Differentiation of Hepatocytes from Pluripotent Stem Cells

Sunil K. Mallanna[1] and Stephen A. Duncan[1]

[1]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin

ABSTRACT

Differentiation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells into hepatocyte-like cells provides a platform to study the molecular basis of human hepatocyte differentiation, to develop cell culture models of liver disease, and to potentially provide hepatocytes for treatment of end-stage liver disease. Additionally, hepatocyte-like cells generated from human pluripotent stem cells could serve as platforms for drug discovery, determination of pharmaceutical-induced hepatotoxicity, and evaluation of idiosyncratic drug-drug interactions. Here, we describe a step-wise protocol previously developed in our laboratory that facilitates the highly efficient and reproducible differentiation of human pluripotent stem cells into hepatocyte-like cells. Our protocol uses defined culture conditions and closely recapitulates key developmental events that are found to occur during hepatogenesis. *Curr. Protoc. Stem Cell Biol.* 26:1G.4.1-1G.4.13. © 2013 by John Wiley & Sons, Inc.

Keywords: hepatocytes • liver development • definitive endoderm • iPSC • hESC

INTRODUCTION

The liver plays a central role in regulating many physiological functions of the body including bile acid production, synthesis of blood coagulation and secreted serum factors, and lipid and carbohydrate metabolism (Si-Tayeb et al., 2010a). It is also the primary organ involved in the metabolism of xenobiotic substances and pharmaceuticals. Liver transplantation is the only definitive therapeutic option available to treat end-stage liver disease. However, the effectiveness of orthotopic liver transplantation is limited by an insufficient number of healthy donor livers and the need for chronic immunosuppression in transplant recipients. Hepatocyte-like cells derived from human pluripotent stem cells offer a future potential alternative to whole-organ transplantation to treat at least a subset of metabolic liver diseases (Thomson et al., 1998; Takahashi et al., 2007; Yu et al., 2007; Behbahan et al., 2011). Although several challenges must be met before human pluripotent stem cell–derived hepatocytes could be utilized for cell therapy, such cells offer immediate use in modeling inborn errors of hepatic metabolism, understanding the molecular basis of liver cell differentiation, and providing a model for drug discovery. This unit describes a protocol to efficiently generate cells with hepatocyte characteristics from human ES or iPS cells (Fig. 1G.4.1; Si-Tayeb et al., 2010b).

NOTE: All procedures using human pluripotent stem cells are to be performed using sterile materials and aseptic technique in a Class II biological safety cabinet and with prior approval from the local Institution Safety Board.

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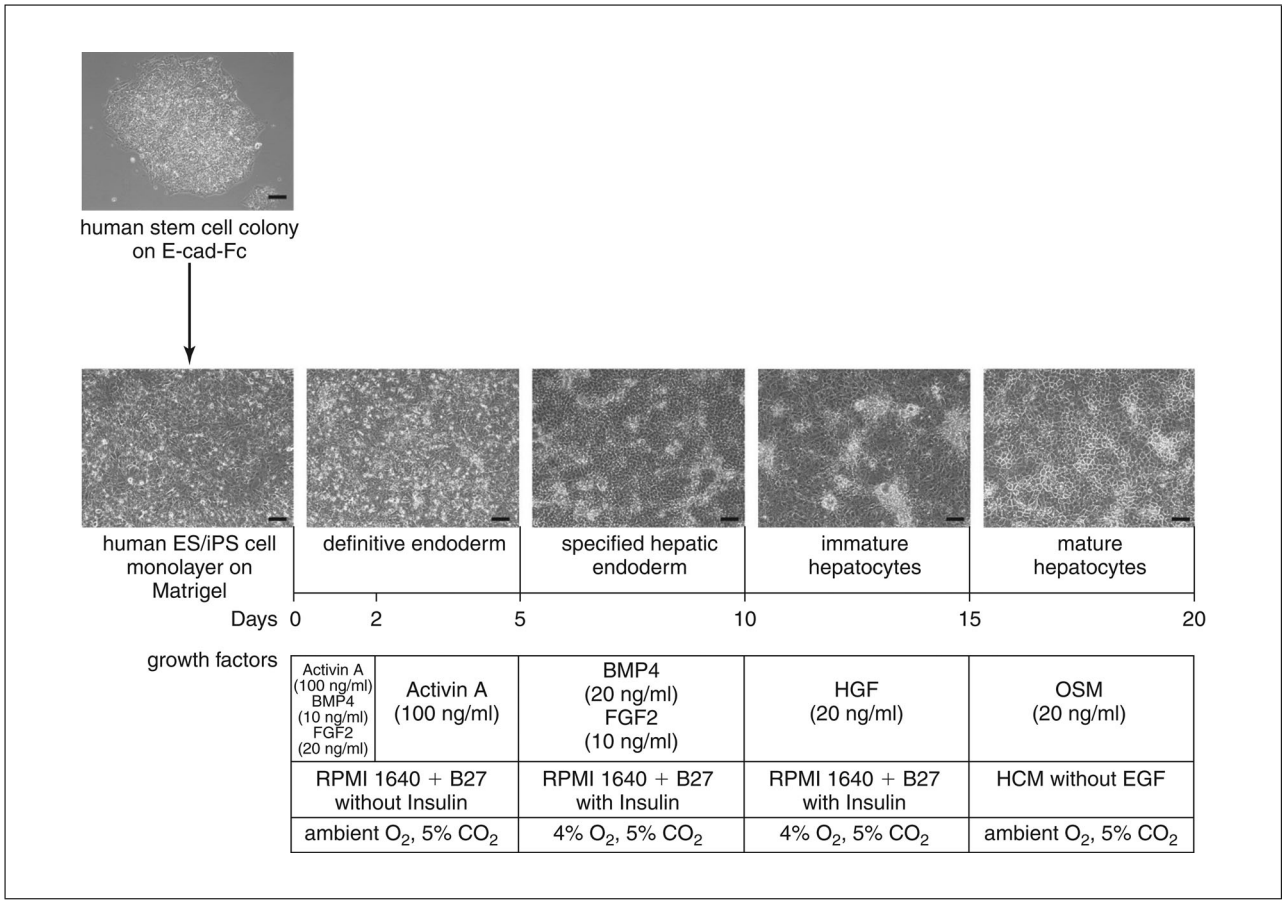

Figure 1G.4.1 Step-wise protocol for the differentiation of human pluripotent stem cells into hepatocyte-like cells. Phase-contrast micrographs of human pluripotent stem cell colony and four key cell types generated during the differentiation protocol (definitive endoderm, specified hepatic endoderm, immature hepatocytes, and mature hepatocytes) are shown. Growth factors used to sequentially generate the four major cells types during differentiation, and their concentrations, are given below respective photomicrographs. Scale bar = 100 μm.

BASIC PROTOCOL

DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TO HEPATOCYTE-LIKE CELLS

This protocol describes the differentiation of human pluripotent stem cells into cells that closely resemble hepatocytes by the sequential addition of growth factors. A successful differentiation should result in 70% to 90% of cells expressing proteins that are characteristic of hepatocytes including albumin and HNF4a.

Materials

Human pluripotent stem cells on either E-cad-FC or Matrigel-coated 100-mm tissue culture dishes (see Support Protocol 1)

Pluripotent stem cell medium: mTeSR1 [STEMCELL Technologies, cat. no. 5850; alternatively, mTeSR can prepared in the laboratory as described previously (Ludwig et al., 2006)] *or* MEF-conditioned stem cell culture medium (see recipe)

DPBS$^-$ (Ca^{++}/Mg^{++}-free DPBS; Invitrogen, cat. no. 14190-136)

DPBS$^-$ with 0.02% EDTA , pH 7.4

Accutase (STEMCELL Technologies, cat. no. 7920), optional

RPMI cell differentiation medium (see recipe)

B27 without insulin (Invitrogen, cat. no. 0050129SA)

Activin A (R&D systems, cat. no. 338-AC-010)

Bone morphogenetic protein 4 (BMP4; R&D, cat. no. 314BP)

Fibroblast growth factor 2 (FGF2; Invitrogen, cat. no. PHG0023)
B27 with insulin (Invitrogen, cat. no. 17504044)
Hepatocyte growth factor (HGF; Peprotech, cat. no. 100-39)
Clonetics hepatocyte culture medium (HCM Bullet kit; Lonza, cat. no. CC-3198)
Oncostatin M (R&D Systems, cat. no. 295-OM-010)

6-well sterile tissue culture plates coated with Matrigel (see Support Protocol 2)
37°C incubator

Plate cells for differentiation

1. Maintain human pluripotent stem cells on either E-cad-FC or Matrigel-coated 100-mm tissue culture dishes using pluripotent stem cell medium, as described in Support Protocol 1.

   Although culturing the pluripotent stem cells on Matrigel is compatible with the differentiation process, we find that E-cad-Fc improves both the efficiency and reproducibility of differentiation by ensuring a homogeneous population of pluripotent cells from which to initiate the process.

2. Examine the pluripotent stem cells under a microscope to ensure that the cell colonies cover no more than ~50% of the surface of the 100-mm tissue culture dish and exhibit minimal morphological evidence of differentiation.

   It is important to ensure that the starting pluripotent stem cells are of high quality and cultured under optimal conditions. Ideally, FACS analyses should reveal that >98% of the cells express the TRA-1-60 epitope.

3. Rinse the plate of pluripotent stem cells with 5 ml sterile $DPBS^-$, discard the rinse, and then incubate the cells for ~2 min with 3 ml $DPBS^-$/0.02% EDTA at room temperature. As soon as the cells begin to detach, remove the $DPBS^-$/0.02% EDTA and flood the plate with 6 ml pluripotent stem cell medium.

   If the pluripotent stem cells are cultured on Matrigel or are difficult to dissociate, they can be removed by incubating at room temperature with 3 ml Accutase.

4. Dissociate the cells into small clusters containing ~3 to 6 cells by pipetting, collect the cells by centrifuging for 5 min at 200 × *g*, room temperature, and then resuspend in pluripotent stem cell medium.

5. Transfer the cell suspension into an appropriate number of 35-mm wells of a 6-well tissue culture plate, which has previously been coated with Matrigel (Support Protocol 2). Culture the cells overnight at 37°C, 4%O_2/5% CO_2.

   The number of cells that produces an optimal differentiation varies from cell line to cell line and should be determined empirically; however, in many cases a 100-mm tissue culture dish whose surface is ~50% covered with stem cell colonies is sufficient to seed 2–3 × 35-mm wells. After overnight culture, the cells should form a monolayer that covers 80% to 100% of the surface of the dish (Fig. 1G.4.1). The cell density at the initiation of differentiation can have a dramatic impact on differentiation efficiency and may need to be determined empirically for each cell line. In addition, if the cells are left for greater than 24 hr before initiation of differentiation, it commonly has a negative impact on the efficiency of differentiation.

Induce differentiation of the cells

6. *Differentiation days 1 and 2:* Replace the culture medium with RPMI medium that has been prewarmed to 37°C supplemented with 2% B27 (without insulin), 100 ng/ml activin A, 10 ng/ml BMP4, and 20 ng/ml FGF2 and culture with daily medium changes for 2 days at 37°C in ambient O_2/5% CO_2.

   If insulin-free B27 is unavailable, 1 μM of the PI-3 kinase inhibitor LY 294002 can be added as an alternative.

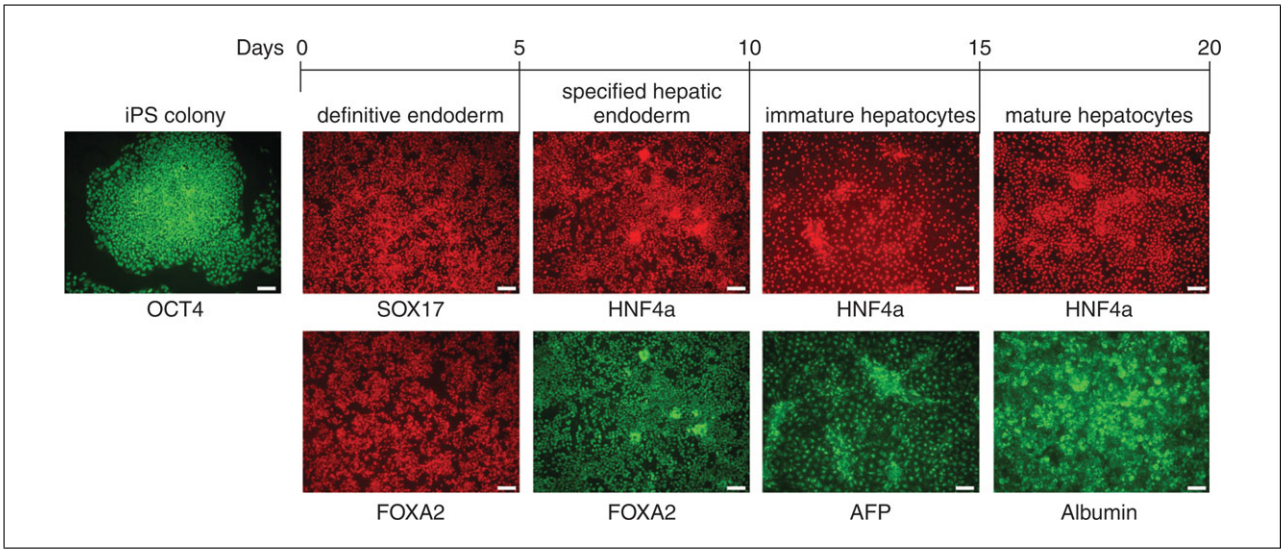

Figure 1G.4.2 Cells generated during the differentiation procedure express characteristic stage-specific markers. Immunocytochemistry was used to identify expression of SOX17 and FOXA2 in definitive endoderm cells, HNF4a and FOXA2 in specified hepatic endoderm cells, HNF4a and AFP in immature hepatocytes, and HNF4a and albumin in mature hepatocytes. OCT4 staining in a representative human iPS cell colony that was used for generating hepatocyte-like cells is shown. Scale bar = 100 μm.

7. *Differentiation days 3 to 5:* Change the culture medium to RPMI/2% B27 (without insulin) containing 100 ng/ml activin A and continue to culture with daily medium changes for an additional 3 days at 37°C, ambient O_2/5% CO_2.

   At the end of this stage of the differentiation, it is crucial that >90% of the cells express proteins that are characteristic of the anterior definitive endoderm including CXCR4, FOXA2, SOX17, and GATA4 (Fig. 1G.4.2). In addition, the presence of proteins associated with pluripotent cells, such as OCT4, should be minimal if detected at all.

8. *Differentiation days 6 to 10:* Induce hepatic differentiation by changing the medium to RPMI/2% B27 (with insulin) supplemented with 20 ng/ml BMP4 and 10 ng/ml FGF2 and continue to culture with daily medium changes for a total of 5 days at 37°C, 4%O_2/5%CO_2.

   After 5 days of culture, the cells should form a continuous monolayer (Fig. 1G.4.1) and 80% to 90% of the cells should express HNF4a (Fig. 1G.4.2) and the levels of GATA4 and SOX17 should have declined.

9. *Differentiation days 11 to 15:* Culture the hepatic progenitor cells for 5 days in RPMI/2% B27 (with insulin) supplemented with 20 ng/ml HGF with daily medium changes at 37°C, 4%O_2/5%CO_2.

   In addition to HNF4a, 80% to 90% of the cells should now express AFP (Fig. 1G.4.2) and lipid droplets are commonly observed within the cytoplasm of the cells. Finally, several mRNAs, which are enriched in fetal hepatocytes, can be detected including fibrinogen alpha chain (FGA), fibrinogen gamma chain (FGG), transferrin (TF), and angiotensinogen (AGT).

10. *Differentiation days 16 to 20:* Replace the medium with Clonetics hepatocyte culture medium (HCM) containing the supplied "singlequots," but omitting the EGF from the HCM "bullet" kit. Supplement the medium with 20 ng/ml Oncostatin-M. Continue to culture the cells for at least 5 days with daily medium changes at 37°C, ambient O_2/5% CO_2.

    By day 20 of the differentiation protocol, the cells should display a morphology that resembles primary hepatocytes with a distinct cuboidal morphology and a large cytoplasmic-to-nuclear ratio (Fig. 1G.4.1). In addition, 70% to 90% of the cells should express albumin

(Fig. 1G.4.2) and the asialoglycoprotein receptor (ASGPR), and the level of albumin secreted into the culture medium can approach >70% of that found for primary hepatocytes. While the cells exhibit many characteristics of adult hepatocytes, it is important to note that they also retain the expression of fetal markers such as AFP, suggesting that they are not fully mature. Moreover, expression of a subset of mature markers such as CYP 3A4 are significantly lower than found in fresh hepatocytes (Fig. 1G.4.3) (Si-Tayeb et al., 2010b; Yu et al., 2012).

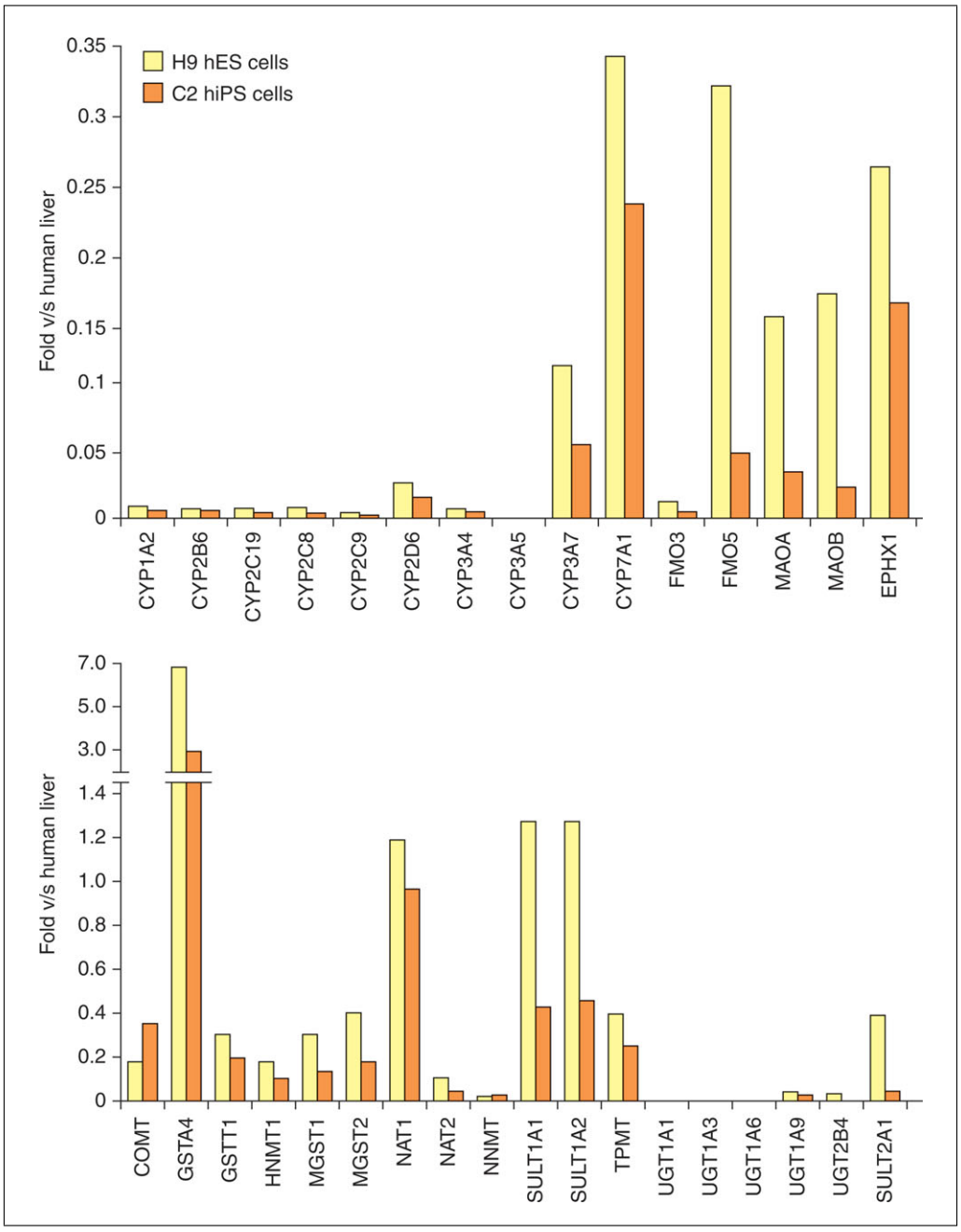

Figure 1G.4.3 Expression levels of phase I and phase II conjugating enzymes in hepatocyte-like cells differentiated from human pluripotent stem cells. The levels of mRNAs encoding phase I and phase II conjugating enzymes in hepatocyte-like cells differentiated from human H9 ES cells and C2 iPS cells was determined by real-time quantitative reverse-transcription polymerase chain reaction and are presented as fold of those found in human liver samples. Modified from Si-Tayeb et al. (2010b).

SUPPORT PROTOCOL 1

CULTURE OF HUMAN PLURIPOTENT STEM CELLS ON AN E-CAD-Fc SUBSTRATE

Although differentiation can be performed using cells cultured on Matrigel or other matrices, we find that the efficiency and reproducibility of differentiation is improved when the pluripotent cells are cultured for several passages on an E-cad-Fc matrix (Nagaoka et al., 2010). The matrix is coated onto nontreated polystyrene cell culture plates, and binding of the cells is facilitated by interactions between E-cadherin on the stem cell's surface and the matrix. E-cadherin is highly expressed on pluripotent stem cells, and expression is commonly repressed as cells differentiate. As a consequence, the E-cad-Fc matrix provides pluripotent stem cells with a selective growth advantage, which produces a relatively homogenous population of pluripotent cells that can be synchronously induced to differentiate. Although E-Cad-Fc matrix can be purified in the laboratory (Nagaoka and Duncan, 2012), a similar substrate called StemAdhere is available commercially.

Materials

StemAdhere (STEMCELL Technologies, cat. no. 7160) *or* E-cad-Fc (prepared as described in Nagaoka and Duncan, 2012)
DPBS^{+} (Ca^{++}/Mg^{++} containing DPBS; Invitrogen, cat. no. 14040-117)
Pluripotent stem cell medium: mTeSR1[(STEMCELL Technologies, cat. no. 5850; mTeSR can be prepared in the laboratory as described previously (Ludwig et al., 2006)], *or* MEF-conditioned stem cell culture medium (see recipe)
DPBS^{-} (Ca^{++}/Mg^{++}-free DPBS; Invitrogen, cat. no. 14190-136)
DPBS^{-}/0.02% EDTA

Sterile nontreated polystyrene cell culture plates (100-mm dishes, Corning, VWR, cat. no.25382-456; 60-mm dishes, Corning, VWR, cat. no. 25382-452; 6-well plates, CellStar BioExpress, T-3026-4; 24-well plates, BD Falcon, VWR, cat. no. 15705-060; 96-well plates, Corning, VWR, cat. no. 25381-056)
27-G needles
37°C incubator

Transfer cells to E-cad-Fc-coated dishes

1. Dilute E-cad-Fc in DPBS^{+} to 15 μg/ml and use the minimal volume necessary to coat suspension culture grade plates for 1 hr at 37°C, at which time the E-cad-Fc can be removed.

   It is essential that nontreated polystyrene cell culture plates be used, to allow the IgG-Fc region of the E-cad-Fc fusion protein to bind to the hydrophobic polystyrene surface. In addition, the hydrophobicity of the plates makes it necessary to vigorously agitate the plates to ensure even covering of the matrix. E-cad-Fc removed from the treated plate can be stored up to several weeks at 4°C and reused for up to three consecutive coatings. If using StemAdhere, follow the manufacturer's directions.

2. Before plating the pluripotent stem cells, remove the E-Cad-Fc substrate and optionally rinse the surface of the plate with 5 ml DPBS^{+}.

3. To initially transfer the pluripotent stem cells from colonies growing either on feeders or Matrigel, collect the cells in pluripotent stem cell medium by dissecting the colonies into small fragments using a 27-G needle and transfer 25 to 30 colony fragments to the E-Cad-Fc-coated plate.

4. Culture the cells in 5 ml pluripotent stem cell medium per 100-mm dish, with daily medium changes for 3 to 5 days at 37°C 4%O_2/5%CO_2.

   Expect that the freshly transferred fragments of colonies often take a few days to flatten out and that larger fragments seem to attach more efficiently than small fragments. It is also important to note that the morphology of the cells may seem different from cells grown

on MEFs or Matrigel in that the cells appear to be more loosely connected with a flatter morphology. This is normal for growth on the E-cad-Fc matrix and is not an indication that the cells are differentiating. Finally, some cell lines require a few consecutive passages to acclimatize to the E-cad-Fc matrix, during which time growth can be slower than expected.

Passage cells already established on E-cad-Fc coated dishes

5. Once the pluripotent stem cell colonies have been established on the E-cad-Fc matrix, they can be passaged using enzyme-free dissociation. Once the colonies reach no more than 50% confluence rinse the plate with 5 ml sterile $DPBS^-$ per 100-mm dish and then incubate with 3 ml $DPBS^-$/0.02% EDTA per 100-mm dish for up to 2 min. As soon as cells begin to detach, flood the dish with pluripotent stem cell medium and collect the cells by centrifuging for 5 min at 200 × *g*, room temperature.

6. Resuspend the cells in an appropriate volume of pluripotent stem cell medium and distribute into wells coated with E-cad-Fc matrix and incubate for 3 to 5 days at 37°C in 4%O_2/5%CO_2 with daily medium changes.

FACS or immunostaining should reveal that 95% to 98% of the cells express pluripotency markers including SSEA3, TRA 1-60, or OCT4. Expect to recover 1.0–2.5 × 10^6 cells from a sub-confluent 100-mm culture dish. The use of enzyme-free dissociation to recover the cells ensures high levels of viability and maintenance of cell surface markers.

COATING TISSUE CULTURE DISHES WITH MATRIGEL

SUPPORT PROTOCOL 2

Matrigel provides a basement membrane matrix for the culture of human pluripotent stem cells and it also provides an attachment surface for the differentiation of these cells to hepatocytes.

Materials

Dulbecco's modified Eagle medium (DMEM):nutrient mixture Ham's F-12 (1:1) (DMEM/F12; Invitrogen, cat. no. 11330)
Matrigel (Geltrex hESC-qualified reduced growth factor basement membrane matrix; Invitrogen, cat. no. A1413301)—diluted to 2 mg/ml in DMEM/F12 medium
Ice

Sterile tissue culture dishes (Corning Bioexpress):

100-mm dishes (cat. no. T-2877-100)
60-mm dishes (cat. no. T-2877-60)
6-well plates (cat. no. T-2989-6)
12-well plates (cat. no. T-2989-12)
24-well plates (cat. no. T-2989-24)
96-well plates (T-2989-96)
37°C incubator

1. Chill 10 ml DMEM/F12 on ice for 30 min.

2. Retrieve a 250 µl aliquot of Geltrex (2 mg/ml) from storage at −80°C and keep on ice.

3. Mix the frozen Geltrex with DMEM/F12 from step 1 and ensure it dissolves by gently tapping the tube over ice.

The matrix must be kept cold to prevent it from forming a gel prior to distribution on a tissue culture plate.

4. Add an appropriate volume (see Table 1G.4.1) of dissolved Geltrex to cover the surface of the tissue culture dish and leave the plates for 1 hr at 37°C.

Table 1G.4.1 Volumes Used for Plating Matrigel

Plates	Volume of Matrigel
100-mm tissue culture dish	4.0 ml per plate
60-mm tissue culture dish	2.0 ml per plate
6-well plate	1.5 ml per well
12-well plate	800 µl per well
24-well plate	400 µl per well
96-well plate	50 µl per well

5. Aspirate any extra liquid, replace with culture medium, and keep the plate in a tissue culture incubator at 37°C until needed.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

MEF-conditioned stem cell culture medium

Dulbecco's modified Eagle medium:nutrient mixture Ham's F-12 (1:1) containing:
20% KO serum replacement (Invitrogen, cat. no. 10828028)
1 mM L-glutamine (Millipore, cat. no. TMS-002-C)
0.1 mM 2-mercaptoethanol (Sigma, cat. no. M6250)
1% non-essential amino acids (Millipore, cat. no. TMS-001-C)
1× penicillin/streptomycin (add from 100×; Millipore, cat. no. TMS-AB2-C)

Add 10 ml medium to a confluent monolayer of mitotically inactivated mouse embryonic fibroblasts in a 100-mm tissue culture dish seeded at a density of 5–8 × 10^5 cell/cm^2. Incubate for 24 hr at 37°C, 4%O_2/5%CO_2 and then collect the medium over the course of 7 days. Conditioned medium should be used immediately or stored at −20°C. Before use, MEF-conditioned pluripotent stem cell culture medium should be supplemented with 10 ng/ml fibroblast growth factor 2 (Invitrogen, cat. no. PHG0023) or 40 ng/ml zbFGF prepared as described elsewhere (Ludwig et al., 2006).

RPMI cell differentiation medium

RPMI 1640, HEPES medium (Invitrogen, cat. no. 22400)
1% non-essential amino acids (Millipore, cat. no. TMS-001-C)
1× penicillin/streptomycin (add from 100× Millipore, cat. no. TMS-AB2-C)
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

One of the earliest descriptions of differentiating human pluripotent stem cells into the hepatic lineage involved the generation of embryoid bodies, which resulted in ∼6% of cells staining positive for albumin (Lavon et al., 2004). Culturing cells in mouse primary hepatocyte conditioned medium increased the number of albumin-positive cells suggesting that exogenous factors secreted by hepatocytes promoted the generation of hepatocyte-like cells from embryoid bodies. Subsequently, several protocols used growth factors with established roles during hepatogenesis to enhance the differentiation of embryoid bodies to hepatocyte-like cells (Schwartz et al., 2005; Duan et al., 2007; Basma et al., 2009; Woo et al., 2012). However, differentiation of

pluripotent cells to hepatocyte-like cells by embryoid body formation is a relatively inefficient approach that in general produces a heterogeneous mixture of cells, often requiring cell sorting to obtain pure populations. A significant advance in growth factor mediated differentiation of human ES cells was described by D'Amour and colleagues, who demonstrated that definitive endoderm could be efficiently induced by the addition of relatively high concentrations of activin A (D'Amour et al., 2005). Minor modifications have been made recently to improve the efficiency of activin A–mediated induction of definitive endoderm by inclusion of growth factors, such as Wnt3A, FGF2, HGF, or BMP4, by inhibiting the PI3Kinase signaling pathway, or by using small molecules to activate BMP/activin receptors (McLean et al., 2007; Hay et al., 2008; Borowiak et al., 2009; Touboul et al., 2010; Chen et al., 2012). Methods for inducing hepatic specification of definitive endoderm cells generated from human ES cells were based on work, primarily by the Zaret laboratory, which revealed that FGFs and BMPs are essential for hepatic specification in mice (Jung et al., 1999; Rossi et al., 2001; Cai et al., 2007). After specification of the hepatic progenitors, a number of different growth factors and approaches have been used to attempt to coax the cells to display functional characteristics that resemble native hepatocytes. The majority of protocols use HGF and OSM (Kamiya et al., 1999, 2001; Cai et al., 2007), but a number of alternatives and modifications have been described that may be more suitable for any given pluripotent stem cell line and this should be determined empirically (Behbahan et al., 2011).

Although arguments have been made that a specific set of conditions yields a higher quality of hepatocytes, quantitative comparisons to fresh hepatocytes unfortunately remain a rarity, and so it is difficult to compare the quality of hepatocytes generated using different protocols. Importantly, to date, no procedure has yielded hepatocytes that can functionally replace a mouse liver parenchyma, as has been demonstrated for fresh primary human hepatocytes (Azuma et al., 2007). This likely reflects the fact that hepatocyte-like cells produced from human pluripotent stem cells in culture retain some fetal characteristics, including the expression of fetal mRNAs such as AFP. In addition, a number of genes that are robustly expressed in adult hepatocytes are expressed at substantially diminished levels in pluripotent stem cell–derived hepatocyte-like cells (Si-Tayeb et al., 2010b; Yu et al., 2012). Such genes include those encoding a subset of cytochrome p450 enzymes, which have important roles in drug toxicity including Cyp3A4 and Cyp2D6. Efforts to improve the quality of hepatocytes generated from pluripotent stem cells are being pursued by many laboratories and include differentiation in three-dimensional cultures, matrix optimization, optimizing each differentiation step, and promoting maturation in vivo (Behbahan et al., 2011; Takayama et al., 2013).

Critical Parameters

The most significant variable that influences efficiency, homogeneity, and reproducibility of differentiation into hepatocyte-like cells is the quality of the starting population of pluripotent stem cells. Extra care should be given to ensuring that the pluripotent stem cells maintain the highest quality in culture. This requires passaging ES/iPS colonies at optimal intervals to ensure that colonies are not overgrown or prematurely passaged. It is important to monitor the growth rate of ES/iPS cell colonies. If the proliferation rate increases or cells undergo morphological changes, the karyotype of the cells should be determined or cells should be re-established from a low-passage frozen aliquot. We have found that passage on an E-cad-Fc matrix helps to maintain a homogeneous population of highly pluripotent cells. Nevertheless, it is important to ensure that >95% of cells express characteristic pluripotency markers before initiating the differentiation protocol. Although the protocol described here has been shown to be effective in inducing the differentiation of a broad repertoire of pluripotent stem cell lines, it is important to realize that different lines commonly exhibit unique characteristics, and empirical optimization of the protocol may be required for any given line.

We have also noted that the quality of growth factors and reagents that are purchased commercially can have a dramatic impact on the efficiency of differentiation. It is, therefore, important to note lot numbers and track when new lots of a given reagent are added to the protocol in order to troubleshoot. The B27 supplement in particular appears to show significant variation between lots. If problems are encountered, it may be worth considering using alternative supplements, such as NS21, which we have found to be a good substitute and can be produced in the laboratory from

Table 1G.4.2 Common Problems and Solutions

Problem	Possible cause	Solution
Non-uniform differentiation characterized by presence of heterogeneous mixture of cells at the end of the differentiation protocol	Poor quality of starting population of pluripotent cells	Routinely confirm pluripotent state of human pluripotent stem cell stock cultures by staining for markers OCT4, TRA-1-60
	Non-uniform plating of dissociated human pluripotent stem cell colonies onto Matrigel-coated plates	Make sure to uniformly disperse cells while plating cells on Matrigel-coated plates so that they form a monolayer
	Low cell density at the initiation of differentiation	Increase the cell density to achieve 80%-100% confluent monolayer at the beginning of differentiation. Cell density at the beginning of the protocol that yields uniform and reproducible differentiations may vary between different cell lines and should be determined empirically.
	Variation in the specific activity of growth factors between different lots	Keep track of lot numbers of all growth factors used in the differentiation protocol. It is not unusual to see variability in the specific activity of a growth factor between different lots. In such cases, check the specific activity of growth factors or obtain different lot.
	Variation in the surface characteristics of different tissue culture treated cell culture plates	If tissue culture plates fail to support differentiation the manufacturer or lot no. should be changed
Increased cell death observed after plating Accutase-dissociated human stem cell colonies resulting in sub-optimal cell confluence at the beginning of differentiation	Prolonged Accutase treatment	Determine the optimum duration of Accutase treatment necessary to dissociate human stem cell colonies. Duration of Accutase treatment may vary between different stem cell lines.
	Improper Matrigel coating of plates	Follow manufacturer guidelines carefully for optimal use of Matrigel and check the shelf life of Matrigel to make sure it has not expired
Spontaneous peeling of cell monolayer during hepatic specification of definitive endoderm cells	When it occurs, peeling is most commonly observed between days 6-8 of the differentiation protocol	Be very gentle while changing the medium. Also, medium changes should be carried out quickly in order to prevent drying of wells.
	Variation in the surface characteristics of tissue culture treated cell culture plates	Change the source of the plates

continued

Table 1G.4.2 continued

Problem	Possible cause	Solution
Low levels of albumin expression/failure to express albumin at the end of the differentiation protocol	Poor efficiency of differentiation that is inherent to a specific cell line	Varying the concentration of different growth factors or prolonging the incubation in hepatocyte maturation medium (HCM + OSM) after day 20 may improve maturation and albumin expression. If modifications to the protocol do not help improve albumin expression, changing the cell line is recommended.

published protocols (Chen et al., 2008). Finally, we have noted that tissue culture plastics from different sources can also impact the efficiency of differentiation and so it is worth avoiding changing manufacturers after the protocol is established.

Troubleshooting

For common problems and solutions, see Table 1G.4.2.

Anticipated Results

This protocol describes generation of hepatocyte-like cells from human ES/iPS cells by sequential addition of growth factors to recapitulate key developmental events functional during in vivo hepatogenesis. Successful completion of the protocol should result in differentiation of human ES/iPS cells into hepatocyte-like cells with expression of liver-enriched proteins including albumin and HNF4a in 70% to 90% of differentiated cells.

Time Considerations

Expansion of human ES/iPS cell colonies necessary to start a medium-scale differentiation usually takes 8 to 10 days. Protocol for generation of hepatocyte-like cells from pluripotent cells takes 21 days. Therefore, one month allows sufficient time to expand and differentiate human pluripotent stem cells into hepatocyte-like cells.

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Key Reference

Si-Tayeb et al., 2010b. See above.

The manuscript describes in detail the differentiation of human pluripotent stem cells into hepatocytes and formed the basis for the Basic Protocol.