Stem cell differentiation protocol

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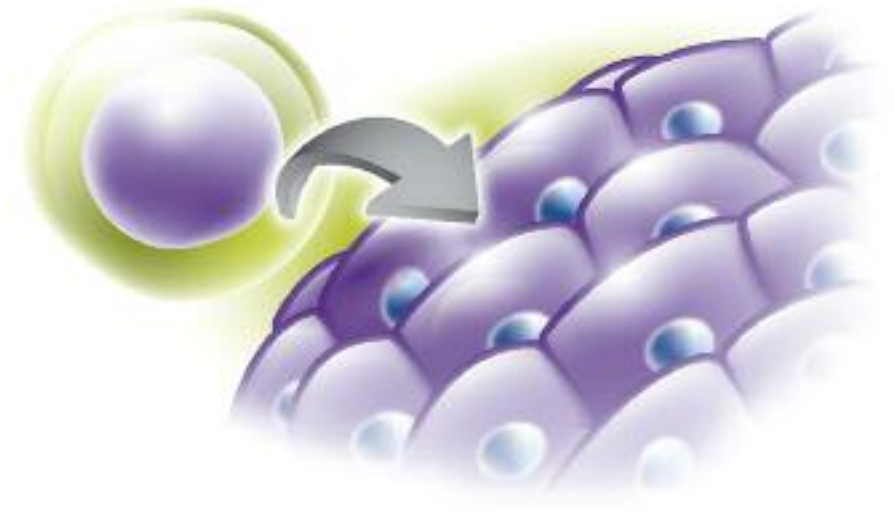
Human embryonic stem cells to hepatic endoderm



The ability to produce a homogenous population of functional cells in large quantities is one of the major challenges for stem cell biology. This is a robust 17 day differentiation protocol to direct human embryonic stem cells (hESCs) towards hepatocyte-like cells.

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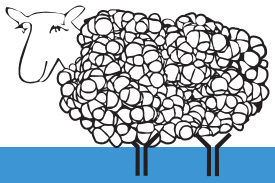
Taken from:

Robust Generation of Hepatocyte-like Cells from Human Embryonic Stem Cell Populations

Claire N. Medine, Baltasar Lucendo-Villarin, Wenli Zhou, Christopher C. West, David C. Hay. J. Vis. Exp. 2011 (56):e2969



Section 1

Initial preparation of all chemical stocks and coating of culture plasticware

All steps to be carried out in a tissue culture hood under aseptic conditions.

1. Preparation of human basic Fibroblast Growth Factor (hbFGF)

1.1. Prepare 10% BSA solution in PBS and filter through a 0.22 µm filter.
1.2. From the 10% BSA solution prepare a 0.2% BSA solution.
1.3. Add 10 ml 0.2% BSA solution/100 µg hbFGF.
1.4. Pre-wet a 0.22 µm filter by filtering 5 ml 10% BSA solution through the filter. Discard the 10 ml BSA wash.
1.5. Filter the hbFGF through the pre-washed filter.
1.6. Aliquot the hbFGF in sterile Eppendorf tubes and store at -20°C.

2. Preparation of Human Activin A Stock Solution

2.1. Add 1 ml of 0.2% BSA into a syringe and pre-wet the filter.
2.2. Dilute the Activin A in 0.2% BSA to a stock concentration of 100 µg/ml.
2.3. Filter the Activin A solution and aliquot in sterile Eppendorf tubes, and store at -20°C.

3. Preparation of Mouse Wnt3a Stock Solution

3.1. Add 200 µl of PBS to a 2 µg vial of Wnt3a to a stock concentration of 10 µg/ml.
3.2. Aliquot in sterile Eppendorf tubes and store at -20°C.

4. Preparation of human Hepatocyte Growth Factor (HGF) Stock Solution (1000X)

4.1. Dilute the HGF in PBS to a stock concentration of 10 µg/ml.
4.2. Filter the HGF solution and aliquot in sterile Eppendorf tubes, and store at -20°C.

5. Preparation of Oncostatin M (OSM) Stock Solution (1000X)

5.1. Dilute the OSM in PBS to a stock concentration of 20 µg/ml.
5.2. Filter the OSM solution and aliquot in sterile Eppendorf tubes, and store at -20°C.

6. Coating of culture plasticware with Matrigel

6.1. Thaw the 10 ml stock bottle of Matrigel overnight at 4°C on ice and then add 10 ml of KO-DMEM (Knock Out-DMEM). Mix well using chilled pipettes and store 1 ml aliquots at -20°C.
6.2. Thaw an aliquot of Matrigel at 4°C for at least 2 hours or overnight to avoid the formation of a gel.
6.3. Add 5 ml of cold KO-DMEM to the Matrigel, mix well with a pipette.
6.4. Make up to 15 ml with cold KO-DMEM and mix using a pipette.
6.5. Add Matrigel to the plate or flask to be coated (see table below)

Recommended volumes of Matrigel for coating typical plasticware for hESC culture:

Plate / Flask	Volume / Well or Flask
12 well plate	0.5 ml per well
6 well plate	1 ml per well
25cm^2 plate	2 ml per flask

6.6. Incubate the coated plate or flask overnight at 4°C or room temperature for 1 hour before use.
6.7. Plates or flasks which have been coated with Matrigel can be stored at 4˚C for up to 1 week. They should be clearly labelled with the date they were coated. Discard any plates or flasks not used within one week.
(a). Before use allow the coated culture container to come up to room temperature inside a tissue culture hood.
(b). Immediately prior to use aspirate the Matrigel and add the cell suspension to the well or flask.

Section 2

Preparation of media for differentiation of hESCs to hepatic endoderm

All media preparation should be carried out in a tissue culture hood under aseptic conditions.

1. Preparation of RPMI:B27 priming medium for endoderm differentiation

1.1. For RPMI-B27 medium, mix RPMI 1640 (500 ml) and B27 (50x, 10 ml).
1.2. Swirl to mix components.
1.3. Add all components to a filter unit and filter under vacuum, store at 4°C.

2. Preparation of SR-DMSO medium for hepatocyte differentiation

2.1. For SR-DMSO medium, mix 80% KO-DMEM, 20% Knock Out Serum Replacement medium (KO-SR), 0.5% L-glutamine, 1% non-essential amino acids, 0.1 mM β-Mercaptoethanol and 1% DMSO.
2.2. Filter the solution under vacuum, store at 4°C and aliquot and store at -20°C if required.
2.3. Use 4 ml per well of a 6-well plate, and 6 ml per T25 flask.

3. Preparation of L15 maturation medium for hepatocyte maturation

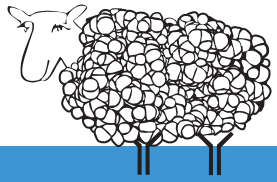
3.1. For L-15 medium, mix 500 ml Leibovitz L-15 medium, tryptose phosphate broth (final concentration 8.3%), heat inactivated foetal bovine serum (final concentration 8.3%), 10 µM hydrocortisone 21-hemisuccinate, 1 µM Insulin (bovine pancreas), 1% L-Glutamine, 0.2% ascorbic acid.
3.2. Filter the solution under vacuum, store at 4°C and aliquot and store at -20°C if required.

4. Preparation of final RPMI:B27 priming medium

4.1. Dispense the required volume of priming medium for the experiment (1 ml per well of a 6-well plate, and 2 ml per T25 flask).
4.2. Add Activin A to a final concentration of 100 ng/ml.
4.3. Add recombinant Wnt3a to a final concentration of 50 ng/ml.
4.4. Mix well and the media is now ready for use.
4.5. This final media should be made up fresh each day.

5. Preparation of final L-15 maturation medium

5.1. Dispense the required volume of L-15 medium for the experiment (4 ml per well of a 6-well plate, and 6 ml per T25 flask).
5.2. Add HGF to a final concentration of 10 ng/ml.
5.3. Add OSM to a final concentration of 20 ng/ml.
5.4. Mix well and the media is now ready for use.
5.5. This final media should be made up fresh each day.



Section 3
Priming hESCs to definitive endoderm

1. Culture hESCs (H1, H9 and RCM-1) and propagate on Matrigel coated plates with mouse embryonic fibroblast MEF-CM (mouse embryonic fibroblast culture media) supplemented with bFGF.

2. Initiate hepatic differentiation when hESCs reach a confluency level of approximately 30%-60% (depending on the hESC line) by replacing the MEF-CM with priming medium (RPMI 1640-B27 supplemented with 100 ng/ml Activin A and 50 ng/ml Wnt3a).

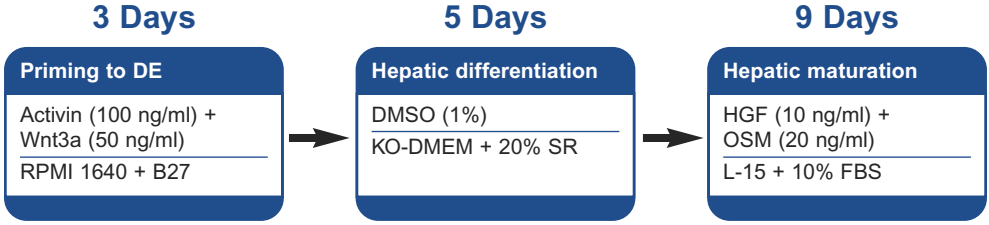
3. The cells are cultured in priming medium for 3 days (changing the medium every 24 hours), and final priming medium with Activin A and Wnt3a is made up fresh each day.

4. After 72 hours in priming medium, change the medium to the second differentiation medium (SR-DMSO) for 5 days (changing the medium every 48 hours).

5. At day 8, culture the cells in maturation and maintenance medium (L-15) supplemented with 10 ng/ml HGF and 20 ng/ml OSM for 9 days (changing medium every 48 hours). Maturation and maintenance medium with HGF and OSM is made up fresh each day.

6. The cells gradually exhibit morphological changes from a spiky/triangular shape to a characteristic liver morphology displaying a polygonal appearance.

7. Schematic for hepato-cellular differentiation:



Notes:

1. All priming, differentiation and maturation media are filtered under vacuum before use.
2. Priming, differentiation and maturation media are stored at 4˚C for no longer than 2 weeks. Assess how much medium is required for the experiment and aliquot the remaining media and store at -20˚C for future use.
3. Matrigel is made up as per the manufacturer's instructions; 1 ml aliquots can be stored at -20˚C until use.
4. Growth factors once made up and aliquoted can be stored at -20˚C and when thawed can be stored at 4˚C for no longer than 2 weeks.