBO	FSQ-101
RNAiso Plus	TaKaRa	9109
RPMI 1640	Gibco	11875093
Skim milk	Sangon Biotech	A600669
SYBR Green Master Mix	Thermo Fisher Scienti	A25742
Torin2	MCE	HY-13002
Tween	Sigma-Aldrich	WXBB7485V

Comments/Description
For hepatic progenitor differentiation
For IF,second antibody
For IF,second antibody
For cell passage
For definitive endoderm formation
For IF and WB, primary antibody
For IF, primary antibody
For IF and WB, primary antibody
For IF, primary antibody
For IF, primary antibody
For definitive endoderm formation
For cell blocking
For definitive endoderm formation
For nuclear staining
For RNA dissolution
For definitive endoderm formation
For cell culture
For hepatic progenitor differentiation
For cell culture
For hepatic progenitor differentiation
For H&E staining
For hepatocyte differentiation
For hepatocyte differentiation
For hepatocyte differentiation
For Indocyanine staining
For hepatic progenitor differentiation
For hepatic progenitor differentiation
For cell culture
For hepatic progenitor differentiation
For cell culture
For cell culture

For hepatocyte differentiation
For CYP450 activity
For PAS staining
For cell differentiation
For WB,second antibody
For primary antibody dilution
For cDNA Synthesis
For RNA Isolation
For definitive endoderm formation
For second antibody preparation
For RT-PCR Analysis
For definitive endoderm formation
For washing buffer preparation

Dear Editor,

Please find attached our manuscript “An efficient method for directed hepatocyte-like cell induction from human embryonic stem cells”. All the authors wish to submit this paper for publication in JOVE-JOURNAL OF VISUALIZED EXPERIMENTS. The manuscript or any parts of its content has not been published previously and is not under consideration in whole for publication elsewhere.

First of all, I want to express my sincere acknowledgement to you for recognizing the merits of our work and recommending revisions to our manuscript. We believe in this protocol can provide stable and efficient method for hepatic differentiation.

The editor’s constructive advice is very useful to improve the quality of our manuscript. We revised our manuscript according to the advice, and all the revised places were highlighted by using the 'track changes' tool in MS Word. I hope that the revised manuscript will meet your needs.

Please feel free to contact me if you have any question.

Thank you for considering our manuscript for publication. We look forward to hearing from you.

Sincerely yours,

Xiaoling Zhou, Ph.D
Stem Cell P2 Laboratory, Research Center for Reproductive Medicine,
Guangdong Provincial Key Laboratory of Infectious Diseases
and Molecular Immunopathology
Shantou University Medical College, China 515041
Tel.: +86 754 88900497
E-mail: xlzhou@stu.edu.cn

Editorial comments:

Changes to be made by the Author(s):

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

Response: Yes.

2. Please provide an email address for each author.

Response: email address for each author.

Qi Zhou 1479288377@qq.com
Xiaoling Xie xxling1995@163.com
Zhiqian Zhong 969482235@qq.com
Pingnan Sun pnsun@stu.edu.cn
Xiaoling Zhou xlzhou@stu.edu.cn

3. Please revise the following lines to avoid overlap with previously published work: 92-93, 95, 96, 153-154, 177-191, 207-218.

Response:

92-93 Thaw the cryopreserved hESCs in a 37°C water bath for 3 min without shaking, then immediately transfer the cells by pipetting into a 15 mL centrifuge tube containing 4 mL prewarmed 37 °C mTesR medium, pipet up and down gently 2 times.

95 Centrifuge the hESCs at 200 x g for 2 min at room temperature (RT).

96 Aspirate the supernatant and gently resuspend the cell in 1 mL mTesR medium.

97-98 Aspirate the DMEM /F12 medium from the plate and seed the cells into a well of 6-well plate at a density of 1 x 105 cells in 2 mL mTesR.

153-154 After 18 days of differentiation, differentiated cells should express characteristic markers of hepatocytes.

177-191

1. Prepare 1X PBST washing buffer by adding 0.1% Tween-20 to PBS.
2. Aspirate medium from adherent cells and wash briefly with PBS at RT on a shaker.
3. Aspirate PBS and incubate cells with ice-cold methanol to fix the cells at -20°C overnight.
4. Remove the methanol and wash the cells 3 times with PBS on a shaker, 10 min each time, at RT.
5. Block cells with 5% bovine serum albumin prepared in PBS for 1 - 2 h at RT on a shaker.
6. Dilute primary antibody in the same solution used for blocking.
7. Incubate the fixed cells in primary antibody solution overnight at 4°C on a shaker.
8. After overnight incubation, wash cells 3 times with PBST, for 10 min each time, at RT on a shaker.
9. Prepare secondary antibody solution in blocking solution at 1:1000.
10. Incubate in secondary antibody solution protected from light for 1 h at RT on a shaker.
11. Aspirate secondary antibody solution and wash cells 3 times with PBST, for 10 min each time, at RT on a shaker.

207-218

5. Block the membrane with 3% bovine serum albumin prepared in TBST for 1 h at RT on a shaker.
6. Incubate in primary antibody solution (1:1000 dilution) overnight at 4 °C on a shaker.
7. After overnight incubation, wash blotting membrane 3 times with TBST for 15 min per time at RT on a shaker.
8. Incubate in secondary antibody solution (1:2000 dilution) for 2 h at RT on a shaker.
9. Discard secondary antibody solution and wash the membrane 3 times with TBST, for 15 min each time, at RT on a shaker.
10. Immunoreactive bands are visualized using a chemiluminescence reagent followed by autoradiography.
11. β-Actin is used as the loading control.

4. 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, alphabets, or dashes.
Resposnsezz: Yes.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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."
Responses: Yes.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Response: Yes.

7. Please include a single line space between each step of the protocol and highlight 3 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.
Response: Yes, we have highlighted the protocol within 3 pages.

8.JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Promega, Matrigel, Accutase, etc.
Response: We have revised it accordingly.

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response: We created in Biorender software.

10. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: We arranged the discussion accordingly.

11. Figure 1: Please include a scale bar for panel B.
Response: We have included a scale bar for panel B.

12. Please sort the materials table in alphabetical order.
Response: We have revised it accordingly.

Reviewer #1:
Minor Concerns:
Please, revised the manuscript according to the following comments:

Line 62: The full name of hiPSCs should be given.
Response: human induced pluripotent stem cells

The authors should discuss if this protocol can be easily applied to other lines, and if not, which steps should be considered and modified. Also, can the cells be cultured with other maintenance medium such as E8 and passaged with other reagents beside accutase, such as EDTA, etc.
Response: This protocol can be easily applied to other lines such as H1, H9. The cells can be passaged with other reagents such as dispase.

Line 108: For cell passaging, the authors mentioned the seeding density. If cells are passaged as single cells why ROCK inhibitor is not added for the first 24h? Do they consider substantial cell death when they decided the seeding density? If ROCK inhibitor is used, does the seeding density need to be reduced since ROCK inhibitor decreases cell death?
Response: When we used accutase, we can passage hESCs into single cells, and there was no obvious cell death.

Line 125: The authors should specify which typical endoderm markers should be analyzed at this stage or refers to a figure.
Response: After 3 days of differentiation, differentiated cells should express markers of definitive endoderm cells, such as FOXA2, SOX17, GATA4, CRCR4, FOXA1 and FDZ8. And the cells express lower pluripotency(OCT4) and mesoderm markers (MSX1, MSGN1, CDX1,EVX1, MESP2, NKX2.5, ISL1).

Line 132: The authors should specify the concentrations of 2-mercaptoethanol and P/S.
Response:100 μM 2-mercaptoethanol and 1X penicillin/streptomycin (P/S)

Line 138: I believe the authors meant the medium needs to be changed every other day and not every 2 days.
Response: Yes, every other day.

Line 139: The authors should specify which typical hepatic progenitor markers should be analyzed at this stage or refers to a figure.
Response: After 8 days of differentiation, differentiated cells should express appropriate markers of hepatic progenitor cells, such as HNF4α, AFP, TBX3, TTR, ALB, NTCP, CEBPA.

Line 143: In which buffer those stock solutions should be prepared?

Response: with DBPS (contain 0.2% BSA)

Line 145: The authors should specify the concentrations of hydrocortisone and P/S.
Response: Prepare Stage III differentiation basic medium (HepatoZYME-SFM (HZM) medium supplemented with 10 μM hydrocortisone-21-hemisuccinate and 1X P/S).

Line 151: Also, here I believe the authors meant the medium needs to be changed every other day.
Response: Yes.

Line 153: The authors should specify which typical hepatocyte markers should be analyzed at this stage or refers to a figure.
Response: After all stages of differentiation, the cells should express the corresponding markers of hepatocytes. Such as AAT, ALB, TTR, HNF4α, NTCP, ASGR1, CYP3A4.

Line 160: How much chloroform?
60 μl chloroform

Line 167: How much DEPC-treated water?
40 μl

Line 177: How much methanol?
500 μL per well of a 24-well plate

Line 181: How much 5% BSA solution? Also, for later solutions the authors need to specify the volumes.
500 μL 5% BSA solution, we also specify the volumes of later solutions.

Sullivan's group published a more recent version of their protocol, which should also be discussed and cited (Mathapati S, Siller R, Impellizzeri AA, Lycke M, Vegheim K, Almaas R, Sullivan GJ. Small-Molecule-Directed Hepatocyte-Like Cell Differentiation of Human Pluripotent Stem Cells. Curr Protoc Stem Cell Biol. 2016 Aug 17;38:1G.6.1-1G.6.18. doi: 10.1002/cpsc.13. PMID: 27532814).
Response: We have cited this reference in the discussion.

Make sure the same abbreviations are used in the main-text as well as the Table.
Response: We did it accordingly.

The Table lacks information for reagents such as DEPC-water, Tween, BSA, antibodies, indocyanine, beyotime kit, PAS staining kit, H&E staining kit, and P450-Glo assay kit.
Response: We have added the information for reagents in the table.

English polishing should be performed.
Responses: Prof. Stanley Lin helped us to revise the English.

Reviewer #2:

Manuscript Summary:

The authors are describing a protocol to differentiate human embryonic stem cells (hESCs) into hepatocyte-like cells. As the authors state in their discussion there are many protocols published, some in great details for example see Robust generation of hepatocyte-like cells from human embryonic stem cell populations. Medine CN, Lucendo-Villarin B, Zhou W, West CC, and Hay DC, https://www.stembook.org/protocols/endoderm. However, they rarely show the morphology of the cells or their density at critical steps (beginning-end of each stage) that are essential for good progression of the differentiation, therefore a video showing these aspects would be of great value to researchers trying to establish that protocol in their lab.

Major Concerns:

On the other hand, the authors have used for this protocol only one hESCs line hES03, the authors should discuss if their protocol can be used with the same efficiency with other hESCs lines and human induced pluripotent stem cells (hiPSCs).

Responses: This protocol can be easily applied to other lines such as H1, H9. However, the efficiency may differ between different cell lines.

Minor Concerns:

Introduction:

Incorrect use of English and meaning:

line 42 ''secreting protein synthesis''line 4, it should be:…including….secretion and synthesis of proteins.

Responses: Revised accordingly.

Line 43: its functions

Responses: Revised accordingly.

Line 50: high degree of self-renewability

Responses: Revised accordingly.

Line 51: ''seed cells'' source cells would be more appropriate

Responses: Revised accordingly.

Line 54: Do the authors mean that in their protocol hESC form embryoid bodies while being differentiated towards endoderm? Could the authors give a reference for that sentence? Or maybe remove it if not necessary

Responses: Removed it.

Line 69: Could the authors give a range of percentage of sox17 compiling

several differentiations in addition to the maximum percentage achieved? Percentage range of hepatocytes obtained would be useful too.
Responses: About 89.7±0.8% SOX17-positive cells. We revised it in the manuscript.

1. Stem cell maintenance
Typographical errors:
Lines 85 and 86: matrige l
Line 90: 4°C f for overnight
Line 93: 15 mLcentrifuge
Line 96: the cells
Response: Revised accordingly.

2. Stem Cell passage And Differentiation
Line 106: 1. prewarmedto 37C.
Response: Revised accordingly.

3. Definitive Endoderm Formation
Line 115 1. Prepare 100 μg/mL activin A and 3 mM CHIR99021 stock solutions. Could the authors explained how they prepare those stock solutions, in which solutions? Filtered?
Response:The Activin A powder was dissolved in 0.2% BSA solution and filtered with a 0.22 μm filter membrane to obtain the stock solution of Activin A. The CHIR99021 powder was dissolved in DMSO to obtain the stock solution of CHIR99021.

Line 125: After 3 days of differentiation, differentiated cells should express markers of definitive endoderm cells. Could the authors precise which markers? Which analysis? Any cut off value? e.g. minimum % of sox17 needed for proceeding?
The markers of definitive endoderm cells were SOX17, FOXA2, CXCR4 etc. Those marker could be analysis by qPCR, IF or WB. The percentage of SOX17 should be as much as possible, usually needed more than 80%.

4. Differentiation of Hepatic Progenitor Cells
Line 131 2. Prepare Stage II differentiation medium containing 1% DMSO, 1X GlutaMAX, 1X non essential amino acid (NEAA) solution, 2-mercaptoethanol and penicillin/streptomycin (P/S) to the appropriate 133 volume of KOSR/knockout DMEM. Could the authors precise which exact concentration of GlutaMAX, NEAA, 2-mercaptoethanol and P/S.
Prepare Stage II differentiation medium containing 1% DMSO, 1X GlutaMAX,

1X non essential amino acid (NEAA) solution, 100 μM 2-mercaptoethanol and 1X penicillin/streptomycin (P/S) to the appropriate volume of KOSR/knockout DMEM.

Line 139 6. After 8 days of differentiation, differentiated cells should express appropriate markers of hepatic progenitor cells. Could the authors precise which markers? Which analysis? Any cut off value?
The markers of hepatic progenitor cells were HNF4a, AFP, TBX3, CEBPA, TTR etc. Those marker could be analysis by qPCR, IF or WB. The percentage of HNF4A should be as much as possible, usually needed more than 80%.

5. Hepatocyte differentiation
Line 143 1. Prepare 10 μg/mL hepatocyte growth factor (HGF), 20 μg/mL Oncostatin (OSM) stock solutions. Could the authors explained how they prepare those stock solutions, in which solutions? Filtered?
The HGF powder was dissolved in 0.2% BSA solution and filtered with a 0.22 μm filter membrane to obtain the stock solution of HGF. The OSM powder was dissolved in 0.2% BSA solution and filtered with a 0.22 μm filter membrane to obtain the stock solution of OSM.

Line 145.2. Prepare Stage III differentiation basic medium (HepatoZYME-SFM (HZM) medium supplemented with hydrocortisone-21-hemisuccinate and P/S. Could the authors precise which exact concentration of hydrocortisone-21-hemisuccinate and P/S.
Prepare Stage III differentiation basic medium (HepatoZYME-SFM (HZM) medium supplemented with 10 μM hydrocortisone-21-hemisuccinate and 1X P/S.

Line 153 6. After 18 days of differentiation, differentiated cells should express appropriate markers of hepatocytes. Could the authors precise which markers? Which analysis? Any cut off value?
The markers of HLCs were AAT, ALB, TTR, NTCP etc. Those marker could be analysis by qPCR, IF or WB. The percentage of Albumin-positive cells usually is more than 90%.

6. RNA Isolation, cDNA Synthesis and RT-PCR Analysis
Line 157 1. Aspirate medium from cells and add the proper amount of RNAiso Plus reagent, (e.g.0.3 mL per well of a 24-well plate), collect the supernatant in an EP tube and let stand at RT for 5 min… Could the authors describe EP? Could the authors provide the primers sequences used?
EP tube is 1.5 ml tube.

7. Immunofluorescence Validation of Differentiation. And 8. Western Analysis
Could the authors provide the antibodies used and their concentrations?

Yes, we provided accordingly. IF concentration is 1：200, and WB is 1：1000.

Representative results:
Line 248: Could the authors describe the typical hepatocyte phenotype?
The typical hepatocyte phenotype is polygon, and the cells were evenly and regularly distributed.

Discussion:
Line 311: The HLCs obtained are actually closer to fetal hepatocytes as AFP is higher expressed than ALB at day 18, this should be noted in discussion.
We have mentioned "The HLCs obtained are actually closer to fetal hepatocytes as AFP is higher expressed than ALB at day 18" here. In order to promote the maturation of HLCs and reduce their heterogeneity, the researchers used two different strategies. One strategy is to use three dimensional (3D) culture, another strategy is to add small molecules that promote differentiation [23003670] [29861165].

Line 325: Activin is essential for this protocol, have the authors observed any variation of efficiency depending on batches, or for other molecules used?
Yes. We observed some variation of differentiation efficiency in different batches.

Figure 1: Could the authors provide higher magnification images for day 3, 8, 14 and 18.
Yes. We revised accordingly.

Figure 2: Could the authors explain AC and give the full name of each gene maybe in the table with the primers sequences for each of them?
Yes. AC means Activin A plus CHIR99021. We arranged primer sequences in Table 2.

Figure 3 A and B: Could the authors provide higher magnification images?
Yes.

Table 1: Typographical error: 2-Mecapto etheanl
Revised accordingly.