Monolayer endoderm differentiation from human ESCs

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Introdution

When embryonic stem cells are differentiated in the presence of activin A in serum-free conditions, an endoderm progenitor population defined by the coexpression of either Brachyury, Foxa2 and c-Kit, or c-Kit and Cxcr4 is generated. Specification of these progenitors with bone morphogenetic protein-4 in combination with basic fibroblast growth factor and activin A results in the development of hepatic populations highly enriched (45–70%) for cells that express the alpha-fetoprotein and albumin proteins.

Protocol

1. Prepare ES cells on matrigel plate to deplete MEFs.
   a. Prepare 1:3 matrigel plate: cool down tissue culture plate and pipettes on ice; thaw 1:3 matrigel in fridge or on ice. Carefully apply matrigel onto plate to cover the bottom and then suck off excessive matrigel. Keep everything cool when plate matrigel. Put matrigel plates on ice for 30 min. Suck off excessive matrigel again and put matrigel-coated plates in 37°C incubator for 30 min.
   b. Treat confluent ES cells with TrypLE for 2–3 min at 37°C, use 4 ml TrypLE for 10 cm dish or 1 ml for 1 well of 6-well plate, and then suck out TrypLE. Wash with wash media (IMDM + Glu + P/S + 0.1% BSA) for 2–3 times. Add 4 ml hES media for 10 cm dish or 1ml for 1 well of 6-well plate, and scrape ES cells off the bottom of plate with cell scraper. Pipette up and down for 3–5 times and check under microscope until you get 5–10-cell clumps.
   c. Plate cells onto matrigel-coated plates. Cells will get ready for differentiation in 1 day @ 1:1 split; 2 days @ 1:2 split and 3 days @ 1:3 split.
2. Setup T0: Differentiation is performed at 5% O_2, 5% CO_2. Cells are ready to go when they are 70–80% confluent. Wash cells twice with wash media and apply appropriate volume of T0 media to cell, such as 10 ml for 10 cm dish, 2 ml for 1well in 6-well dish.
3. Wash cells with wash media and change media everyday until T5. It's normal to see huge cell death in the first several days. Detailed differentiation media recipe is attached in the next page.
4. To proceed to liver differentiation, dissociate T5 cells with Accutase @ 37°C for 3 min. Rinse with wash media twice. Add 1 ml wash media to each well of 6-well plate and scrape cells off the dish. Collect T5 cells,

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spin down @ 1200 rpm for 3 min. Resuspend cells in T5 media and seed onto 1:6 matrigel-coated 6-well plate @ 250K cells/well.

5. Feed cells every two days with different differentiation media (recipe below) and harvest between 18–25 days for analysis.

Reagents and preparation

Reagent	Supplier	catalog #
Matrigel	BD Biosciences	354230
DMEM/F12	Cellgro	10-092-CV
Knockout™ Serum Replacement (KOSR)	Invitrogen	10828-028
β-Mercaptoethanol	Sigma	M3148
L-Ascorbic Acid	Sigma	A4544-25G
Monothioglycerol	Sigma	M6145
TrypLE	Invitrogen	12604013
DNAse I Bovine Pancreas	VWR	80510-412
Hams F12 media	Invitrogen	10-080-CV
L-Glutamine	Invitrogen	25030-081
Penicillin/Streptomycin	Invitrogen	15070-063
N2 supplement	Invitrogen	17502048
B27 Supplement	Invitrogen	17504044
Non-Essentail Amino Acids	Invitrogen	11140-050
BSA	Sigma	A1470
γ-Secretase Inhibitor	Calbiochem	565771
Vitamin K (VK)	Sigma	M2518
Dexamethasone	Sigma	D4902
DMSO	Sigma	C6164
Chir 99021	Stemgent	04-0004

Cytokines	Supplier/Catalog #	Buffer	Stock Conc.
hBMP-4	(R&D Systems# 314-BP)	H_2O, 4 mM HCL, 0.1% BSA	10 ug/mL
hbFGF	(R&D Systems# 233-FB)	PBS, 0.1% BSA,1 mM DTT	10 ug/mL
hVEGF	(R&D Systems# 293-VE)	PBS, 0.1% BSA	5 ug/mL
ActivinA	(R&D Systems# 338-AC/CF)	PBS, 0.1% BSA	10 ug/ml
EGF	(R&D Systems# 236-EG)	PBS, 0.1% BSA	100 ug/ml
HGF	(R&D Systems# 294-HG)	PBS, 0.1% BSA	10 ug/ml
TGFα	(R&D Systems# 239-A-100)	PBS, 0.1% BSA	20 ug/ml
Oncostatin M (OSM)	(R&D Systems # 295-OM-010)	PBS, 0.1% BSA	100 ug/ml

L-ASCORBIC ACID (AA) *(SIGMA # A-4544)*

Prepare a stock solution of 5 mg/mL in cold TC-H_2O. Leave on ice and vortex periodically until completely dissolved. Filter sterilize, aliquot and store at −20°C. Use once and discard.

MONOTHIOGLYCEROL (MTG) *(SIGMA# M-6145)*

The amounts of MTG indicated in our protocols are recommended concentrations. However, it is important to test each new batch of MTG as there is variability between them. MTG should be aliquoted (1 mL) and stored frozen (−20°C). When aliquots are thawed, they can be used for several experiments and then discarded. Aliquoting of MTG is strongly recommended as it minimizes the amount of oxidation due to repeated opening.

∗MATRIGEL (REDUCED FACTOR) *(BD# 354230)*

Each batch of matrigel has its own unique levels of endotoxin and protein concentrations. We find that the endotoxin levels should not be higher than 2 endotoxin units/mL and the protein levels should range between 7 to 10 mg/mL. If the protein levels are higher than this you may need to dilute the matrigel more than 1:1. This is determined by observing the hESC colony morphology and the ability of the hESCs to differentiate into the lineage required of them.

Caution: When working with matrigel, all tubes, plates and pipettes should be pre-chilled, as matrigel solidifies at room temperature.

MATRIGEL1:1 PREPARATION

1. Thaw frozen bottles of matrigel on ice overnight in the cold room. We normally thaw 6×5-mL bottles per batch.
2. The next day, make a 50% working stock by adding an equal volume of IMDM+P/S to each bottle. Resuspend gently with a pre-chilled 5 mL pipette.
3. Leave the bottles on ice all day to allow the matrigel to completely equilibrate with IMDM.
4. Pool 3 bottles of 1:1 matrigel (30 mL) into a pre-chilled 50 mL tube. Gently mix with a chilled 10 mL pipette and aliquot.
5. Transfer 2.5 mL into pre-chilled and pre-labelled 4-mL snap cap tubes
6. Store aliquots at −20°C

hES Media (500 ml)

DMEM/F12: 400 ml
KOSR: 100 ml
NEAA: 5 ml
L-glutamine: 5 ml
Pen/strep: 5 ml
β-Mercaptoethanol: 3.5 ul
bFGF: 10 ng/ml (50 ul of 100 ug/ml stock)
L-Ascorbic Acid: 50 ug/ml –add fresh 5 mg/ml stock at each media change

Detailed differentiation media:

T0 Media			
Basic Media	RPMI		
Ingredient	Stock	Volume	Final
Glutamine	100x	10 ul/ml	1x
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Chir 99021 (Stemgent)	10 mM	0.2 ul/ml	2 uM
Activin A	100 ug/ml	1 ul/ml	100 ng/ml

T1 and T2 MEDIA			
Basic Media	RPMI		
Ingredient	Stock	Volume	Final
Glutamine	100x	10 ul/ml	1x
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
BMP4	10 ug/ml	0.025 ul/ml	0.25 ng/ml
bFGF	10 ug/ml	0.5 ul/ml	5 ng/ml
Activin A	100 ug/ml	1 ul/ml	100 ng/ml
VEGF	25 ug/ml	0.4 ul/ml	10 ng/ml

T3 and T4 MEDIA			
Basic Media	SFD		
Ingredient	Stock	Volume	Final
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
BMP4	10 ug/ml	0.025 ul/ml	0.25 ng/ml
bFGF	10 ug/ml	0.5 ul/ml	5 ng/ml
Activin A	100 ug/ml	1 ul/ml	100 ng/ml
VEGF	25 ug/ml	0.4 ul/ml	10 ng/ml

SFD: Recipe for 1 L

750 ml: homemade IMDM (Invitrogen 12200-069) (1×10 L) (+ Glutamine, + 25 mM HEPES+P/S)
250 ml: Hams F12 (Mediatech 10-080-CV)
5 ml: N2-supplement (Invitrogen 17502-048)
10 ml: B27 supplement w/o retinoic acid (Invitrogen 12587-010)
5 ml: 10% BSA in PBS (Sigma A1470, Cohn analog) (Lot tested)

Liver Differentiation Media:

DE T5 and T6 MEDIA			
Basic Media	SFD		
Ingredient	Stock	Volume	Final
Glutamine	100x	10 ul/ml	1x
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
BMP4	10 ug/ml	1 ul/ml	10 ng/ml
bFGF	10 ug/ml	0.5 ul/ml	5 ng/ml
Activin A	100 ug/ml	0.5 ul/ml	50 ng/ml
VEGF	10 ug/ml	1 ul/ml	10 ng/ml

DE T7-T12 MEDIA			
Basic Media	SFD		
Ingredient	Stock	Volume	Final
Glutamine	100x	10 ul/ml	1x
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
BMP4	100 ug/ml	0.5 ul/ml	50 ng/ml
bFGF	100 ug/ml	0.1 ul/ml	10 ng/ml
VEGF	25 ug/ml	0.4 ul/ml	10 ng/ml
EGF	50 ug/ml	0.2 ul/ml	10 ng/ml
TGFα	50 ug/ml	0.4 ul/ml	20 ng/ml
HGF	100 ug/ml	1 ul/ml	100 ng/ml
DEX	50 uM	2 ul/ml	100 nM

T13-T18			
Basic Media	SFD		
Ingredient	Stock	Volume	Final
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
bFGF	100 ug/ml	0.1 ul/ml	10 ng/ml
VEGF	25 ug/ml	0.4 ul/ml	10 ng/ml
EGF	50 ug/ml	0.2 ul/ml	10 ng/ml
HGF	100 ug/ml	1 ul/ml	100 ng/ml
OSM	100 ug/ml	0.2 ul/ml	20 ng/ml
DEX	50 uM	4 ul/ml	200 nM
VK	10 mg/ml	0.6 ul/ml	6 ug/ml
DMSO	dry	10 ul/ml	1%
gSI	1 mM	1.5 ul/ml	1.5 uM

Liver T19-T25			
Basic Media	SFD		
Ingredient	Stock	Volume	Final
MTG	13 ul/ml	3 ul/ml	4.5×10-4 M
Ascorbic Acid	5 mg/ml	10 ul/ml	50 ug/ml
HGF	100 ug/ml	1 ul/ml	100 ng/ml
OSM	100 ug/ml	0.2 ul/ml	20 ng/ml
DEX	50 uM	2 ul/ml	100 nM
VK	10 mg/ml	0.6 ul/ml	6 ug/ml