

Development of Hepatocyte-like Cell Derived from Human Induced Pluripotent Stem cell as a Host for Clinically Isolated Hepatitis C Virus

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This unit describes protocols to develop hepatocyte-like cells (HLCs) starting from mesenchymal stem cells (MSCs) as a natural host for hepatitis C virus (HCV). These include the preparation of MSCs from bone marrow, the reprogramming of MSCs into induced pluripotent stem cells (iPSCs), and the differentiation of iPSCs into HLCs. This unit also incorporates the characterization of the resulting cells at each stage. Another section entails the preparations of HCV. The sources of HCV are either the clinically isolated HCV (HCVser) and the conventional JFH-1 genotype. The last section is the infection protocol coupled with the measurement of viral titer. © 2017 by John Wiley & Sons, Inc.

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SIGNIFICANCE STATEMENT

This series of protocols cover the essential techniques in cell and molecular biology involved in maintaining a complete hepatitis C viral life cycle in cell culture model. Only recently can we propagate hepatitis C virus from infected serum in non-cancerous host cells. An emphasis will be put on the generation of the host “hepatocyte-like cells (HLCs)”. Mesenchymal stem cells are served as the precursors for induced pluripotent stem cells, which in turn, are served as immediate precursors for HLCs. The outcomes of these protocols can provide a ground work for not only the study of hepatocyte-pathogen interactions, but also carcinogenesis, pharmacological and immunological applications of HLCs.

INTRODUCTION

The human primary hepatocytes are ideal natural host cells for liver-targeting pathogens such as hepatitis B (Kaneko et al., 2016; Lindenbach & Rice, 2005), hepatitis C (Sa-Ngiamsuntorn et al., 2016) malarial parasite (Ng et al., 2015) and dengue viruses (Lang, Vera, Cheng, & Tang, 2016). The difficulty in obtaining these cells and their limited life span restricted the study of long term host-pathogen interactions and xenobiotic metabolism. Hepatitis C virus is a single positive-stranded RNA virus belonged to the genus of *Hepacivirus* and *Flaviviridae* family (Simmonds et al., 2005). Chronic HCV infection led to cirrhosis and hepatocellular carcinoma (Chisari, 2005). An estimated 130-150 million people globally have chronic HCV infection and there is no effective vaccine to protect against HCV. Recently, the treatment of HCV using direct-acting antivirals (DAA) drugs such as sofosbuvir and simeprevir could eradicate HCV infection. However, several DAA drugs resistance have been reported (Imhof & Simmonds, 2011; Jazwinski & Muir, 2011). The development of effective HCV culture models is critical for designing efficacious anti-HCV strategies. The studies on HCV life cycle and anti-HCV drugs relied on human hepatocellular carcinoma cell lines such as Huh7 and their derivative clones. Only HCV genotype 2a (JFH-1) could be propagated from Huh7 derived cells (Catanese & Dorner, 2015; Wakita et al., 2005). The application of hepatocellular carcinoma cells as a host for HCV could not fully mimic primary human hepatocytes. The genotype and phenotype of cancer cell are abnormal. Huh7 cells also lose their contact inhibition dissimilar to the primary hepatocytes which mostly present in quiescent stage. Most hepatocellular carcinoma cells usually lack several liver enzymes e.g., CYP450s, other phase I, II, and drug transporters that make them inadequate for the anti-HCV drug screening.

Human induced pluripotent stem cells (iPSCs) can be reprogramed from somatic cells through ectopic expression of Oct4, Sox2, Klf4, and c-MYC (Takahashi & Yamanaka, 2006; Takahashi et al., 2007). These cells actively entered cellular division and can be differentiated into functional hepatocyte-like cell (HLCs) (Chun, Byun, & Lee, 2011) and other lineages. The applications of HLCs derived from either iPSCs or embryonic stem cells as natural hosts for HCV were recently reported (Schwartz et al., 2012; Si-Tayeb et al., 2012). These differentiated cells expressed essential liver functions and achieved mature hepatocytes. HLCs also expressed known HCV host receptors involved in HCV entry (Claudin-1, Occludin, SR-BI, CD81) and supported complete life cycle of classical HCV genotype 2a up to 30 days (Sa-Ngiamsuntorn et al., 2016; Wu et al., 2012). HLCs were promptly taken as HCV hosts. The infected cells could host full viral life cycle after the transfection/infection with HCVcc and HCVser. HLCs could sustain the replication of not only JFH-1 HCV but various wild-type HCV derived from patients' sera.

This unit describes overall procedures for generation of hepatocyte-like cell as a natural host hepatitis c virus production and drug metabolism. The unit begins with a Basic Protocol 1, which explains procedures for isolation human mesenchymal stem cell (MSC) from aspirated bone marrow. Basic Protocol 2 describes the further maintenance and culture of isolated MSCs. Lentiviral particles that carry the reprogramming factors (Oct4, Sox2, Klf4, and c-MYC) are produced using plasmid co-transfection into HEK293T described in a Basic Protocol 3). To generate the iPSCs, MSCs are used as precursor cells for cellular reprogramming following the Basic Protocol 4. The iPSCs are characterized by various method such as alkaline phosphatase staining, immunofluorescent staining and pluripotent genes expression using reverse transcription PCR. Series of iPSCs characterization method are described in the Basic Protocol 5. Basic Protocol 6 describes hepatic induction of iPSCs to functional hepatocyte-like cell. Basic Protocol 7 describes the characterization of the differentiated cells using the following methods: Periodic acid-Schiff staining of glycogen, hepatocyte-selective gene expressions by real-time qPCR,

CYP450s activities by luciferase-based assay and cellular hepatitis C receptors on HLCs by immunofluorescent staining. Basic Protocol 8 and 9 describe the applications of HLCs as a host for HCV infection and replication. Basic Protocol 10 demonstrates the infectivity titer of either HCVcc or HCVser in HLCs. At the moment, only HLCs can serve as host cells for wild-type HCV (HCVser).

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All the cell culture reagents listed are stored, reconstituted and used as specified in the product data sheet supplied by the manufacturer unless specifically stated.

NOTE: Recombinant proteins and chemical inhibitors/activators should be stored at –20°C or –80°C after reconstitution.

THE ISOLATION OF HUMAN MESENCHYMAL STEM CELL FROM BONE MARROW

BASIC PROTOCOL 1

Human mesenchymal stem cell (MSC) can be isolated from aspirated bone marrow of healthy donor. This protocol describes the procedure to separate the fraction of mononuclear cells using Ficoll density gradient centrifugation. Ficoll is a neutral, highly branched, high-mass, hydrophilic polysaccharide which dissolves readily in aqueous solutions. After centrifugation, the mononuclear cells are seeded and MSCs will appear within 7 days. Two weeks after seeding, MSCs display spindle-shaped morphology (Figure 4A.13.1). The pure population of MSCs can be obtained after 1 to 2 passages of subculture.

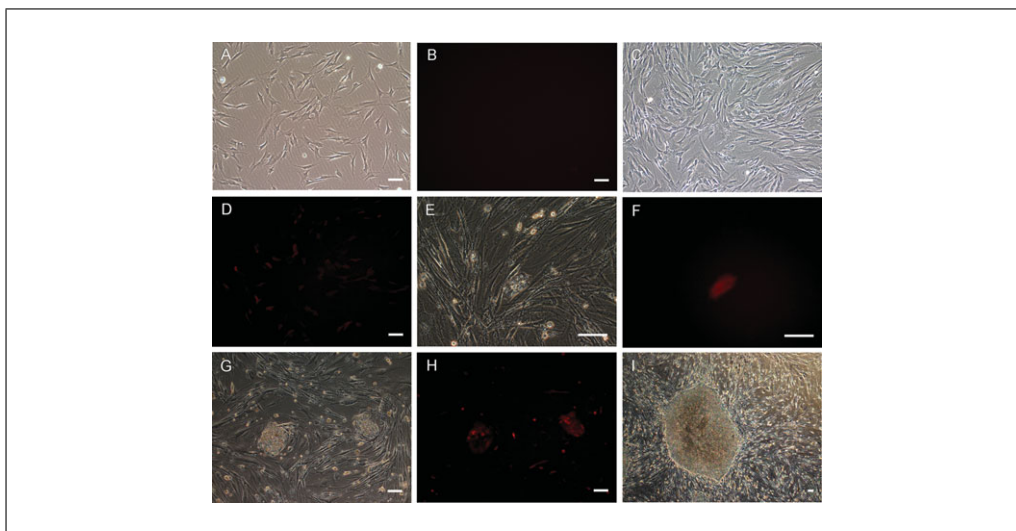


Figure 4A.13.1 The generation of iPSC from MSC. MSC isolated from a healthy donor at the 2nd passage showed a spindle-shape (A). MSCs were OSKM-dTomato negative before the reprogramming (B). MSCs after being transduced with polycistronic OSKM-dTomato lentivirus for 2 days were observed under a light microscope (C) and were positive for dTomato under a fluorescence microscope (D). A small colony of epithelium-like cells strongly expressing dTomato was observed on day 5 after the transduction (E, F). The cell colonies expanded and became tightly packed (G). More colonies expressing dTomato could be clearly observed on day 11 (H). After achieving fully reprogramming, the stable colonies were observed (I). Scale bars = 100 μm.

Manipulation of Potency

4A.13.3

Materials

Phosphate-buffered saline (DPBS) without calcium and magnesium
Ficoll (GE Healthcare Life Sciences) or IsoPrep (Robbins Scientific)
Trypan Blue solution, 0.85% in saline (Sigma)
MEM Alpha modification (α -MEM, Hyclone, cat. no. SH30265.02) supplemented with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences)
Penicillin G sodium (Sigma, cat. no. P7794)
Streptomycin (Sigma, cat. no. S6501)

50- and 15-ml polypropylene centrifuge tubes
Centrifuge
Sterile Pasteur pipettes
Neubauer hemacytometer
75-cm² flasks
Inverted microscope

The isolation of MSCs from human bone marrow

1. Collect human bone marrow from healthy donors or from a routine pathological diagnosis of patients. The procedure and the informed consent form should be approved by the institutional review board (IRB).

Bone marrow isolated from a young donor is preferred since the quality of MSC such as viability, expansion and life-span depend on donor's age. The aspirated bone marrow should be preserved in heparin solution (25 IU/ml) to prevent blood coagulation.

2. Transfer bone marrow aspirate (5 ml) into 50-ml centrifuge tube. Dilute bone marrow with PBS at a ratio of 1:4 to reconstitute the volume up to 20 ml.
3. To each of two 15-ml conical centrifuge tubes, add 3 ml of Ficoll – Hypaque reagent (IsoPrep) followed by the gentle overlay of the 10 ml of diluted bone marrow over the Isoprep. The solvent phase junction between the Isoprep layer and the bone marrow layer should not be disrupted.

This is an important step for isolating the mononuclear cell. Do not shake the tube or disrupt the solvent interface between the Isoprep solution and the diluted bone marrow. If the solvent interface is disturbed, the gradient centrifugation would fail to isolate the MSCs.

4. Centrifuge the solution for 30 min at $1000 \times g$, 20°C with the brake off.
5. After centrifugation, the mononuclear cell layer would present as a white ring just above the interface between the Isoprep layer at the bottom and the diluted bone marrow layer on top. The white ring could be collected immediately using a sterile Pasteur pipette and transferred into a new 50-ml conical centrifuge tube.

Before collecting the white ring solution, remove the plasma solution in the upper layer by aspiration and gently pipette the white ring solution which contain the mononuclear cell into the conical centrifuge tube.

6. Wash the white pellet from step 5 three times, each time with three-fold volume of PBS and then centrifuge for 10 min at $1500 \times g$, room temperature.
7. Evaluate the viability of the isolated cells using trypan blue exclusion assay. Mix the cell suspension (10 μ l) with 10 μ l trypan blue and lay over a hemacytometer. The cell viability should be over 90%.
8. Resuspend the cell pellet in 20 ml MSC culture medium (α -MEM supplemented with 10% FBS, $1 \times$ penicillin, and streptomycin).

The cell density should be seeded at 10^6 - 10^7 cells per one 75-cm² cell culture flask

9. Transfer the cell suspension into 75-cm² cell culture flasks and incubate the cell suspension in a humidified incubator with 5% CO₂. Replace the culture medium for the first time on day 4 or 5 followed by every 3 to 4 days thereafter.
10. After 7 days, the adherent cells could appear and show spindle-shaped morphology when observing under an inverted microscope. The colony should be clearly observed on the culture surface. The possible contamination of the culture cell with some hematopoietic cells such as red blood cells and blood stem cell could be eliminated during successive cell passaging.

The MSC cell can be characterized by immunostaining for anti-CD105, anti-CD90, and anti-CD73 and the cell population can be analyzed using a flow cytometer (Wongkajornsilp, Sa-Ngiamsumtorn, & Hongeng, 2012).

THE MAINTENANCE OF HUMAN MESENCHYMAL STEM CELL (MSC)

This protocol aims to propagate MSCs for further experiments or for frozen storage. The isolated MSCs can be enzymatically passaged following this protocol up to 10 times without the acquisition of cellular senescence. The maximum passage or life span of cultured MSCs could vary depending on the individual donor. The optimized time for harvesting MSCs was at the early passages (3rd-5th) as a precursor cell for cellular reprogramming.

Materials

Human MSCs in 75-cm² flasks (see Basic Protocol 1)
Phosphate-buffered saline (DPBS) without calcium and magnesium.
Trypsin-EDTA, 0.25% (w/v), 1 mM EDTA in PBS
MEM Alpha modification (α -MEM, HyClone, cat. no. SH30265.02) supplemented with 10% fetal bovine serum (FBS; HyClone GE Healthcare Life Sciences)
Penicillin G sodium (Sigma, cat. no. P7794)
Streptomycin (Sigma, cat. no. S6501)
Trypan blue

Inverted microscope
Hemocytometer
Centrifuge

Subculture and expansion of MSC

Using the MSC isolation protocol, cells are expected to reach 70% to 80% confluence within approximately 1 week. Confluent MSCs are trypsinized and seeded as a monolayer as follows.

1. Aspirate the old cell culture medium from the 75-cm² culture flask and wash the cell culture by adding 4 ml PBS
2. Aspirate the PBS and replace with 4 ml of 0.05% trypsin/EDTA.
3. Place the flask in the 37°C incubator for 2 to 3 min, and inspect the monolayer using an inverted microscope with 10 \times objective lens. The adherent cells should be detached from the cell surface. Additional incubation at 37°C for 2 to 3 min might be necessary if adherent cells have not detached.

When first subculturing the MSCs, carefully monitor the trypsin/EDTA incubation time because the MSC may be tightly adhered to the culture surface.

BASIC PROTOCOL 2

Manipulation of Potency

4A.13.5

4. Inactivate the trypsin by adding 4 ml MSC culture medium with 10% FBS (α -MEM supplemented with 10% FBS, penicillin and streptomycin) to the flask and gently resuspend the detached cells. Transfer the reconstitution into a 50-ml conical centrifuge tube, and centrifuge for 10 min at $1000 \times g$, 20°C .
5. Discard the supernatant from the cell pellet and resuspend with 1-2 ml prewarmed MSC culture medium with 10% FBS (α -MEM supplemented with 10% FBS, penicillin and streptomycin).
6. Mix 10 μl of the cell suspension with 10 μl of trypan blue and count with a hemacytometer. Cell viability should be greater than 90%.

MSC cell density per 75-cm² flask should be around $2\text{-}5 \times 10^6$ cells.

7. Incubate the cells in 5% CO_2 at 37°C .
8. Replace the MSC culture medium twice a week and subculture once a week.

The MSCs at the early passage (i.e., 1st to 4th) can be cryopreserved in liquid nitrogen for long-term use.

BASIC PROTOCOL 3

THE PREPARATION OF LENTIVIRAL VECTORS FOR REPROGRAMMING MSCS TO IPSCS

This protocol describes the production of lentiviral vectors for delivering the reprogramming genes (OSKM). To increase the safety of lentivirus, the essential components of viral production are split into multiple plasmids (three for 2nd generation, for 3rd-generation systems). The components of reprogramming vectors are as follows: (1) lentiviral transfer plasmid encoding Oct4, Sox2, Klf4, and c-MYC. These sequences are inserted between the LTRs region that enable host genome integration; (2) packaging plasmids; and (3) envelope plasmid. The procedure for producing infectious transgenic lentivirus consists of (a) co-transfection of three or four plasmids into HEK293T cells, (b) collection of conditioned medium containing the virus and (c) purification and concentration of the virus. The concentrated virus can then be used to infect the MSCs. Biosafety should always be considered with respect to the precise nature of experiments being performed. Your biosafety office can provide more information on your institution's best practices with regard to manipulating infectious virus. The NIH also has additional information on lentiviral safety considerations.

Materials

Lentiviral plasmids:

pRRL.PPT.SF.hOKSM.idTomato.preFRT (polycistronic OSKM-dTomato or equivalence)

pMDLg/pRRE (packaging plasmid; contains Gag and Pol; Addgene plasmid # 12251)

pRSV-REV (Rev, Addgene plasmid # 12253)

pMD2.G (VSV-G, Addgene plasmid # 12259)

NucleoBond Xtra Midi Kit (MN, cat. no. 740410.10).

Transformed *E. coli* culture

Luria broth medium (see recipe)

HEK293T cell (CRL-3216, ATCC)

DMEM/high glucose (HyClone, cat. no. SH30243.02)

Fetal bovine serum (FBS; GE Healthcare Life Sciences)

Penicillin G sodium (Sigma, cat. no. P7794).

Streptomycin (Sigma, cat. no. S6501)

Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific, cat. no. 31985088)

X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics)

Lenti-X concentrator (Takara Bio)
Phosphate-buffered saline (DPBS) without calcium and magnesium

10-cm culture dishes
1.5-ml microcentrifuge tubes
Pipettes and micropipettes
37°C, 5% CO² incubator
20-ml syringes
Sterile syringe filter (0.45 μm Sartorius)
Centrifuge

Preparation of Lentiviral particles

1. Extracting the lentiviral plasmid DNA using NucleoBond Xtra Midi Kit from an overnight transformed *E. coli* culture grown in 250 to 500 ml LB medium. The 250 or 500 ml overnight LB culture should yield 0.5 to 1.0 mg plasmid DNA.

Do not culture the E. coli in LB medium for more than 18 hr. The over growth of E. coli in an orbital shaker can reduce the yield of plasmid DNA.

2. Adjusting the concentration of the lentiviral plasmid DNA in a sterilized 1.5-ml microcentrifuge tube. For polycistronic OSKM-dTomato lentivirus transduction using pRRL.PPT.SF.hOKSM.idTomato.preFRT (lentiviral vector containing hOCT4, hKLF4, hSOX2, hMYC, and a fluorescent reporter gene, dTomato) or other polycistronic OSKM equivalences, pMDLg/pRRE, pRSV-REV and pMD2.G (VSV-G)

The plasmids concentration can be measured using Nanodrop spectrophotometer and could be adjusted to 1 μg/μl or appropriate concentration.

Preparing packaging cells

3. Approximately 24 hr before transfection, seed HEK293T cells at a density of 4 × 10⁶ cells in 10 ml DMEM, 10% FBS, 1 × penicillin and streptomycin onto a 10-cm culture dish. Gently shake the cell culture dish side to side to evenly distribute the cells. Monitor the confluence daily and transfect the cells when the adherent cells are 70% to 80% confluent.

If cell detachment during transfection is observed, use cell culture dishes precoated with poly-D-lysine prior to the seeding of HEK293T.

Transfection of the lentiviral plasmids to the packaging cells

4. On the transfection day, gently remove the culture medium from the 10-cm cell culture dish and replace with 10 ml DMEM supplemented with 10% FBS, penicillin and streptomycin.
5. In a sterile 1.5-ml microcentrifuge tube, add 10 μg pRRL.PPT.SF.hOKSM.idTomato.preFRT or equivalent mixed thoroughly with 3.75 μg pMDLg/pRRE Gag, Pol plasmid, 3.75 μg pRSV-REV Rev plasmid, and 2.5 μg of pMD2.G vesicular stomatitis virus G envelope plasmid (Addgene) and adjust the total volume to 500 μl using Opti-MEM medium.
6. In a 1.5-ml microcentrifuge tube, add 440 μl of Opti-MEM medium and 60 μl X-tremeGENE HP DNA transfection reagent and mix by pipetting 2 to 3 times.

The undiluted X-tremeGENE HP DNA transfection reagent should be pipetted directly into Opti-MEM medium without any contact with the sides of the microcentrifuge tube as this may reduce transfection efficiency.

7. Gently mix the plasmid solution from step 5 with the solution from step 6 by pipetting and incubate the transfection reagent/DNA complex for 15 min at room

temperature. Add the solution condition (1-ml) onto the 10-cm culture dish drop by drop using a micropipette to cover the entire culture area.

8. Incubating the transfection complex over the packaging cells on the cell culture dish in 5% CO₂ at 37°C overnight (16 to 18 hr).
9. Approximately 24 hr after transfection, replace the cell culture medium with 10 ml DMEM, 10% FBS containing 1 × penicillin/streptomycin and incubate at 37°C, 5% CO₂.

Isolation of lentiviral particles from the culture supernatant

10. At 48 hr after transfection, harvest the conditioned medium using a 20-ml syringe and filter through a 0.45-μm filter to remove cell debris.

The conditioned medium containing lentivirus can be collected at 48 hr and 72 hr after transfection. The medium can be pooled and kept at 4°C for one week. Freeze the lentiviral particles at -80°C for long-term storage.

11. To the concentrated lentivirus, add 1 volume of Lenti-X concentrator to 3 volume of clarified supernatant from step 10. Gently mix the solution by inversion. Incubate the mixture at 4°C for 1 hr. Centrifuge the solution for 1 hr at 1500 ×g, 4°C. After centrifugation, an off-white pellet will be visible.

The lentivirus in conditioned medium can be concentrated with sucrose gradient ultracentrifugation using 10% (w/v) sucrose for 4 hr at 90,000 ×g.

12. Gently reconstitute the pellet in Dulbecco's phosphate-buffered saline (DPBS) and store up to 1 year at -80°C in single-use aliquots.

Please avoid freezing and thawing lentivirus more than one time. The freeze-thaw cycle reduces the viral infectivity titer.

BASIC PROTOCOL 4

REPROGRAMMING MSCS TO iPSCS

Several types of cells have been used to generate iPSCs by transducing with reprogramming factors. The most common somatic cells that have been extensively reprogrammed are fibroblasts. However, human fibroblasts have some limitations such as a short life span, genetic instability, and requiring expansion into several passages before reprogramming. Human MSCs are multipotent stem cell-derived from aspirated bone marrow, which can be passaged up to 10 times without genetic instability and gene mutation. This protocol describes the procedures to infect MSCs with lentivirus carrying Oct4, Sox2, Klf4, and c-MYC including the selectable marker dTomato. The transduced MSCs can be observed by the red fluorescence of dTomato and forming colonies after 29 days post-transduction. Fully reprogramming colony can be selected by staining colonies with TRA-1-60 and observing the silencing of dTomato. After the 15th passage, the iPSCs can be transferred to feeder-free culture in E8 medium and can be used as precursor cells for hepatocyte differentiation.

Materials

Human mesenchymal stem cell (MSC) passage 3rd – 5th

Lentivirus

MSC growth medium (see recipe)

Hexadimethrine bromide or polybrene (Sigma, cat. no. H9268)

Sodium butyrate (Sigma, cat. no. B5887)

Inactivated MEF (mouse embryonic fibroblast, PMEF-CFX, Merck Millipore)

iPSC medium [DMEM/F12, 20% Knockout Serum Replacement (KO-SR), 110 mM 2-mercaptoethanol, 1 × non-essential amino acid (NEAA), 1 × GlutaMAX, 1 × penicillin, streptomycin, and 10 ng/ml basic fibroblast growth factor, bFGF]

DMEM/F12 (HyClone, cat. no. SH30261.02)
 KnockOut Serum Replacement (Thermo Fisher Scientific, cat. no. 10828028)
 2-Mercaptoethanol (Thermo Fisher Scientific, cat. no. 21985023)
 MEM Non-Essential Amino Acids (100×; NEAA; Thermo Fisher Scientific, cat. no. 11140050)
 GlutaMAX Supplement (Thermo Fisher Scientific, cat. no. 35050061)
 Penicillin G sodium (Sigma, cat. no. P7794)
 Streptomycin (Sigma, cat. no. S6501)
 Recombinant Human FGF-basic (bFGF; Peprotech, cat. no. 100-18B)
 TRA-1-60 Antibody (SC-21705, SantaCruz)
 Phosphate-buffered saline (DPBS) without calcium and magnesium
 Goat anti-Mouse IgG/IgM (H+L) Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. no. A-10684)
 Geltrex LDEV-Free, hESC-Qualified (Thermo Fisher Scientific, cat. no. A1413301)
 Essential 8 Medium (Thermo Fisher Scientific, cat. no. A1517001)
 mTeSR 1 (Stem Cell Technologies, cat. no. 05850)
 Y-27632 (Cell Guidance Systems, cat. no. SM2)
 Versene solution (0.48 mM EDTA; Thermo Fisher Scientific, cat. no. 15040066)
 Fetal bovine serum (FBS; GE Healthcare Life Sciences)
 EmbryoMax 0.1% Gelatin Solution, Merck Millipore, cat. no. ES-006-B)

6-well plates
 10-cm cell culture dishes
 Stereomicroscope
 3.5-cm dishes

Reprogramming MSCs to iPSCs

1. One day before the infection, seed MSCs at 1×10^5 cells on each well of a 6-well plate (Fig. 4A.13.1A,B). Infect the MSCs with the lentivirus at a MOI of 0.5 through spinfection by centrifuging for 30 min at $1000 \times g$, 32°C , in MSC growth medium (α -MEM supplemented with 10% FBS, penicillin and streptomycin) containing $4 \mu\text{g/ml}$ polybrene.
2. After 16 to 18 hr post-infection, replace the medium with 2 ml MSC growth medium without polybrene, and maintain the infected MSCs in this culture medium for 5 days. Add 0.5 mM sodium butyrate into the culture medium on days 2 to 11 post-infection.

Adding sodium butyrate or valproic acid solution to the transduced MSCs could enhance cellular reprogramming.

3. On day 4, split the infected MSCs and plate from each 6-well plate onto 10-cm cell culture dishes pre-seeded with Mitomycin C-inactivated mouse embryonic fibroblast (i-MEF) feeder cells at a density of 3.5×10^4 cells/cm². Maintain the infected cells in MSCs growth medium for 24 hr (Fig. 4A.13.1C).

Coat the surface of 10-cm dish with EmbryoMax 0.1% Gelatin Solution before seeding i-MEF. The recommended i-MEF density is 4×10^4 cells/cm².

4. After 24 hr, replace the culture medium with 10 ml iPSC medium containing DMEM/F12, 20% Knockout Serum Replacement (KO-SR), 110 mM 2-mercaptoethanol, 1% non-essential amino acid (NEAA), $1 \times$ GlutaMAX, $1 \times$ penicillin-streptomycin, and 10 ng/ml basic fibroblast growth factor (bFGF). The culture medium should be replaced daily.

The iPSC culture medium should be prepared in advance without basic fibroblast growth factor (bFGF). After adding bFGF, the medium must be spent within 1 week.

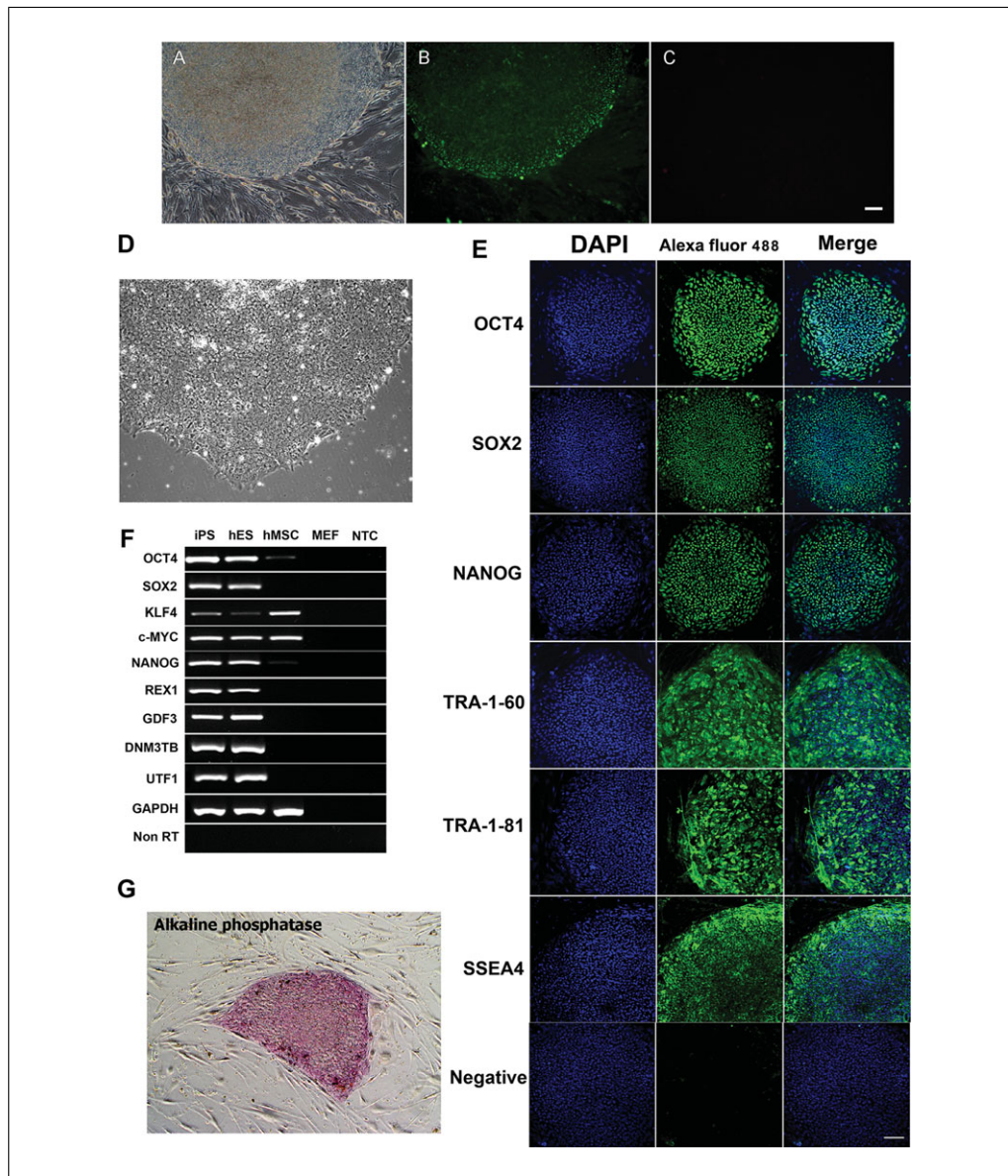


Figure 4A.13.2 iPSC colonies were live-stained with TRA-1-60 antibody, a pluripotency surface marker. The iPSC colony was grown on MEF for 25 days after transduction (A) and live-stained with TRA-1-60 antibody followed by Alexa 488 conjugated goat anti mouse IgG. The TRA-1-60-positive colony was verified under fluorescence microscope (B). The dTomato disappeared in iPSCs (C). The iPSCs on Geltrex exhibited high nucleate-to-cytoplasm ratio with prominent nucleoli (D). Immunofluorescent staining for OCT4, SOX2, NANOG, TRA-160, TRA-1-81, and SSEA4 followed by the Alexa fluor 488 conjugated secondary antibody confirmed the identity iPSCs after the reprogramming from MSCs (E). Nuclei were localized by DAPI (blue). Scale bars = 100 μ M. The expressions of pluripotency markers were determined by RT-PCR. iPSCs exhibited similar expression profile of pluripotent markers to that of embryonic stem cells (ESCs) (G).

- Observe the human ES-like colonies. The colonies should appear on day 29-30 post-infection (Fig. 4A.13.1E-G). Select the ES-like colonies by picking one colony at a time and transfer a single colony onto a 6-well plate pre-seeded with inactivated i-MEF (Fig. 4A.13.2).

Pick the ES-like colonies using a stereomicroscope in the biosafety cabinet to avoid contamination. Transfer an individual colony from a single reprogramming cell onto each well of 6-well plate in order to get an identical iPSC line.

6. Perform preliminary identification of the reprogramming cells by staining the colony with anti-human TRA-1-60 antibody at a 1:100 dilution in iPSC medium for 30 min in CO₂ incubator. After the incubation, gently wash the colonies with 2 ml PBS, and then add Alexa fluor 488-conjugated goat anti-mouse IgG and/or IgM at a 1:1000 dilution and incubate for 30 min. Wash the colonies twice, each time with 2 ml iPSC medium (Fig. 4A.13.2A-B).

Stain the live cells using aseptic technique in the class II biosafety cabinet. Antibodies, reagents, and PBS should be sterile to prevent the contamination in iPSCs.

7. Observe the TRA-1-60-positive colonies under a stereomicroscope and mark the colony by drawing a circle on the bottom of the plate with a permanent pen to further subculture. After the 15th passage, the iPSC colonies can be picked and maintained with feeder-free culture using Geltrex coated-plate and mTeSR1 medium or in house E8 medium with daily medium replacement (Fig. 4A.13.2D).

After transferring iPSCs to feeder-free condition, cultured iPSCs in mTeSR1 and 10 μ M Y-27632 should yield viable colonies.

8. Under feeder-free conditions, passage the iPSCs by incubating with diluted Versene solution (0.5 mM EDTA) for 2-5 min or until the round cells appear. Remove the EDTA solution and reconstitute the iPSC with mTeSR1 or E8 medium plus 10 μ M Y-27632 before seeding onto a new Geltrex-coated 3.5-cm dish.

The iPSC cells should reach 80-90% confluence prior to subculturing. Screen the spontaneous differentiated cells using an inverted microscope. Mark and remove the colony before subculture. Avoid bringing iPSCs to 100% since spontaneous differentiation can ensue.

THE SCREENING OF HUMAN-INDUCED PLURIPOTENT STEM CELLS

Human-induced pluripotent stem cell (iPSCs) can be characterized by several methods such as pluripotent gene expressions, alkaline phosphatase activity, silencing of exogenous reprogramming factors, immunostaining of pluripotent markers, karyotype analysis and in vitro and in vivo differentiation. However, all of these methods are time-consuming and labor intensive. In this unit, we describe some recommended screening protocols of iPSCs. These include three fundamental methods for rapidly characterizing iPSCs: (1) the alkaline Phosphatase (AP) activity, (2) immunofluorescent staining, and (3) RT-PCR. However, all newly established iPSCs should undergo complete characterization.

Materials

- iPSC line (see Basic Protocol 4)
- Alkaline Phosphatase Detection Kit (Merck Millipore, cat no. SCR004) containing:
 - Naphthol/Fast Red Violet Solution
 - 1 \times Rinse Buffer
- 4% paraformaldehyde (PFA) fixing solution (see recipe)
- Blocking solution (see recipe)
- Primary antibodies: NANOG, SOX2, SSEA4, OCT4, TRA-1-60, TRA-1-81, alpha fetoprotein (AFP), alpha-smooth muscle actin, and β -III tubulin)
- Tween-20
- Goat anti-Mouse IgG / IgM (H+L) Secondary Antibody, Alexa Fluor 488 (Thermo Scientific, cat. no. A-10684)
- Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 (Thermo Scientific, cat. no. A-11008)
- Hoechst 33342 (Thermo Scientific, cat. no. H3570)
- Fluorescence Mounting Medium (Dako, cat. no. S302380)

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Manipulation of Potency

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illustra RNAspin Mini Kit (GE Healthcare Life Sciences, cat. no. 25-0500-71)
ImProm-II reverse transcription system (Promega, cat. no. A3800)
KAPA2G Fast HotStart ReadyMix PCR Kit (Kapa Biosystems, cat. no. KK5603)
Gel electrophoresis apparatus and reagents including:
Molecular-biology-grade agarose (Vivantis, cat. no. PC0701)
10× Tris-Acetate-EDTA (TAE) Buffer (Vivantis, cat. no. PB0940)
RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, cat. no. 21141)
6× Loading Dye with SDS (Vivantis cat. no. NM0416)
DNA size ladder (Vivantis, cat. no. NL1401)

24-well plate
Phase-contrast inverted microscope
Fluorescence microscope
Thermal cycler

Alkaline Phosphatase (AP) staining

AP activity is detected using Alkaline Phosphatase Detection Kit following the manufacturer's instructions.

- 1a. Culture iPSCs for 5 days prior to measuring AP activity, at low to medium cell density.
- 2a. On day 5, aspirate the medium and fix the iPSCs with 4% PFA fixing solution for 1 to 2 min.
- 3a. Aspirate the fixative and rinse with 1 ml 1× Rinse Buffer and then add enough Naphthol/Fast Red Violet Solution to fully cover each well (e.g., 0.5 ml for a well of a 24-well plate). Incubate for 15 min in the dark at room temperature.
- 4a. Aspirate the staining solution and rinse each well with 1 ml 1× Rinse Buffer. Cover the cells with 1× PBS to prevent drying and then count the number of colonies expressing AP (red iPSC colonies) using a phase-contrast inverted microscope (Fig. 4A.13.2G).

Detection of pluripotent markers using immunofluorescent staining

- 1b. Culture iPSCs for five days prior to analyzing pluripotent proteins, at low to medium cell density.
- 2b. On day 5, aspirate the culture medium and fix the colonies with 4% PFA fixing solution for 15 min at room temperature. Incubate iPSC colonies with permeabilizing/blocking solution containing 0.2% Triton X-100, 3% BSA and 10% FBS in PBS for 45 min at room temperature.

If the secondary antibody is anti-goat species, replace the 2% normal goat serum to donkey serum or other species. The fixed colony can be kept at 4°C in blocking solution up to 1 to 2 weeks. Seal the cell culture plate or dish with parafilm to prevent liquid vaporizing and specimen drying.

- 3b. The primary antibodies used for the iPSC characterization are as follow: NANOG, SOX2, SSEA4, OCT4, TRA-1-60, TRA-1-81, alpha fetoprotein (AFP), alpha-smooth muscle actin, and β -III tubulin.
- 4b. Dilute primary antibodies in blocking solution at 1:100, incubate with iPSC colonies overnight at 4°C, and then wash the colonies three times, each time with 1 ml of 0.1% Tween-20 in PBS.

The primary antibody concentration should be optimized from 1:50 to 1:100 depending on individual lot and supplier. Incubation time can be shortened to 60 min at 37°C in a moist chamber.

- 5b. Dilute secondary antibodies in blocking solution at 1:1000, incubate with iPSC colonies after stained with primary antibody, and then wash the colonies three times, each time with 1 ml of 0.1% Tween-20 in PBS. Counterstain the nucleus with Hoechst 33342 diluted in PBS at 1:1000 for 15 min at room temperature.

Protect the cell samples from direct sun light or fluorescent lamp before observation under a fluorescence microscope by carrying the staining cell in a black box or wrapping by aluminum foil.

- 6b. After extensive washing, mount the cell samples with mounting medium, examine the positive colonies under a fluorescence microscope, and photograph (Fig. 4A.13.2E).

Mount the cell sample with a coverslip and seal the sample with glue or nail polish. Secure the cell sample at 4°C in the dark can preserve the sample for further analysis.

Detection of pluripotent genes using reverse transcription PCR (RT-PCR)

- 1c. Extract the total RNA from iPSCs using illustra RNAspin Mini Kit with DNase I treatment.
- 2c. Covert the 200 ng of total RNA to cDNA using oligo-dT primer and ImProm-II reverse transcription system following the manufacturer's instructions.
- 3c. Dilute cDNA to 1:10 ratio and use as a template for the PCR reaction. Mix 2 µl of diluted cDNA with the PCR master mix solution containing KAPA2G Fast HotStart ReadyMix PCR Kit with 5 µM of each pluripotent gene primers (see Table 4A.13.1).
- 4c. Amplify the PCR products with a thermal cycler using temperature condition as follows:

1 cycle:	3 min	95°C (initial denaturation)
40 cycles:	30 sec	95°C (denaturation)
	30 sec	60° to 65°C (annealing)
	30 sec	72°C (extension)
1 cycle:	5 min	72°C (final extension).

- 5c. Detect the specific amplification of PCR products using gel electrophoresis with 1% agarose gel (Fig. 4A.13.2F).

THE DIFFERENTIATION OF IPSC TO HOMOGENEOUS HEPATOCYTE-LIKE CELLS

In this protocol, we describe the procedure to derive hepatocyte-like cells (HLC) from iPSCs using direct differentiation. The differentiated cells develop a homogeneous population of hepatoblast-like cells, which can be differentiated to functional hepatocytes (Fig. 4A.13.3A). This protocol can be applied to differentiate the conventional hES cell lines into HLCs. The procedure uses defined culture medium devoid of serum and non-defined factors and contains four steps, which mimic embryonic liver development stages. The procedure begins with expansion of iPSCs in cell culture plate followed by the first stage differentiation protocol. The iPSCs are differentiated into definitive endoderm cells. In the second stage, differentiated cells are developed into anterior definite endoderm cells. During the third stage, the endodermal cells turned into hepatic progenitors. In the fourth stage, the HLCs undergo functional maturation and exhibit liver specific markers, such as albumin secretion, glycogen storage, cholesterol metabolism,

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4A.13.13

Table 4A.13.1 The Primer Sequences for Conventional PCR Used

Primer name	Sequences (5'—3')
hKILF4-F	ATTAATGAGGCAGCCACCTGG
hKLF4-R	CTCCCGCCAGCGGTTATTTCG
hOCT4-F	TGTACTCCTCGGTCCCTTTC
hOCT4-R	TCCAGGTTTTCTTTCCCTAGC
hSOX2-F	GCTAGTCTCCAAGCGACGAA
hSOX2-R	GCAAGAAGCCTCTCCTTGAA
hCMYC-F	CGGAACTCTTGTGCGTAAGG
hCMYC-R	CTCAGCCAAGGTTGTGAGGT
hNANOG-F	CAGTCTGGACACTGGCTGAA
hNANOG-R	CTCGCTGATTAGGCTCCAAC
hGDF3-F	TCCTGGAGATACTGGTCAAAGAA
hGDF3-R	GAGCATCTTAGTCTGGCACAG
hREX1-F	TGCTCACAGTCCAGCAGGTGTTT
hREX1-R	TCTGGTGTCTTGTCTTTGCCCGTT
hUTF-1-F	CCGTCGCTGAACACCGCCCTGCTG
hUTF-1-R	CGCGCTGCCAGAATGAAGCCCCAC
hDNMT3B-F	AAGTCGAAGGTGCGTCTGTGC
hDNMT3B-R	CCCCTCGGTCTTTGCCGTTGT
hGAPDH-F	GAAGGCTGGGGCTCATT
hGAPDH-R	CAGGAGGCATTGCTGATGAT

and cytochrome P450 enzymes after changing the medium to Williams' medium E, 1% DMSO, and prototypic CYP450 inducers.

Materials

iPSC line (see Basic Protocol 4)
 Geltrex LDEV-Free, hESC-Qualified (Thermo Fisher Scientific, cat. no. A1413301)
 Essential 8 Medium (Thermo Fisher Scientific, cat. no. A1517001)
 Endoderm differentiation basal medium (see recipe)
 Endoderm commitment medium (see recipe)
 RPMI 1640 medium (HyClone, cat. no. SH30255.01)
 B-27 Supplements (Thermo Fisher Scientific, cat. no. 17504044)
 Activin A (Cell Guidance Systems, cat. no. GFH6AF)
 Recombinant Human FGF-10 (Peprotech, cat. no. 100-26)
 Recombinant Human BMP-4 (Peprotech, cat. no. 120-05ET)
 Recombinant Human Oncostatin M (Cell Guidance Systems, cat. no. GFH356)
 Recombinant Human HGF (Peprotech, cat. no. 100-39H)
 Williams' Medium E (Thermo Fisher Scientific, cat. no. 12551032)
 Dimethyl sulfoxide (Sigma, cat. no. D2650)
 HBM Basal Medium (Lonza, cat. no. CC-3199)
 6-well plates

1. Culture the characterized iPSCs 10^6 cells /well on a Geltrex-coated 6-well plate in 2 ml of E8 medium to 60% confluence prior to the start of hepatic lineage development protocol.

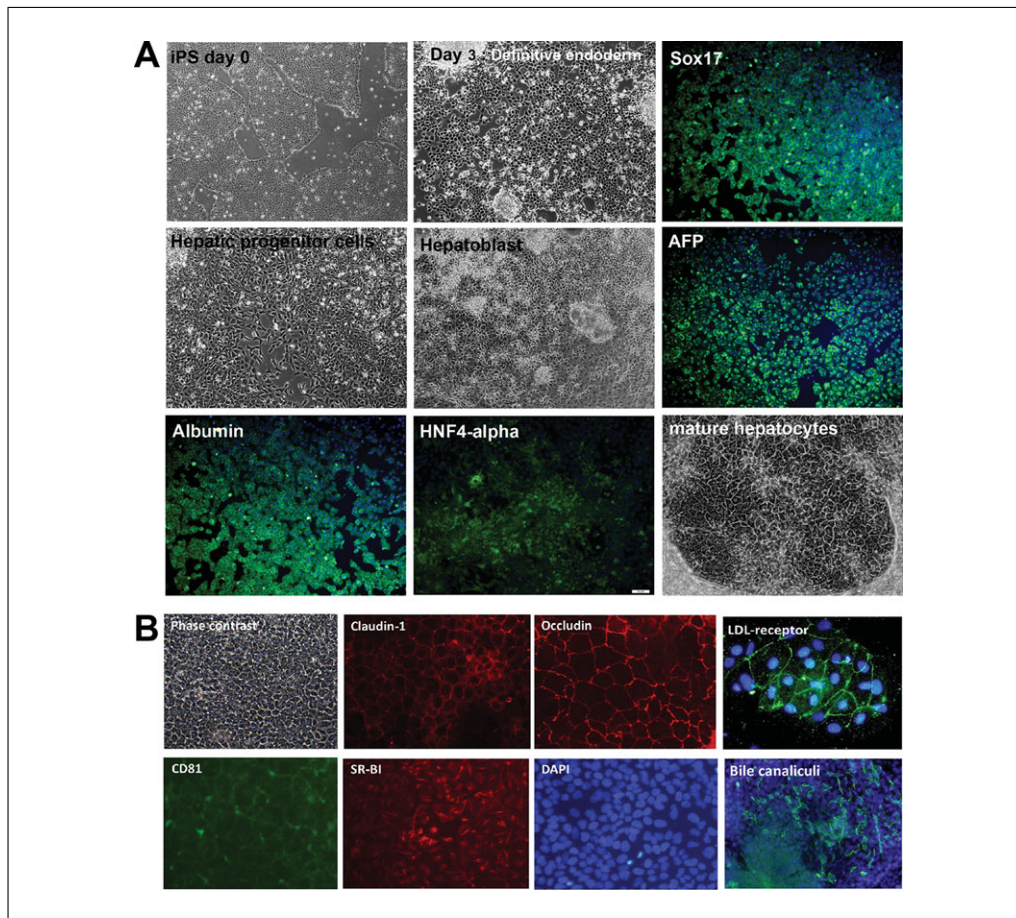


Figure 4A.13.3 The direct differentiation of iPSCs into the fully functional homogeneous HLCs and the characterization of HCV cell specific receptors. The iPSCs in feeder-free condition at 60% confluence were differentiated into definitive endoderm with round-shaped morphology. During the differentiation, cells were positive for Sox17. During anterior definitive endoderm (ADE) development, the differentiated cells underwent epithelial to mesenchymal transition (EMT). The ADE cells with oval-shape served as hepatic progenitors. The differentiated cells were positive for α -fetoprotein, albumin, and HNF-4 α . The homogeneously mature HLCs exhibited polygonal morphology, cord-like structure with bile canaliculi and hepatic sinusoidal-like structures (A). The immunofluorescent staining of six major HCV receptors (claudin-1, occluding, LDL-receptor, CD81, SR-BI, and bile canaliculi) were observed in the HLCs (B).

Bringing iPSCs higher than 60% confluence could decrease the homogenous endoderm differentiation and induce cell clumping.

- Maintain the cultured cells in endoderm differentiation medium DMEM/F12: IMDM (1:1), 2 mg/ml PVA, 2% (v/v) concentrated lipids, 100 ng/ml activin A, 10 ng/ml bFGF, 10 ng/ml BMP-4, 1 μ M LY294002, 1 \times Insulin-Transferrin-Selenium and 3 μ M CHIR99021 for 24 hr.

PVA needs to be dissolved in 50 ml of IMDM by mixing 0.5 g PVA in a 50-ml tube with DMEM/F12: IMDM and placed on a tube roller overnight at 4°C. Sterilize endoderm differentiation basal medium using a 0.22- μ m filtration device. Medium should be warmed to 37°C before use.

- Further maintain the cultured cells in 2 ml endoderm commitment medium DMEM/F12: IMDM (1:1), 2 mg/ml PVA, 2% (v/v) concentrated lipids, 100 ng/ml activin A, 100 ng/ml bFGF, 10 ng/ml BMP-4, 1 \times Insulin-Transferrin-Selenium, and 1 μ M LY294002 for 24 hr.

Adjust the optimal LY294002 concentration from 1 μ M to 10 μ M, some previous publications use 10 μ M LY294002. In our experiment, this concentration induced cell death.

4. Culture the endoderm-like cells in 2 ml anterior definitive endoderm differentiation medium: RPMI/B27 with 50 ng/ml activin A for 3 days with a daily medium replacement.

Anterior definitive endoderm is committed over a 3-day period with medium changes every 24 hr until the third day. Typical morphology of cells undergoing differentiation can be obtained (Fig. 4A.13.3A).

5. Maintain the differentiated cells in 2 ml hepatic lineage specification medium (RPMI/B27, 20 ng/ml BMP-4 and 10 ng/ml FGF10) for 4 days with daily medium replacement.

6. Culture the differentiated cells in 2 ml HBM Basal Medium (HBM; 30 ng/ml oncostatin M and 50 ng/ml HGF) for 10 days with medium replacement every 2 days.

Medium should be changed every 2 days during the maturation phase of the differentiation protocol. Morphological characteristics of hepatocytes such as polygonal shape, multi-nucleated cells, and canaliculi-like structures appear at approximately day 5 onward of this step along with the gene expression of immature fetal hepatocytes such as AFP (Fig. 4A.13.3A).

7. For functional hepatocyte induction, culture hepatocyte-like cells in 2 ml Williams' Medium E, 1 % DMSO for 3 to 5 days.

Induction of cytochrome P450 expressions and activities with 1% DMSO could be detected within 3 to 5 days of this stage. Protein secretion, cytochrome activity, LDL uptake, and glycogen storage are reliably detected and measured between day 20 and 28 after differentiation. Cell viability beyond this time can vary depending on cell types and cell lines; therefore, the authors recommend screening several maturation media.

BASIC PROTOCOL 7

CHARACTERIZATION OF HEPATOCYTE-SPECIFIC MARKERS

To ensure that iPSCs achieve complete hepatic differentiation, this protocol selects some distinct markers to confirm hepatic phenotypes. Mature hepatocytes accumulate glycogen in cytoplasm and are measured using the Periodic Acid-Schiff staining (PAS) assay. The expression of selective hepatocyte markers e.g., albumin, α -fetoprotein, cytokeratin 18, G-6-PD, HNF-4 α , tyrosine aminotransferase, and cytochrome P450 could be used to determine the maturation of HLC (Sa-ngiamsuntorn et al., 2011). In this protocol, we describe the procedure used to evaluate the hepatic markers using quantitative real-time PCR to benchmark HLC against the primary hepatocyte. Cytochrome P450 activities can be measured using luciferase-based assay (Sa-ngiamsuntorn et al., 2016). The resulting cells in this unit still express significant levels of α -fetoprotein and also express markers of adult hepatocytes such as albumin, α -fetoprotein, cytokeratin 18, G-6-PD, HNF-4 α , and tyrosine aminotransferase. The CYP450s expression and activity increasingly responded to the prototypic inducers in the same fashion to primary hepatocyte. In the last section of this unit, we describe the protocol to detect hepatitis C host specific receptors such as Claudin-1, Occludin, SR-BI, CD81 using reverse transcription PCR and immunofluorescent staining.

Materials

- Hepatocyte-like cell (HLCs; see Basic Protocol 6)
- Human mesenchymal stem cells (MSCs; see Basic Protocol 2)
- 95% ethanol
- 5% formaldehyde
- Periodic Acid-Schiff (PAS) Kit (Sigma, cat. no. 395B) containing:

Periodic acid solution
Schiff's reagent
Hematoxylin solution
dH₂O
HepaRG cell line (Thermo Fisher Scientific, cat. no. HPRGC10)
illustra RNAspin mini Kit (GE Healthcare Life Sciences, cat. no. 25-0500-71)
ImProm-II reverse transcription system (Promega, cat. no. A3800).
Real-time PCR primer sets (1st BASE)
Gene-specific primers (see Table 4A.13.2) KAPA SYBR FAST qPCR Kit Master Mix (2×) (Kapa Biosystems, cat. no. KK4600)
Primary human hepatocyte (Thermo Fisher Scientific, cat. no. HMCPTS)
Rifampicin (Sigma, cat. no. R3501)
Omeprazole (Sigma, cat. no. O104)
Phenobarbital (Sigma, cat. no. P5178)
Ethanol
P450-Glo 1A1, 2B6, 2C9 and 3A4 assay kits (V8751, V8321, V8791, V9001; Promega,)
Williams' Medium E (Thermo Fisher Scientific, cat. no. 12551032)
Luciferin-CEE, Luciferin-2B6, Luciferin-H, and Luciferin-IPA (P450-Glo assay kit, Promega)
Luciferin detection reagent
Fetal bovine serum (FBS; GE Healthcare Life Sciences)
Fluorescent-conjugated antibodies
Mayer's hematoxylin (Sigma, cat. no. MHS1-100ML)

35-mm dishes
4- or 8-well plates, optional
Light microscope
Vector NTI (version 11.5; Invitrogen)
Mix3005P qPCR system (Agilent Technologies)
Collagen type IV-coated 6-cm dish (IWAKI)
96-well opaque white luminometer plate (Nunc)
Spectrofluorometer
24-well plates

Periodic Acid-Schiff staining of glycogen (PAS assay)

1. Culture 2×10^5 hepatocyte-like cells (HLCs) or MSCs for negative control on 35-mm dish for 3 days before performing the PAS assay
Seed HLCs and MSCs in 4- or 8-well chamber slides instead of a 35-mm dish if you need to stain cell samples in the Coplin jar.
2. Discard the culture medium and fix the adherent cells in 1 ml of 95% ethanol/5% formaldehyde for 1 min, and then rinse the cells under running tap water for 1 min.
3. Immerse the cells in periodic acid solution for 5 min, rinse the sample three times, each time with 1 ml dH₂O, immerse the sample in Schiff's reagent for 15 min, and rinse with running tap water for 5 to 10 min.
4. Counterstain the samples with 0.5 ml Mayer's hematoxylin for 1 min, rinse the samples with water, and examine the samples under light microscope.

Detection of hepatocyte-selective gene expressions

5. Extract the total RNA from iPSCs, HLCs, or HepaRG using illustra RNAspin Mini RNA Isolation Kit following the manufacturer's instructions.

6. Immediately, construct cDNA from the total RNA using the ImProm-II reverse transcription system following the manufacturer's instructions.

The total mRNA can be stored for several weeks at -70°C . Immediately, converting mRNA to cDNA is highly recommend because mRNA is not stable at room temperature.

7. The hepatocyte markers and cytochrome P450 markers are albumin, α -fetoprotein, cytokeratin 18, G-6-PD, HNF-4 α , tyrosine aminotransferase and major 7 CYPs isozymes. All gene-specific primers including a house keeping gene (GAPDH) are designed using vector NTI version 11.5 (Invitrogen). All primers are listed (Table 4A.13.2).
8. Amplify the specific genes using KAPA SYBR FAST qPCR Kits. In each reaction, mix 50 ng of cDNA with 15 μl of the master mix of the KAPA SYBR FAST qPCR kits and 5 μM of a pair of specific primers. Amplify the target DNA with a Mx3005P qPCR System using the following temperature profile: first step: 95°C for 3 min; second step: 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and final step: melting curve analysis. Confirm PCR amplicons using size and melting curve analysis.

Agarose gel electrophoresis is needed to check the authenticity of the PCR products. If the researcher use SYBR green master mix from others suppliers, one should run no template control (NTC) to optimize the PCR condition.

9. Calculate gene expression levels using the $\Delta\Delta\text{Ct}$ method and normalize to the endogenous GAPDH expression. Transform the $\Delta\Delta\text{Ct}$ into fold change using the formula: fold change = $2^{-\Delta\Delta\text{Ct}}$.

The detection of CYP450 activities

10. Culture HLCs, HepaRG, and primary human hepatocyte on the collagen type IV-coated 6-cm dish at a density of 10^6 cells/well.
11. Incubate the culture cells with a cocktail of 20 μM rifampicin, 50 μM omeprazole, 1 mM phenobarbital, and 88 μM ethanol. Incubate all treated cells for 48 hr with daily medium change.
12. Measure CYP1A1, CYP2B6, CYP2C9, and CYP3A4 enzyme activities in all cultured cells using the P450-glo 1A1, 2B6, 2C9, and 3A4 assays (V8751, V8321, V8791, V9001). After a 2-day incubation period, incubate the cells with Williams' Medium E supplemented with 100 μM Luciferin-CEE, Luciferin-2B6, Luciferin-H for 3 to 4 hr or 3 μM Luciferin-IPA for 30 to 60 min.
13. Transfer 50 μl of the medium to 96-well opaque white luminometer plate. Add the luciferin detection reagent to each well, incubate at room temperature for 20 min, measured with a spectrofluorometer.
14. The relative luminescence unit (RLU) could be calculated as follows: $\text{RLU} = (\text{LU treated-LU blank})/(\text{LU untreated-LU blank})$.

The detection of cellular hepatitis C receptors on HLCs

15. Monitor cellular receptors essential for HCV infection such as Claudin-1, Occludin, SR-BI, and CD81 using quantitative RT-PCR and immunofluorescent technique. Seed mature HLCs on a 24-well plate and maintain until reaching 80% confluency in Williams' Medium E, 10% FBS.
16. Fix and stain HLCs with fluorescent-conjugated antibodies raised against Claudin-1, Occludin, SR-BI, or CD81 as described in the section "Detection of pluripotent markers using immunofluorescent staining" (Fig. 4A.13.3B).

Table 4A.13.2 Primer Sets and Conditions Used in Quantitative Real-Time PCR (qPCR)

Gene	Genbank Accession	Sense primer '5' → 3' (Tm°C)	Antisense primer 3' → 5' (Tm°C)	Amplicon size (bp)	Annealing temp. (°C)	Putative function
ALB	NM_000477	TGAGAAAACGCCA GTAAGTGAC (56.5)	TGCGAAATCATCC ATAACAGC (54.7)	265	60	Albumin
AFP	NM_001134	GCTTGGTGGTGG ATGAAACA (57.2)	TCCTCTGTTATTT GTGGCTTTTG (54.6)	157	60	α-fetoprotein
CK18	X12881	GAGATCGAGGC TCTCAAGGA (57.9)	CAAGCTGGCC TTCAGATTC (55.8)	357	60	Cytokeration 18
G6PD	U01120	GCTGGAGTCCTGTC AGGCATTGC (58.1)	TAGAGCTGAGGCG GAATGGGAG (63.1)	349	60	Glucose-6-phosphate dehydrogenase
HNF-4α	AY680696	GCCTACCTCAAA GCCATCAT (56.4)	GACCCTCCCAG CAGCATCTC (62.9)	256	60	Hepatocyte nuclear factor 4α
TAT	NM_000353	TGAGCAGTCTGTCC ACTGCCT (62.3)	ATGTGAATGAGGAGG ATCTGAG (54.9)	338	60	Tyrosine aminotransferase
CYP2B6	NM_000767	ATGGGGCACTG AAAAAGACTGA (58.0)	AGAGGCGGGGA CACTGAATGAC (63.5)	283	60	CYP2B6
CYP2D6	NM_000106	CTAAGGGAACG ACACTCATCAC (56.6)	GTCACCAGGAAAG CAAAGACAC (58.1)	289	60	CYP2D6
CYP2C9	NM_000771	CCTCTGGGGCA TTATCCATC (57.1)	ATATTTGCACAGT GAAACATAGGA (52.9)	137	60	CYP2C9
CYP2C19	NM_000769	TTCATGCCT TTCTCAGCAGG (56.8)	ACAGATAGTG AAATTTGGAC (47.9)	277	60	CYP2C19
CYP3A4	AK298451	GCCTGGTGCT CCTCTATCTA (57.6)	GGCTGTTGACC ATCATAAAAGC (56.0)	187	60	CYP3A4
CYP1A2	AF182274	ACCCAGCTG CCCTACTTG (61.8)	GCGTTGTGT CCCTGTGTTG (57.4)	101	60	CYP1A2
CYP2E1	NM_000773	ACCTGCCCATG AAGCAACC (62.8)	GAAACAACCTCC ATGCGAGCC (58.9)	246	60	CYP2E1
UGT1A1	BC128414	GGAGCAAAAGG CGCCATGGC (65.6)	GTCCCTCTGC TGCAGCTGC (66.6)	178	60	Uridine diphosphate glucuronyltransferase 1A1
OATP2	AJ132573	GCCCACGC GTCCGACT (63.8)	ACAGAGCTGCCAA GAACATCT (57.6)	277	60	Organic anion transporting polypeptide 2
Claudin-1	NM_021101	GTGGAGGATTTA CTCCTATGCCG (59.1)	ATCAAGGCAC GGGTTGCTT (59.1)	165	60	Claudin-1
Occludin	NM_001205255	ACAGGCCTGA TGAATTGCCA (58.5)	GTGAAGGCACG TCCTGTGT (59.3)	218	60	Occludin
SR-B1	NM_005505	TGCACTATGCCC AGTACGTC (58.7)	TAGGCCTGAAT GGCCTCCTT (60.3)	148	60	Scavenger receptor class B type I
CD81	NM_004356	ACCTCCTGTATCTG GAGCTGG (60.0)	TTGGCGATCTGGT CCTTGTTG (59.4)	235	60	Cluster of Differentiation 81
ApoE	XM_005258867	CGCTTTTGGA TTACCTGCG (58.8)	GGGGTCAGTTG TTCCTCCAG (59.8)	158	60	Apolipoprotein E
miR-122	NR_029667.1	ACACTCCAGC TGGGTGGAGTGT GACAATCC (65.7)	TGGTGTCTGTGG AGTCG (48.5)	66	60	MiroRNA 122
SEC14L2	NM_012429	GGGATCCTTTA AGAGGCGGG (55.9)	GTCATCTGGA TTCGGCAGGG (55.9)	262	60	SEC14-like protein 2
GAPDH	NG_009349.4	GAAATCCCATCACC ATCTTCC (55.0)	AAATGAGCCCCAG CCTTCTC (59.6)	124	60	Glyceraldehyde-3-p dehydrogenase

**PRODUCTION OF HEPATITIS C VIRUS DERIVED FROM CELL CULTURE
(HCVcc), JFH-1**

The full-length genome of JFH-1 has been cloned into the pUC DNA vector under the transcription control of T7 promoter (Kato et al., 2001). The length of pJFH-1 is ~12.4 kb and the pUC DNA vector contains the ampicillin resistance gene. The pJFH-1 plasmid can be transformed and propagated in *E. coli* in medium with 100 µg/ml ampicillin. To propagate the HCVcc, purified pJFH-1 DNA is linearized at the end of HCV sequence with the restriction enzyme *Xba*I to synthesize the genomic JFH-1 RNA from in vitro transcription. The HCV JFH-1 RNA is then transfected into Huh7 cells or HLCs to allow HCV replication and virion production. The virus released into the medium can be titered with infectious virion as focus forming unit (ffu) per ml (Lindenbach et al., 2005).

Huh7 and Huh7-derived hepatocytes (e.g., Huh7.5 and Huh7.5.1) have been used to produce HCV infectious particles up to 10⁴ to 10⁶ ffu /ml after transfection with JFH-1 RNA or infection with HCVcc. Huh7 and their derivatives are hepatocellular carcinoma isolated from a Japanese male with well-differentiated hepatocellular carcinoma (Nakabayashi, Taketa, Miyano, Yamane, & Sato, 1982). HLCs derived from iPSCs can also host HCV production after transfection with JFH-1 RNA or infection with HCVcc and usually produce 10⁶ to 10⁷ ffu /ml. The advantages of HLCs as a natural host for HCV production/infection are their non-malignant origin and their capacity to produce higher HCV titer than those from classical Huh7-derived hepatocytes. In the following procedure, this unit describe the HCVcc propagation protocols using Huh7 cell and HLCs as hosts for viral production using liposome-based transfection.

Materials

- JFH-1 plasmid propagated in *E. coli*
- Luria broth (LB; see recipe)
- Ampicillin (Sigma, cat. no. A1593)
- NucleoBond Xtra Midi Kit (MN, cat. no. 740410.10)
- FastDigest *Xba*I (Thermo Fisher Scientific, cat. no. FD0684)
- Buffered phenol, pH 7 to 8
- 25:24:1 (v/v) phenol/chloroform/isoamyl alcohol
- Chloroform (Merck Millipore)
- 3 M sodium acetate, pH 7.0
- Isopropanol (Merck Millipore)
- UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, cat. no. 10977015)
- 75% (v/v) ethanol
- 100% (v/v) ethanol (Merck Millipore)
- TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, cat. no. K0441) containing:
 - 5× TranscriptAid Reaction Buffer
 - ATP/CTP/GTP/UTP mix
 - TranscriptAid enzyme mix
- Hepatocyte-like cell (HLCs)
- Huh7 cell
- DMEM/F12 (HyClone, cat. no. SH30261.02)
- Fetal bovine serum (FBS; GE Healthcare Life Sciences)
- Penicillin G sodium (Sigma, cat. no. P7794)
- Streptomycin (Sigma, cat. no. S6501)
- Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific, cat. no. 31985088)
- X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics)
- Sucrose (Sigma, cat. no. S0389)

Centrifuge
Pipettes
1.5-ml microcentrifuge tubes
Vortex mixer
Nanodrop spectrophotometer
200- μ l PCR tubes
10-cm cell culture dishes
Micropipettes
37°C, 5% CO₂ incubator
0.45- μ m sterile syringe filter (Sartorius)

Linearization of JFH-1 plasmid DNA

1. Extract the JFH-1 plasmid DNA from *E. coli* cultured in LB broth with 100 μ g/ml ampicillin overnight in 250 ml-Erlenmeyer flask with NucleoBond Xtra Midi Kit following the manufacturer's instructions.

The E. coli could not be cultured in LB medium more than 18 hr. The overgrowth of E. coli in orbit shaker can reduce the yield of plasmid DNA.

2. Digest the JFH-1 plasmid with *Xba*I restriction enzyme. For normal digestion, incubate 20 μ g pJFH-1 DNA overnight at 37°C. For fast digestion, incubate 20 μ g pJFH-1 plasmid for 1 to 2 hr at 37°C. The *Xba*I cuts the JFH-1 plasmid DNA at the 3' end of the HCV genome, permitting the ensuing in vitro transcription.

Linearized pJFH-1 DNA should be confirmed using agarose gel electrophoresis with the fragment size of 12.4 kb.

Purification of linearized plasmid JFH-1 template

3. Reconstitute the total volume of linearized DNA solution to 400 μ l using TE buffer, pH 7.5.
4. Add 500 μ l buffered phenol (pH 7 to 8) and mix the sample vigorously using a vortex mixer.

Phenol and chloroform are hazardous and require use of protective gloves and eyewear.
5. Centrifuge the sample for 10 min at maximum speed, room temperature.
6. Pipette the upper aqueous solution containing linearized JFH-1 DNA into a new 1.5-ml microcentrifuge tube, then add one equal volume of 25:24:1 (v/v) phenol/chloroform/isoamyl alcohol and mix the sample vigorously using a vortex mixer.
7. Centrifuge the sample again for 10 min at maximum speed, room temperature.
8. Pipette the upper aqueous solution containing linearized JFH-1 DNA into a new 1.5-ml microcentrifuge tube, add one equal volume of chloroform, and mix the sample vigorously using a vortex mixer.
9. Centrifuge the sample again for 10 min at maximum speed, room temperature.
10. Pipette the upper aqueous solution containing linearized JFH-1 DNA into a new 1.5-ml microcentrifuge tube, add 1/10 volume of 3 M sodium acetate, pH 7.0 to achieve a final concentration of 0.3 M sodium acetate (add 44 μ l of 3 M sodium acetate at pH 7.0 to 400 μ l sample), and mix the sample vigorously using a vortex mixer.
11. Add one volume of isopropanol to the microcentrifuge tube and mix the sample vigorously using a vortex mixer. The DNA sample could be precipitated using 2 volumes of ethanol.

Depending on sample volume and maximum tube capacity, one volume could also precipitate the DNA using 2 volumes of ethanol.

12. Incubate the plasmid solution for 1 hr at -20°C to allow plasmid DNA precipitation. To achieve complete precipitation, the overnight incubation is recommended for DNA less than 5 μg being digested.
13. Precipitate the DNA by centrifuging for 30 min at maximum speed, 4°C , remove the isopropanol, and wash the pellet with 500 μl of 75% ethanol.
14. Centrifuge the DNA sample for 5 min at maximum speed, room temperature. Discard 75% ethanol, wash the pellet with 500 μl of 100% ethanol, and centrifuge the DNA sample for 5 min at maximum speed, room temperature. Discard 100% ethanol and aspirate the leftover liquid that may present on the tube. Air dry the DNA pellet at room temperature.
15. Reconstitute the DNA pellet in nuclease-free water and measure DNA concentration using Nanodrop spectrophotometer. The reconstituted volume of nuclease-free water depends on the amount of DNA being digested.

Normally, one microgram per microliter is recommended. The DNA sample could be stored at -20°C for further use.

In vitro transcription of JFH-1 HCV DNA

16. Transcribe the JFH-1 mRNA from linearized plasmid using TranscriptAid T7 High Yield Transcription Kit following the manufacturer's instructions.
17. Combine the following reaction components at room temperature in the order as described: In a new 200- μl PCR tube, mix 4 μl 5 \times TranscriptAid Reaction Buffer, 8 μl ATP/CTP/GTP/UTP mix, 1 μg linearized JFH-1 DNA and 2 μl TranscriptAid enzyme mix. Adjust the total volume to 20 μl with DNase/RNase-free distilled water.
18. Mix the sample thoroughly by hand, spin down to collect all drops, and incubate for 2 hr at 37°C .
19. Purify the JFH-1 RNA using chloroform extraction and ethanol precipitation of RNA. Add 115 μl DNase/RNase-free distilled water and 15 μl of 3 M sodium acetate solution, pH 5.2, into the 20 μl solution from step 18 and mix thoroughly using a vortex mixer.

Purification methods include but are not limited to these methods. All methods have their particular benefits and all are convenient and effective ways to recover RNA. The authors usually employ phenol/chloroform extraction and isopropanol precipitation.

20. Extract the JFH-1 RNA with an equal volume of 1:1 phenol (pH 4.7)/chloroform mixture and then add another equal volume of chloroform. Collect the aqueous phase and transfer the solution into a new 1.5-ml tube.
21. Add 2 volumes of ethanol to the sample to precipitate JFH-1 RNA. Incubate at -20°C for at least 30 min and collect the pellet by centrifugation.
22. Discard the supernatant and rinse the pellet with 500 μl cold 70% ethanol and reconstitute the RNA with 20 μl of DNase/RNase-free distilled water.
23. Store the JFH-1 RNA at -20°C or -70°C for long-term storage up to 6 months.

Preparing hepatocytes to host HCVcc by transfection with JFH-1 RNA

24. At 2 days prior to transfection, seed 1.5×10^6 HLCs or Huh7 cells onto 10-cm cell culture dish, maintain the culture cells in 10 ml DMEM/F12, 10% FBS, with $1 \times$

streptomycin and penicillin. The optimum cell number is depending on the number of transfections to be performed. Each transfection will require 4×10^6 cells. On a 10-cm dish at 80% confluency, HLCs and Huh7 are approximately 5×10^6 cells.

25. On the transfection day, gently remove the culture medium from the 10-cm cell culture dish and replace with 10 ml DMEM, 10% FBS.
26. In a sterile 1.5 ml microcentrifuge tube, mix 15 μ g JFH-1 RNA with Opti-MEM medium and adjust the total volume to 500 μ l with Opti-MEM medium.
27. In another 1.5 ml microcentrifuge tube, add 440 μ l Opti-MEM to a tube and pipet 60 μ l X-tremeGENE HP DNA transfection reagent directly into the Opti-MEM.

Do not pipette X-tremeGENE HP DNA transfection reagent directly into microcentrifuge tube because the transfection efficiency will decrease.

28. Gently mix the plasmid solution from step 26 with the solution from step 27 by pipetting and incubating the transfection reagent/DNA complex for 15 min at room temperature. Add the solution condition (1 ml) onto the 10-cm culture dish drop by drop using a micropipette to cover the entire culture area.

Mix the solution condition again by moving the cell culture dish up-down and left-right to ensure that the plasmid solution covers the entire culture area.

29. Incubate the cell culture dish overnight (16 to 18 hr) in a 37°C, 5% CO₂ incubator.
30. Approximately 24 hr after transfection, replace the cell culture medium with 10 ml DMEM/F12, 10% FBS, 1 \times penicillin/streptomycin and incubate at 37°C, 5% CO₂.
31. Collect the conditioned medium every 3 days and keep at 4°C. Conditioned medium can be harvested within 2 weeks after transfection.
32. Clarify the conditioned medium containing HCVcc by centrifuging for 10 min at 500 \times g, 4°C, to remove cell debris, and filter the supernatant through a 0.45- μ m cellulose acetate filter.
33. Concentrate HCVcc to 5 \times using sucrose gradient ultracentrifugation. Briefly mix one volume 10% sucrose solution with 4 volumes conditioned medium, and then centrifuge for 2 hr at 100,000 \times g, 4°C. Discard the supernatant and collect the HCVcc pellet.
34. Transfer the reconstituted HCVcc into an individual 1.5-ml microcentrifuge tube for a single freeze/thaw cycle and store up to 1 year at -70°C for further use.

PRODUCTION OF HCVcc FROM INFECTIOUS VIRUS

HLCs and Huh7 cells can host HCVcc derived from JFH-1 RNA and produced infectious virions. Depending on host cells and HCV genotypes, maximal titers could be obtained on day 7 to day 12 from HCVcc-infected HLCs and Huh7 cells with an MOI of 0.01 ffu/cell. This protocol aims to produce HCVcc starting from the infectious virions, instead of from the classical transfection with JFH-1 RNA. The advantages of this infection protocol over the classical transfection protocol consist of: (1) the infection protocol is simplified; (2) the infection protocol spends less time and resources; and (3) the infection protocol allows higher titer of HCVcc. The protocol described in this unit has been optimized to generate infectious JFH-1 HCVcc from HLCs and Huh7 cells. Huh7 cells normally produce HCVcc with titers ranging from $10^4 - 10^6$ ffu/ml. HLCs produced higher titers with a range from $10^7 - 10^8$ ffu/ml. Huh7 cells were incapable for hosting HCV derived from patient serum (HCVser), but HLCs can readily host HCVser that lead to rising HCV titers to $10^4 - 10^6$ ffu/ml (Fig. 4A.13.4C-D).

**BASIC
PROTOCOL 9**

**Manipulation
of Potency**

4A.13.23

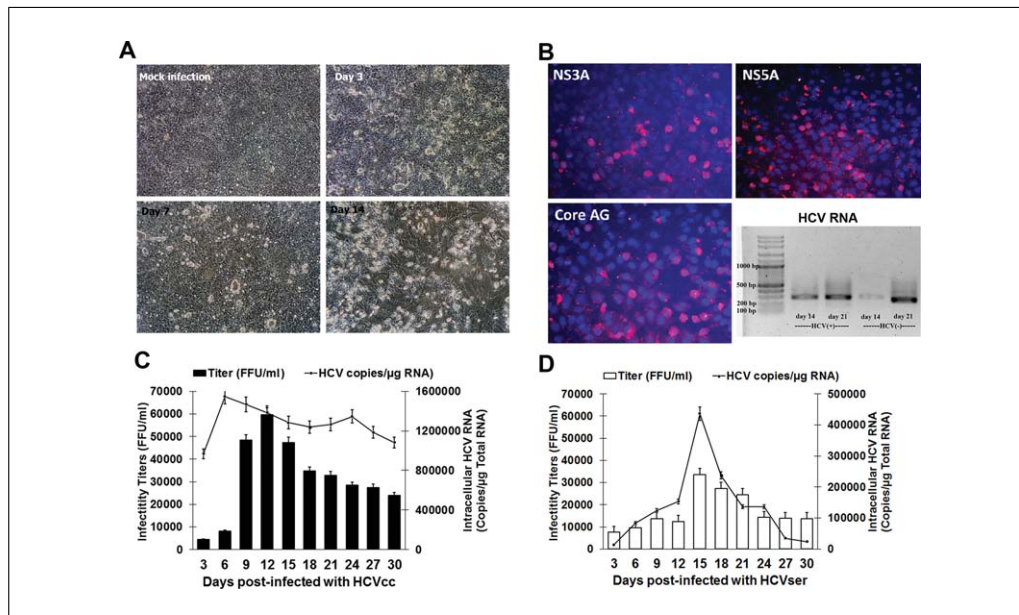


Figure 4A.13.4 The Infection of HLCs with HCV and the measurement of HCV titer. The CPE appeared in the cytoplasm of HLC after the infection with HCVser for 7 days (A). HCV proteins (NS3A, NS5A, and HCV core antigen) in infected HLCs were detected using immunofluorescent staining, while the positive and negative RNA strands were evaluated with RT-PCR (B). The infectivity titers of HCVcc (C) and HCVser (D) were measured as fluorescent focus forming units (FFU/ml), while the intracellular HCV RNA was determined by quantitative real-time PCR (RNA copies/μg of total RNA).

Materials

Hepatocyte-like cell (HLCs; see Basic Protocol 6)
 Huh7 cell line
 DMEM/F12 (HyClone, cat. no. SH30261.02)
 Fetal bovine serum (FBS; GE Healthcare Life Sciences)
 Penicillin G sodium (Sigma, cat. no. P7794)
 Streptomycin (Sigma, cat. no. S6501)
 Hepatitis C virus derived from cell culture with titer $> 10^5$ ffu/ml (JFH-1 HCVcc) (see Basic Protocol 8)
 Phosphate-buffered saline (DPBS) without calcium and magnesium
 Trypsin-EDTA, 0.05% w/v, 1 mM EDTA in PBS
 Sucrose (Sigma, cat. no. S0389)

10-cm cell culture dishes
 50-ml conical tubes
 0.45-μm sterile syringe filter (Sartorius)

1. Seed 1.5×10^6 Huh7 cells or HLCs on a 10-cm cell culture dish in 10 ml DMEM/F12, 10% FBS, $1 \times$ streptomycin and penicillin. The culture cell should reach 10% confluency the next day.
2. Discard the culture medium and infect with HCVcc at MOI of 0.01 ffu/cell in 10 ml of DMEM/F12, 10% FBS, $1 \times$ streptomycin and penicillin. Approximately 2×10^6 Huh7 cells or HLCs and 20,000 ffu of HCVcc are required for infection.
3. The infected cells should reach 90% confluency within 1 or 2 days post-infection. Collect the conditioned medium and transfer into a 50-ml conical tube, wash cell once with 5 ml PBS and trypsinize the cells using 0.05% trypsin-EDTA in PBS. Add 5 ml DMEM/F12, 10% FBS to inhibit the trypsin activity.

4. Reconstitute the trypsinized cells to a final volume of 10 ml with DMEM/F12, 10% FBS and transfer to the conical tube containing the conditioned medium. Adjust the volume to 50 ml by add 30 ml of DMEM/F12, 10% FBS into the conical tube.
5. Seed 2×10^7 infected Huh7 cells or HLCs in 10 ml of DMEM/F12, 10% FBS, $1 \times$ streptomycin and penicillin onto 10-cm cell culture dishes (10 ml/dish).
6. Observe the cytopathic effect (CPE) in the HLC and Huh7 cells. HCVcc-infected cells usually exhibit CPE on day 6 to 8 post-infection. Collect the conditioned medium daily and store at 4°C prior to ultracentrifuging. The maximum JFH-1 viral titer are usually achieved on day 7 to 12 post-infection.
7. After harvesting HCVcc, add 10 ml of DMEM/F12, 10% FBS, $1 \times$ streptomycin and penicillin to each dish and bring the culture cells to incubator.

Conditioned culture medium can be harvested every 3 days into conical centrifuge tubes. Virus-containing medium can be stored at -80°C for future determination of viral titers.
8. Clarify the conditioned medium containing HCVcc by centrifuging for 10 min at $500 \times g$, 4°C . To remove cell debris, filter the supernatant through a $0.45\text{-}\mu\text{m}$ cellulose acetate filter.
9. Concentrate HCVcc to $5 \times$ using sucrose gradient ultracentrifugation. Briefly, mix one volume 10% sucrose solution with 4 volumes conditioned medium, centrifuge for 2 hr at $100,000 \times g$, 4°C , discard the supernatant, and collect the HCVcc pellet.
10. Transfer the reconstituted HCVcc into several 1.5-ml microcentrifuge tubes to allow a single freeze/thaw cycle, and store up to 1 year at -70°C for further use.

HCVcc OR HCVser INFECTIVITY TITER ANALYSIS

Viral infection can be measured by several techniques. For cytolytic viruses such as coronavirus and herpes viruses, the most frequently used technique is plaque assay on cell culture in monolayers using inert overlay or non-inert overlay with methylcellulose or agarose (Cooper, 1961). Viral infectivity titers are calculated as plaque-forming units (pfu/ml) based on the number of plaques presented in the cell monolayer culture. However, HCVcc is a non-cytolytic virus and does not form plaques on HLC monolayers. The HCVcc could form a small foci of HCV protein (e.g., core antigen), which can be detected by immunofluorescent staining of 4% PFA fixed infected cells in monolayers. The number of positive cells is calculated for HCV infectivity titers as focus-forming units (ffu/ml). The protocol described in this unit uses immunofluorescent staining to determine the HCV core antigen in the HCV-infected cells (Zhong et al., 2005).

Materials

- Hepatocyte-like cell (HLCs; see Basic Protocol 6) (From iPSCs differentiation protocol)
- Huh7 cell line
- DMEM/F12 (HyClone, cat. no. SH30261.02)
- Fetal bovine serum (FBS; GE Healthcare Life Sciences)
- Penicillin G sodium (Sigma, cat. no. P7794)
- Streptomycin (Sigma, cat. no. S6501)
- Hepatitis C virus derived from cell culture with titer $> 10^5$ ffu/ml (JFH-1 HCVcc)
- Hepatitis C virus derived from patient serum with titer $> 10^5$ ffu/ml (HCVser)
- Ice
- Methylcellulose overlay (see recipe)

**BASIC
PROTOCOL 10**

**Manipulation
of Potency**

4A.13.25

Paraformaldehyde fixing solution (see recipe)
Phosphate-buffered saline (DPBS) without calcium and magnesium.
Triton X-100 (Sigma, cat. no. X100)
Bovine serum albumin (HyClone, GE Healthcare Life Sciences, cat. no. SH30574.02)
Normal goat serum (Thermo Fisher Scientific, cat. no. 31872)
Primary antibodies: HCV core antigen, NS3, NS5A, NS5B (Santa Cruz)/Tween-20 (Sigma, cat. no. P9416)
Goat anti-Mouse IgG / IgM (H+L) Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. no. A-10684)
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. no. A-11008)
Hoechst 33342 (Thermo Fisher Scientific, cat. no. H3570)
Fluorescence mounting medium (Dako, cat. no. S302380)
50% (v/v) glycerol
Williams' Medium E (Thermo Fisher Scientific, cat. no. 12551032)
 α -tocopherol (Merck Millipore, cat. no. 613420)
Sucrose (Sigma, cat. no. S0389)

8-well chamber slide (Thermo Fisher Scientific, cat no. 154534)
37°C water or dry bath
Pipettes
Fluorescence microscope
Parafilm
6-well plates
50-ml conical tubes
0.45- μ m sterile syringe filter (Sartorius)

HCV infectivity titer analysis

1. Seed 4×10^3 Huh7 cells or HLCs on each well of the 8-well chamber slide in 100 μ l DMEM/F12, 10% FBS with $1 \times$ penicillin and streptomycin.
2. Immediately thaw HCVcc or HCVser samples at 37°C in water or dry bath; keep the viral samples on ice.
3. Prepare a series of three to five 10-fold dilutions of each viral sample in chilled fresh cell culture medium (DMEM/F12, 10% FBS with $1 \times$ penicillin and streptomycin) in triplicate.
4. Discard the cell culture medium from the HLCs or Huh7 cells on 8-well chamber slide, pipette 50 μ l of each viral dilution into each individual well and incubate cells for 24 hr at 37°C in CO₂ incubator.
5. Overlay 150 μ l DMEM/F12, 10% FBS with $1 \times$ penicillin and streptomycin containing 0.25% methylcellulose on each well and incubate the cells for 24 hr at 37°C in CO₂ incubator.
6. Approximately 3 days post-infection, discard the culture medium, and add 300 μ l 4% PFA solution (pH 7) to each well.
7. Incubate for 15 min at room temperature in moist chamber, discard the fixing solution, and gently wash the cells three times, each time with 200 μ l PBS.
8. Incubate the infected cells with permeabilizing/blocking solution containing 0.2% Triton-X 100, 3% BSA, and 10% FBS in PBS for 30 min at room temperature.
9. Dilute primary antibodies (anti-HCV core antigen) in blocking solution (1:1000, incubate with infected cells overnight at 4°C, wash the colonies three times, each time with 200 μ l of 0.1% Tween-20 in PBS.

10. Dilute secondary antibodies in blocking solution (1:1000), incubate with the primary antibody-stained infected cells, and wash the cell samples three times, each time with 200 μ l of 0.1% Tween-20 in PBS. Counterstain the nucleus with Hoechst 33342 diluted in PBS at 1:1000 for 15 min at room temperature.
11. After extensive washing, adding 20 μ l of fluorescence mounting medium and overlay each well with 100 μ l of 50% (v/v) glycerol. Visualize the cell samples immediately with a fluorescence microscope, quantify the foci. Chamber slides can be stored for 1 month at 4°C. For extended storage, the authors recommend using parafilm M wrapping to minimize evaporation.
12. Calculate HCV titer as focus-forming units ffu/ml under a fluorescence microscope or from a photograph (Fig. 4A.13.4B). Select triplicate culture wells with an adequate number of foci to perform a reliable count.

The authors prefer to select wells with 10 to 50 distinct foci.

13. Using the average number of foci in the triplicate wells, calculate the titer in ffu/ml using the following formula:

$$\text{Titer (ffu/ml)} = \text{foci number} \times (1/\text{dilution factor of HCV}) \\ \times (1/\text{volume plated in ml})$$

For example, if an average of 30 foci were observed at 10^2 dilution after infection with 50 μ l of HCVcc, then the ffu/ml = 30 ffu \times (1/ 10^2) \times (1/0.05 ml) = 6×10^4 ffu/ml.

Infection of serum-derived HCV (HCVser) to hepatocyte-like cells

14. Collect the leftover HCV-positive sera from chronic HCV patient. The collection of leftover blood specimen should receive an approval from the IRB. Select the serum from HCV patient with HCV load $> 10^6$ IU/ml to infect HLCs.

Carrying infectious specimens should follow the NIH or institutional guidelines.

15. For HCVser infection, seed HLCs on a 6-well plate in Williams' Medium E, 10 % FBS until reaching 80% confluency.
16. Pretreat HLCs with 5 μ M α -tocopherol for 24 hr prior to incubation with HCVser. Add 50 μ l of patient serum in 2 ml culture medium.

Pretreated HLCs with α -tocopherol lowers lipid peroxidation from patient serum and enhances HCVser infectivity.

17. After 24 hr incubation, wash the HLCs five to six times, each time with 1 ml of 0.1% BSA in PBS, and reconstitute with 2 ml Williams' Medium E, 10 % FBS. Replace and collect conditioned medium twice a week for one month.

The maximum titers should be obtained on day 7 to 14 from HLCs infected with HCVser.

18. Observe the cytopathic effect (CPE) in HLCs. HCVser-infected cells usually exhibit CPE on day 7 to 9 post-infection. Syncytial cells would form within 2 weeks after infection (Fig. 4A.13.4A).
19. Pool the conditioned medium in a 50-ml conical centrifuge tube and filter through a 0.45- μ m filter to remove cell debris.
20. The HCVcc derived from HCVser can be concentrated using sucrose gradient centrifugation and stored up to 1 year at -70°C for further use.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipe (unless otherwise indicated).

Blocking solution (for immunofluorescent staining)

400 ml phosphate-buffered saline (PBS) without calcium and magnesium, prepare with deionized or double-distilled H₂O without Ca²⁺ and Mg²⁺)
15 g bovine serum albumin, Fraction V
2.5 ml 10% (v/v) Triton X-100 (Sigma, cat. no. X100)
50 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
Bring to 500 ml total volume with PBS (without CaCl₂ and MgCl₂)
Filter sterilize using a 0.2- μ m cellulose acetate filter
Store up to 1 year at 4°C

Final composition: 1× PBS/3% BSA/0.5% Triton X-100/10% FBS.

Endoderm commitment medium

1:1 DMEM/F12:IMDM
2 mg/ml PVA
2% (v/v) concentrated lipids
100 ng/ml activin A
100 ng/ml bFGF
10 ng/ml BMP-4
1× Insulin-Transferrin-Selenium
1 μ M LY294002
Store up to 1 week at 4°C

Endoderm differentiation basal medium (DMEM/F12: IMDM, 500 ml)

Dissolve 0.5 g Polyvinyl Alcohol (PVA; Sigma, cat. no. P8136, Sigma, MO) 0.5 g in 50 ml DMEM/12 in a 50-ml conical tube overnight at 4°. Mix the dissolved PVA with 200 ml DMEM-F12 (GE HyClone, cat. no. SH30261.02), 250 ml IMDM (HyClone, cat. no. SH30228.01), 5 ml concentrated lipids chemically defined (Thermo Fisher Scientific, cat. no. 11905031), 20 μ l thioglycerole, 1× Insulin-Transferrin-Selenium (Thermo Fisher Scientific, cat. no. 41400045), 5 ml penicillin/streptomycin 5 ml (penicillin, Sigma, cat. no. P7794; streptomycin, Sigma, cat. no. S6501). Store up to 1 month at 4°C.

To prepare 40 ml endoderm differentiation medium, Mix endoderm differentiation basal medium with 100 ng/ml activin, 10 ng/ml bFGF, 10 ng/ml BMP-4, 1 μ M LY294002 and 3 μ M CHIR99021 in 50-ml tube. Store up to 1 week at 4°C.

Gelatin, 0.1% (w/v)

Dissolve 0.5 g of gelatin (from porcine) in 500 ml deionized water and autoclave. Store up to 6 months at room temperature.

Gelatin coated-flasks/plates

Prior to addition of MEFs, coat all plates and flasks with enough 0.1% (w/v) gelatin solution to cover the surface. Remove gelatin after 5 min.

Hepatocyte basal medium (500 ml)

HBM Basal Medium (Lonza, cat. no. CC-3199) prepared according to the manufacturer's specifications including bullet kit. Medium should be warmed to 37°C prior to use. The precipitation could be found in the solution at 4°C. Store up to 1 month at 4°C.

hESC medium

DMEM/F12 containing:

20% (v/v) Knockout Serum Replacement (KOSR)
10 mM non-essential amino acids
2 mM L-glutamine or 1 × GlutaMAX
1 × penicillin/streptomycin (add from 200× stock)
50 mM of 2-mercaptoethanol
10 ng/ml bFGF
Store up to 1 week at 4°C

Luria broth (LB), 500 ml

5 g tryptone, 2.5 g yeast extract, 5 g NaCl and dissolve in 500 ml of deionized H₂O.
Autoclave using liquid cycle. Store up to 1 month at 4°C

MEF medium

DMEM containing:
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine or 1 × GlutaMAX
1 × penicillin/streptomycin (add from 200× stock)
Store up to 4 weeks at 4°C

Methylcellulose overlay, 0.25%

Add 1 volume 2% methylcellulose stock solution (see recipe) to 7 volumes of DMEM/F12, 10% FBS, with 1 × streptomycin and penicillin. Mix well and store up to 1 month at 4°C in the dark.

Methylcellulose overlay stock solution, 2%

Preparing by add 10 g of methylcellulose (Methocel MC, Fluka Bio-Chemika, cat. no. 64625) to 500 ml water in a sterile 1-liter bottle containing a stir bar. Stir at 4°C until methylcellulose partially goes into solution (~4 hr). Do not heat; methylcellulose will only dissolve in cold solutions. Sterilize by autoclaving. Allow solution to return to room temperature (methylcellulose will solidify), shake vigorously to break up clumps, and transfer to 4°C overnight. It will not be possible to stir the methylcellulose, but it will gradually go into solution. Store up to 6 months at 4°C.

MSC culture medium

MEM Alpha modification containing:
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine or 1 × GlutaMAX
1 × penicillin/streptomycin (add from 200× stock)
Store up to 4 weeks at 4°C

Paraformaldehyde (PFA) fixing solution, 4%

20 g paraformaldehyde (PFA), 400 ml phosphate-buffered saline (PBS) without calcium and magnesium. Adjust pH to 7.4 with 10 N NaOH (PFA will not well dissolve into solution until pH is 7.4). Aid dissolution by heating solution in fume hood (do not let solution rise above 65°C). Bring to 500 ml total volume with DPBS (without CaCl₂ and MgCl₂). Filter sterilize using a 0.22-μm filter, wrap in aluminum foil, and store up to 1 year at 4°C.

Recombinant human basic fibroblast growth factor (bFGF)

Reconstitute lyophilized bFGF to a final concentration of 10 μg/ml in PBS containing 0.1% (w/v) bovine serum albumin (BSA). Store at -80°C according to manufacturer's instructions. Store up to 1 year at -80°C.

RPMI-B27 differentiation medium (500 ml)

490 ml RPMI 1640

10 ml B27

5 ml NEAA

5 ml Penicillin/streptomycin

Medium should be warmed to 37°C prior to use.

Store up to 3 weeks at 4°C

Small molecules

Dissolve LY294002 and CHIR99021 in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM. Store up to 1 year at –80°C.

COMMENTARY

Background Information

Hepatitis C virus is a tropic positive-stranded RNA virus causing acute and chronic hepatitis that could develop into hepatocellular carcinoma (Chisari, 2005). HCV infection is the leading cause of life-threatening chronic liver disease and affects ~3% of the world population (Gondeau et al., 2014). Current *in vivo* model of HCV is limited to humans and chimpanzees owing to ethical concerns, economics, and the technical boundaries of *in vivo* experiment (Billerbeck, de Jong, Dorner, de la Fuente, & Ploss, 2013). Different *in vitro* models that support the entire HCV life cycle (entry, replication, assembly, release) and the host reacting innate immune response have been demonstrated (Steinmann & Pietschmann, 2013). However, our current knowledge of HCV life cycle is largely dependent on the molecular clone JFH-1 (HCV genotype 2a) that replicates in a few hepatocellular carcinoma cell lines with resulting infectious particles (HCVcc) production (Saeed et al., 2015).

Hepatocytes can host HCV infection and allow infectious viral production (Zhong et al., 2005). Since the discovery of HCV in 1989, a major burden impeding HCV study has been the lack of robust hepatocyte culture model to grow HCV derived from patient serum (HCVser) (Saeed et al., 2015). The significant improvement has been made with the isolation of a HCV genotype 2a from Japanese patient suffering with fulminant hepatitis (JFH-1). The JFH-1 HCV together with human hepatocellular carcinoma cell line, Huh7 or its derivatives brought a promising cell culture model to study the entire HCV life cycle (Wakita et al., 2005). The application of hepatocellular carcinoma as a natural HCV host could not completely mimic primary human hepatocyte. Cancer cells usually lack some specific factors necessary for the repli-

cation of HCV derived from patient serum. The newly identified factor, SEC14L2 promotes HCV infection by enhancing vitamin E-mediated protection against lipid peroxidation. The exogenous expression of SEC14L2 in Huh7.5 cell line allowed HCVser to replicate in hepatocellular carcinoma cell line (Colpitts & Baumert, 2015). Even though the lacking of essential host factor for HCVser infection was solved, the impairment of various liver functional enzymes such as CYP450s and other phase I, II, and III drug metabolizing enzymes make the hepatoma cells awkwardly unfit] for the assessment of anti-HCV drug interaction and metabolism (Blight, McKeating, Marcotrigiano, & Rice, 2003).

Human-induced pluripotent stem cell (iPSC) can be generated from the somatic cells using exogenous expression of the reprogramming factors (Oct4, Sox2, Klf4, and c-MYC). The iPSCs can differentiate into other cells, thus serve as a new model for drug discovery, disease modeling, and toxicity assessment (Greenbaum, 2010). Recently, several investigators reported that iPSC can develop into functional hepatocyte-like cell (HLCs) that exhibited various functions associated with mature hepatocytes (Roy-Chowdhury, Wang, Guha, & Roy-Chowdhury, 2016). The applications of HLC derived from pluripotent stem cell as a natural host for HCV have been reported (Yoshida et al., 2011). Differentiated HLCs exhibited important liver functions and achieved closely mature hepatocytes, including α -fetoprotein, albumin, phase I and phase II drug metabolizing enzymes. Importantly, HLCs also expressed known HCV host receptors involved in HCV entry (Claudin-1, Occludin, SR-BI, CD81) and supported entire life cycle of HCV genotype 2a up to 21 d (Wu et al., 2012). Nonetheless, the maturity, homogeneity and long-term stability of these HLCs have not been revealed. The HLCs not

only carried less CYP450 expression than that of the primary hepatocytes, but retained several fetal hepatocyte markers. Recently, we reported a new model of natural HCV infection using HLCs and identified the crucial factors SEC14L2, an α -tocopherol transfer protein required for HCVser infection. These HLCs reproducibly express overall hepatic functions comparable to the primary human hepatocyte (Sa-Ngiamsumtorn et al., 2016).

Critical Parameters and Troubleshooting

Isolated MSCs from human bone marrow

Collecting the bone marrow from a healthy donor could be performed by certified surgeons. All procedures should receive an approval from IRB. Immediately preserve the bone marrow in heparin solution to prevent blood coagulation. Do not shake the tube or disrupt the solvent interface between the Iso-prep solution and the diluted bone marrow to avoid low MSC yield.

Culture of human mesenchymal stem cell

During trypsinization, do not incubate MSC in trypsin/EDTA longer than 10 min. Over incubation compromises cell viability after cell passaging. The optimal split ratios are between 1:3 to 1:6. MSCs in the early passage could be cryopreserved in liquid nitrogen. Do not use MSC in the late-passage or in senescence stage (passage > 10) as a cell source for reprogramming. MSCs in late-passage usually have unstable karyotype.

Preparation of lentiviral vectors for reprogramming MSCs to iPSCs

All of the following steps in this unit should be performed in a class II tissue culture cabinet. Lentivirus requires the use of a Biosafety Level 2 facility. Pseudotyped lentiviruses packaged from HIV-1-based vectors are capable of infecting human cells. Handling with infectious virus should follow the institutional guidelines for biosafety. The following information is a brief description of Biosafety Level 2.

Summary of Biosafety Level 2

1. Practices:
 - Standard microbiological practices
 - Limited access to work area
 - Biohazard warning signs posted
 - Minimize production of aerosols
 - Decontaminate potentially infectious wastes before disposal
 - Use precautions with sharps (e.g., syringes, blades)

- Biosafety manual defining any needed waste decontamination or medical surveillance policy
2. Safety equipment:
 - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA filter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is uncirculated
 - PPE: protective laboratory coats, gloves, face protection as needed
 3. Facilities:
 - Autoclave available for waste decontamination
 - Chemical disinfectants available for spills

Lentiviral plasmids should be prepared with molecular biology grade reagents to avoid chemical contamination. Each plasmid should be verified with restriction map analysis.

Differentiation of human iPSC to homogeneous hepatocyte-like cell

After being passaged and split at a ratio of between 1:6 to 1:10 for 6 to 8 days, hPSCs will be 80% confluent and ready for passaging. The GSK-3 inhibitor (CHIR99021) is added only on the first day of differentiation. The endodermal differentiation takes 3 days. Replace the differentiation medium every 24 hr until the third day. Hepatic differentiation takes 4 days. Hepatic maturation can be monitored with the expression levels of HNF-4 α , AFP and albumin using quantitative real-time PCR. Particularly, HNF-4 α expression should be expressed in 90% of the cell population. Additional incubation with 1% DMSO in Williams' Medium E could increase CYP450s expression. Authors have also found that the quality of growth factors and reagents being purchased commercially can affect the efficiency of differentiation.

Production of hepatitis C virus derived from cell culture (HCVcc), JFH-1

The critical point for the production of infectious HCVcc using in vitro-transcribed HCV RNA was the quality and quantity of full-length HCV RNA genome. The JFH-1 expression plasmid vector contains a T7 promoter that drives the transcription of an approximate 9.6 kb RNA genome. However, the transcription should be optimized to perfect the synthesis of large HCV transcript. The authors recommend that the in vitro-transcription should be monitored with gel electrophoresis to ensure RNA quality. The incidence of a large

smear of RNAs in different sizes rather than a single band (with minimal smear of incomplete products) would indicate degradation or incomplete *in vitro* transcription. This could negatively affect intracellular HCV RNA replication after the transfection. The treatment with an RNase inhibitor and the optimization of incubation time during the transcription can improve the integrity of the HCV RNA genome. If longer RNA strand are detected, it might indicate that the plasmid DNA is not completely linearized and can interfere the transcription of the desired product. This problem can be resolved using either the linearized template DNA band prepared from agarose gel electrophoresis or another RNA template.

Hepatocyte-like cells and Huh7 cells

The most important factor in the generation of high-titer HCV stocks is the quality of host cells during production. Under ideal cellular conditions, Huh7 cell should produce JFH-1 HCVcc titers up to 10^5 ffu/ml, while HLCs could achieve the titers between 10^6 - 10^7 ffu/ml. However, mycoplasma contamination, non-optimized cell density, varied lots of serum and chemical reagents can negatively impact HCVcc titers. After treating mature HLCs with 1% DMSO, passaging HLCs is not possible because DMSO would induce growth arrest. These cells carry the hepatic markers for months and closely mimic the primary human hepatocyte.

Production of HCVser from patient sera

Only HLCs and Huh7.5 cells expressing SEC14L2 allow HCV production after being infected by patient serum (HCVser). The infectivity can be enhanced using 5-10 μ M α -tocopherol pre-treated host cells or adding α -tocopherol together with the serum during the infection process. The lipid oxidation in lipoprotein particles destroys the envelope of HCVser. This lipid peroxidation could be prevented using α -tocopherol. The titer of HCV particles derived from patient serum typically was 10^4 - 10^5 , and this was significantly inferior to transfected host cell with JFH-1 HCV RNA. The HCV particles should be stored at -70°C for long-term usage in a single use aliquot to avoid several freeze/thaw cycles.

HCV infection

To ensure the reproducibility and reliability of the future experiments, a single HCVcc stock sufficient to complete the entire experiment should be produced and the kinetics

of infection should be evaluated. The HCV stock and handling techniques should also be optimized.

Suboptimal viral titers

HCV is typically described as a noncytolytic virus. HCVcc and HCVser infected HLCs exhibit cytopathic effect (CPE) as identified by decreasing cell growth and round-shaped morphology with syncytial formation. The levels of CPE appeared differently among different HLCs, Huh7, and their derivative clones. The beginning of CPE frequently coincides with maximal viral titer. Therefore, collecting HCV particles during the initial onset of HCV-induced CPE is recommended. CPE usually appear in 2 weeks after the transfection with HCV RNA or infection with HCVser.

Anticipated Results

The series of protocols presented in this unit describe the production of functional hepatocyte-like cell as host cells for hepatitis C (HCV) studies. The procedure comprises three major stages: (1) the reprogramming of MSCs to iPSCs; (2) the differentiation of iPSCs to mature HLCs; and (3) the infection of HLCs with HCV. The minor protocols also describe the characterization and detection of iPSCs, HLCs, HCV host cell receptors and HCV titer including the production of HCV infectious particles. Successful reprogramming of MSCs to iPSCs establishes 2 - 3 stable iPSC lines and these cells can be further differentiated into hepatic and other lineages. The hepatic induction protocol can generate expandable immature HLCs from iPSCs. These cells can be readily induced to functional HLCs using 1% DMSO and the defined condition. The mature HLCs express all hepatic markers, albumin, AFP, CK18, TAT, major CYP450s enzyme, and HCV cell receptors. Following the differentiation protocol should generate 10^7 mature hepatocytes from 6×10^5 iPSCs on a 10-cm dish.

Production of HCV in HLCs cells using either transfection of *in vitro*-transcribed JFH-1 RNA or the infection of Huh7 cells with HCVser should yield titers between 10^4 - 10^7 ffu/ml. Maximal JFH-1 titers in HLCs should be achieved on day 18 post transfection or day 12 post infection. On day 3 post-infection, HLCs are fixed and stained with anti-HCV protein (HCV core antigen). Numerous positive foci could be detected in cytoplasm of clusters of 5 to 10 cells using immunofluorescent straining.

Time Considerations

The preparation of the primary culture of MSC from the aspirated bone marrow should take 2 weeks. During the 2nd-4th passage, MSCs which have been passaged one a week should be ready for reprogramming to iPSCs. It should take approximately 3 weeks before the transfection. The preparation of lentiviral plasmid from *E. coli* culture frequency takes 3 to 4 days to get purified plasmid DNA. The production of lentivirus carrying poly-cistronic OSKM takes one week starting from seeding HEK293T until the purification of the lentiviral particles. Reprogramming MSC to iPSC takes 2 to 3 months to achieve stable iPSC clones. Differentiating iPSCs to immature HLC takes 27 days and mature HLCs can be yielded after incubation with 1% DMSO for a week. The production of HCVcc from HCV RNA should take 2 to 3 weeks, starting from linearizing JFH-1 plasmid until concentrating HCVcc. The assessment of HCV titer would start at 2 to 3 weeks after infecting HLCs with HCVcc or HCVser.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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