Mouse Thyroid Directed Differentiation Protocol – Kotton Laboratory

INTRO
This protocol describes the methods to derive, sort and plate primordial thyroid progenitors from mouse iPSCs/ESCs. As published in:

REAGENTS
A) cSFDM (complete serum free differentiation media):

<table>
<thead>
<tr>
<th>Volume for 500 ml</th>
<th>Final concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 ml IMDM</td>
<td>75%</td>
<td>Invitrogen 12440</td>
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<tr>
<td>125 ml Ham’s F12</td>
<td>25%</td>
<td>Cellgro 10-080-CV</td>
</tr>
<tr>
<td>5 ml B-27 (with RA) supplement</td>
<td>1%</td>
<td>Invitrogen 17504-44</td>
</tr>
<tr>
<td>2.5 ml N-2 supplement</td>
<td>0.5%</td>
<td>Invitrogen 17502-048</td>
</tr>
<tr>
<td>3.3 ml BSA (7.5% stock)</td>
<td>0.05%</td>
<td>Invitrogen 15260-037</td>
</tr>
<tr>
<td>1 ml Primocin (100 µg/ml stock)</td>
<td>200 ng/ml</td>
<td></td>
</tr>
<tr>
<td>5 ml Glutamax 100X</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>500 µl Ascorbic Acid (50 mg/ml stock)</td>
<td>50 µg/ml</td>
<td>Sigma A4544</td>
</tr>
<tr>
<td>1.5 ml MTG (from 26 µl in 2 ml IMDM)</td>
<td>4.5x10^-M</td>
<td>Sigma M6145</td>
</tr>
</tbody>
</table>

B) Growth factors and chemical inhibitors:
Activin A, R&D 338-AC
rnNoggin, R&D 1967-NG
SB431542, Sigma S4317
rhBMP4 R&D 314-BP
rhFGF2, R&D 233-FB
rhFGF10, R&D 345-FG
rmlIGF-1, R&D 791-MG-050
rhEGF, R&D 236-EG
bTSH, LA BioMed AFP8755B
ITS (Insulin-Transferrin-Selenium), ThermoFisher 41400045
Dexamethasone, Sigma D4902
Rho-associated kinase (ROCK) inhibitor, Tocris Y-27632
Heparin Sodium Salt, Sigma H4784, 0.05% Trypsin-EDTA (1X), gibco 25300-054
ES-qualified FBS, HyClone SH30070.03
DMEM (1X), gibco 11995-065

PROTOCOL
1) MEF depletion, LIF withdrawal, and embryoid body (EB) formation (Day 0 to Day 2.5)
   A. Trypsinize 1 well of a 6-well plate containing undifferentiated mESCs growing on a layer of MEFs in mESC media supplemented with LIF:
      1. Wash the well with PBS x1 and add 1ml of pre-warmed 0.05% trypsin for 10-30 sec (Tip: pipet to detach cells in 30 sec to decrease the trypsin exposure time.)
      2. Pipet a few times and get close to a single cell suspension.
3) Transfer the cell suspension to a 15ml conical tube containing 1ml ESC-qualified FBS and fill the tube with DMEM.
4) Centrifuge at 300g x 5 min.
5) Discard the supernatant and re-suspend the pellet in 10ml mESC media.
6) Transfer the suspension to a p100 tissue culture dish and incubate for 30-45 min; MEFs will attach to the dish while mESCs will mostly stay in the supernatant. Remove carefully the supernatant and transfer to a 15ml conical tube.
7) Collect the mESCs by centrifuging the supernatant at 300g x 5 min.
8) Discard the supernatant and re-suspend the cell pellet in 1ml of cSFDM.
9) Count the cells.

B. For starting the differentiation, add 5x10^5 cells to a p100 Petri dish containing 10ml of cSFDM. Carefully shake the plate to homogenize suspension and place in the incubator for 60h to allow embryoid body formation. (Optional: you may want to harvest a pellet of day 0 cells for RNA controls, and you may want to keep undifferentiated mESCs going in parallel for FACS or other negative controls.)

2) Definitive endoderm induction (Day 2.5 to Day 5):

A. Collect the EBs by transferring the content of the p100 Petri dish to a 15ml conical tube and either spin at 300g x 1 min or let the EBs settle by gravity for 3-4 min. The latter usually helps to get rid of debris sometimes observed at this stage. Discard the supernatant by careful aspiration.

B. Cells at this stage (EBs) are very sensitive to trypsin. Have cold 0.05% trypsin as well as cold ESC-qualified FBS ready (on ice). Add 1 ml of cold trypsin and re-suspend the EBs carefully but fast while keeping the conical tube on ice. Transfer to a water bath at 37°C for 1 min; shake the tube while in the water bath. Immediately, move the tube back on ice, pipet 2-3 times with a P1000 pipette, add 1ml of cold ESC-qualified FBS, and pipet 2-3 times to mix well. Working very fast at these steps is key to avoid toxicity due to overexposure to trypsin. Fill the conical tube with media (IMDM or cSFDM) to dilute the FBS and trypsin and centrifuge at 300g x 5 min; if cell/DNA clumps are observed due to overexposure to trypsin, use a cell strainer before centrifugation. Discard the supernatant, re-suspend cells in 1ml cSFDM and count them.

C. Transfer 0.5-1 x10^6 cells to a p100 Petri dish containing 10ml of cSFDM + Activin A at 50 ng/ml (1:200 dilution of a 10 µg/ml stock). Depending on the size of your experiment, you may elect to prepare more than one p100 Petri dishes at this stage. Shake the dish(es) to homogenize and place in the incubator for 60h.

3) Anterior foregut endoderm (Day 5 to Day 6):

A. Transfer the content of the p100 Petri dish(es) to a 15ml conical tube. Transfer 1-2ml to a separate tube to be used for evaluation of CKIT/CXCR4 co-expression to assess sufficient definitive endoderm induction (see CKIT/CXCR4 staining protocol).

B. Let the rest of the EBs settle or collect them by centrifugation at 300g x 1 min. Re-suspend the content of each conical tube in 10ml of “anteriorization media” composed of cSFDM + 100 ng/ml rmNoggin + 10 µM SB431542 (1:100 dilution of a 10 µg/ml mNoggin stock and 1:1000 dilution of a 10 mM SB431542 stock). Incubate for 24h in p100 Petri dish(es).

4) Nkx2-1 induction/specification (Day 6 to Day 14)

A. Prepare specification media composed of cSFDM supplemented with 10 ng/ml rhBMP4 (1:1000 dilution of a 10 µg/ml stock), 250 ng/ml rhFGF2 (1:1000 dilution of a 100 µg/ml stock) and 100 ng/ml heparin sodium salt (1:10,000 dilution of a 1 mg/ml stock). Supplementation of specification media with ROCK inhibitor (10 µM Y-27632) during the first 24 hours of specification, greatly increases survival after single cell suspension.

B. Cells on Day 6 can be plated on specification media either as EBs or as a single cell suspension. In either case, 6-well plates are coated with 0.1% gelatin for 20 min prior to seeding, as cells will adhere to the plate at this stage.

C. For plating cells as EBs, separate an aliquot of 1ml of the preparation assuring EBs are homogeneously re-suspended. This aliquot will serve to estimate the cell number in the EBs. Trypsinize the aliquot of EBs (using same steps as on Day 2.5) before counting. Using this count, plate EBs estimating 2x10^5 cells per well of a 6-well plate (approximately 2x10^4 cells/cm²).
D. For plating cells as single cell suspension, trypsinize the whole preparation of EBs (as described previously) and plate 1x10^5-2x10^5 cells per well of a 6-well plate.

E. For either EBs or single cell suspension, 2ml media per well of a 6-well plate is usually enough. Specification media is changed every 2 days initially and daily as the cells get more confluent. If media turns yellow in less than 24h by Day 14-15, the cells will survive poorly to the sort and could consider plating less cells per well on Day 6.

5) Sorting Nkx2-1+ thyroid progenitors and expansion in 3D Matrigel (Day 14 to Day 30)

A. Prepare FACS buffer:
   1. PBS or HBSS 1x
   2. 2% ESC-qualified FBS
   3. ROCK inhibitor (10 µM Y-27632)

B. Aspirate the old media (consider washing with serum-free media once prior to the next step).

C. Add 1ml warm 0.05% trypsin to each well at room temperature and start pipetting in 15-30 sec.

D. Pipet a few times to make a single cell suspension. Transfer the volume to a 15ml conical tube on ice containing 1ml cold ESC-qualified FBS (1ml ESC-qualified FBS for each well you are harvesting).

E. Centrifuge at 300g x 5 min.

F. Discard the supernatant and re-suspend in FACS buffer. Approximately 0.5ml per harvested well gives a cell density appropriate for cell sorting on the MoFlo.

G. Filter through a strainer (30-40µm) x 1.

H. Add Propidium Iodide (1:500 of 1mg/ml stock) or Calcein Blue (1:1000 dilution of 1mg/ml stock) to the FACS sample and transfer to polypropylene (white top) FACS tubes.

I. Prepare collection tubes with FACS buffer supplemented with ROCK inhibitor.

J. For 3D expansion, thaw Matrigel (Corning 356231) on ice at this time.

K. Sort Nkx2-1+/PI negative or Nkx2-1+/Calcein Blue+ cells.

L. Spin collected samples at 300g (optional 500g if difficult to recover) x 7min in either Eppendorf or 15ml conical tubes depending on the volume.

M. Discard the supernatant, leaving ~50µL to re-suspend cells by gentle tapping, and place Eppendorf or conical tube on ice.

N. For 3D expansion, re-suspend cells at 620 cells per µl of Matrigel using cold 200µl pipette tips. Take care to (a) avoid bubbles and (b) distribute the cells evenly in the Matrigel.

O. Slowly pipette the Matrigel + cells into the center of a 24-well plate (60-80 µl/drops, typically 70 µl).

P. Place the 24-well plate in the incubator for 20 min.

Q. Add “Differentiation I” media (cSFDM supplemented with 250 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100ng/ml Heparin Sodium Salt, 50 ng/ml mIGF-1, 10 µg/ml insulin, 25 ng/ml hEGF, 1 mM bTSH) for 8 days after sorting. For 48 hours, ROCK inhibitor 10µM (Y-27632) was included in the media.

R. From day 22-28, add “Differentiation II” media (cSFDM supplemented with 250 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100ng/ml Heparin Sodium Salt, 50 ng/ml mIGF-1, 5 µg/ml ITS (Insulin, transferrin, selenium), 25 ng/ml hEGF, 1 mM bTSH).

S. On days 28-30 (or longer), add ‘Thyroid Maturation’ media (Ham’s F12 supplemented with 15 mM HEPES, 0.8 mM CaCl2, 100 ng/ml Heparin Sodium Salt, 0.25% BSA, 50 ng/ml mIGF-1, 5 µg/ml ITS, 25 ng/ml hEGF, 50 nM Dexamethasone, and 1 mM bTSH).

T. Re-feed every two days with respective media.

**VERSION HISTORY**

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
<th>Author</th>
</tr>
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<tr>
<td>2017-04-26</td>
<td>Drafted by Maria Serra.</td>
<td>MS</td>
</tr>
<tr>
<td>2017-05-25</td>
<td>Edited/updated by Jinyoung Choi and transferred to CReM letterhead</td>
<td>JC</td>
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