Title	McEwen - Pancreatic differentiation
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Submitted by -	Sunita D'Souza
Adapted from -	Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells M. Cristina Nostro,[1] Farida Sarangi,[1] Shinichiro Ogawa,[1] Audrey Holtzinger,[1] Barbara Corneo,[2] Xueling Li,[3] Suzanne J. Micallef,[3] In-Hyun Park,[4] Christina Basford,[5] Michael B. Wheeler,[5] George Q. Daley,[6] Andrew G. Elefanty,[3] Edouard G. Stanley,[3] and Gordon Keller[1*] Development. 2011 March 1; 138(5): 861–871. doi: 10.1242/dev.055236 PMCID: PMC3035090
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Pancreatic – Insulin producing β cells protocol (adapted from Nostro et al., 2011)

INTRODUCTION

The generation of insulin-producing β-cells from human pluripotent stem cells is dependent on efficient endoderm induction and appropriate patterning and specification of this germ layer to a pancreatic fate. In this study, we elucidated the temporal requirements for TGFβ family members and canonical WNT signaling at these developmental stages and show that the duration of nodal/activin A signaling plays a pivotal role in establishing an appropriate definitive endoderm population for specification to the pancreatic lineage. WNT signaling was found to induce a posterior endoderm fate and at optimal concentrations enhanced the development of pancreatic lineage cells. Inhibition of the BMP signaling pathway at specific stages was essential for the generation of insulin-expressing cells and the extent of BMP inhibition required varied widely among the cell lines tested. Optimal stage-specific manipulation of these pathways resulted in a striking 250-fold increase in the levels of insulin expression and yielded populations containing up to 25% C-peptide+ cells.

FLOWCHART

REAGENT LIST

Reagents

	Reagent	Company	Catalogue #
1.	anti-CXCR4-phycoerythrin (1:100)	BD	551966
2.	anti-CD31-phycoerythrin (1:100)	BD	553373
3.	anti-CD117-allophycocyanin (1: 100)	Caltag	CD11705
4.	anti-KDR–allophycocyanin (1: 10)	R&D	FAB357A
5.	HPi3 (1:20)	Novus Biologicals	NBP1-18947
6.	HPα2 (1:20)	Novus Biologicals	NBP1-18950
7.	HPx1 (1:20)	Novus Biologicals	NBP1-18951
8.	HPd1 (1:20)	Novus Biologicals	NBP1-18953
9.	anti-mouse IgG-phycoerythrin (1:100)	Jackson ImmunoResearch	715-006-150
10.	goat anti-human SOX17 (1:40)	R&D	BAF1924
11.	goat anti-FOXA2 , clone M20 (1:50)	SantaCruz	SC6554
12.	rat anti-human C-peptide (AB1921) (1:300)	BCBC consortium	AB1921
13.	mouse anti-GCG (1:500)	Sigma	G2654
14.	donkey anti-goat IgG-Alexa 488 (1:400),	Invitrogen	A11055
15.	goat anti-mouse allophycocyanin (1:200).	R&D	F0101B
16.	16% paraformaldehyde	Electron Microscopy Sciences	50980487
17.	rat anti-human C-peptide, AB1921, 1:1000	Beta Cell Biology Consortium	AB1921
18.	goat anti-human glucagon, C-18, 1:500	Santacruz	Sc-7779
19.	mouse anti-SST (AB1985; 1:500	Beta Cell Biology Consortium	AB1985
20.	goat IgG (Sigma)	Jackson ImmunoResearch	005-000-003
21.	mouse, rabbit or rat IgG	Jackson ImmunoResearch	015-000-003 / 005-000-003 / 012-000-003
22.	goat anti-mouse IgG-PE	Jackson ImmunoResearch	115-115-208
23.	donkey anti-rat IgG-Cy3	Jackson ImmunoResearch	712-166-153
24.	rabbit anti-mouse Alexa 350, 1:200	Invitrogen	A-21062

REAGENT PREPARATION

STOP MEDIUM

		Final Conc.	For 40 mL
hESC WASH Medium		50%	20 mL
FCS		50%	20 mL
+/- Matrigel (1:1) *	*(BD# 356 230)*	1:800	100 uL

DNASE I (VWR, Cat # 80510-412, 10MU)

1. Want final concentration to be 1mg/ml

2. $10MU \times \frac{1X10^6\ U}{1\ MU} \times \frac{1mg}{65150\ U} = 153mg$

3. In the hood transfer powder to a 125 ml bottle
4. Bring the volume up to 153 ml with ice cold sterile water
5. Let dissolve on ice for 1-2 hours
6. Filter and aliquot 1ml/eppendorf
7. Store at –20.
8. Filter sterilize, aliquot in 1 mL amounts and store frozen at -20ºC
9. Use aliquots once and discard excess

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 -20ºC. 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 (-20ºC). 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

TRANSFERRIN *(ROCHE# 10 652 202)*

The amounts of Transferrin indicated in our protocols are recommended concentrations. However, it is important to test each new batch of transferrin as there is variability between them. It should be aliquoted (2 mL) and stored at 4ºC.

L-Glutamine (Gibco# 25030)

TRYPSIN-EDTA (Invitrogen# 25-053-CL)

		Company	Catalogue #	Buffer	Stock conc.
1.	Wnt3a	*R&D Systems*		PBS, 0.1%BSA, 0.5% CHAPS, 1mM EDTA	50ug/mL
2.	ActivinA	*R&D Systems*	*338-AC/CF*	PBS, 0.1%BSA	10ug/mL
3.	hBMP-4	*R&D Systems*	*314-BP*	H20, 4mM HCL, 0.1%BSA	10ug/mL
4.	hbFGF	*R&D Systems*	*233-FB*	PBS, 0.1%BSA,1mM DTT	10ug/mL
5.	hVEGF	*R&D Systems*	*293-VE*	PBS, 0.1%BSA	5ug/mL
6.	hFGF10	*R&D Systems*	*345FG/CF*	PBS, 0.1%BSA	100ug/mL
7.	hNOGGIN	*R&D Systems*	*3344/NG*	PBS, 0.1%BSA	100ug/mL
8.	Dorsomorphin	Sigma	*P5499*	DMSO	1mM
9.	SB431542	Sigma	*S4317*	DMSO	20mM
10.	L-685,458	*ToCris*	*2627*	DMSO I think now it is	10mM

				R&D	
11.	RA	*Sigma*	*R2625*	DMSO	1mM

Serum Free Differentiation (SFD) Media

Reagent	**Stock conc.**	**Working conc.**	**Per ml**	**1000ml**
IMDM	Cellgro	10-016-CV	75%	750ml
Ham's F12	Cellgro	10-080-CV	25%	250ml
Penicillin/Streptomycin P/S	Invitrogen	15070-063	1%	10ml
N2 Supplement	Invitrogen	17502-048	1%	5ml
B27 Supplement	Invitrogen	12587-010	1%	10ml
7.5% BSA in PBS	10ug/ml	A9647	0.05%	6.66ml

RPMI (Gibco# 31800) supplemented with antibiotics, 10mM HEPES and 1mM Pyruvate.

(A) Day 0 Stage 1 MEDIUM

Reagent	**Stock conc.**	**Working conc.**	**Per ml**
RPMI	1X	1X	1ml
Glutamine	100X	1%	10ul
MTG	26λ/2mLs	3ul/ml	3ul
Ascorbic acid	5mg/ml	50ug/ml	10ul
BMP4	10ug/ml	0.25ng/mL	0.025
bFGF	10ug/ml	5ng/mL	0.5ul
ActA	50ug/mL	100ng/mL	2ul
VEGF	5ug/mL	10ng/mL	2ul

(B) Day 1-2 Stage 1 MEDIUM

Reagent	**Stock conc.**	**Working conc.**	**Per ml**
RPMI	1X	1X	1ml
Glutamine	100X	1%	10ul
MTG	26λ/2mLs	3ul/ml	3ul
ActA	5ug/mL	100ng/mL	2ul
WNT3a	50ug/mL	25ng/mL	0.5ul

(C) Day 3,5 Stage 2 MEDIUM

Reagent	**Stock conc.**	**Working conc.**	**Per ml**
SFD	1X	1X	1ml
Glutamine	100X	1%	10ul
MTG	26λ/2mLs	3ul/ml	3ul
FGF10	100ug/mL	50ng/mL	0.5ul
WNT	50ug/mL	3ng/mL	0.06ul
Dorsomorphin	1mM	0.75uM	0.75ul

* Dorsomorphin is required for H1 and H9 differentiation. Other human pluripotent stem cell lines may not require Dorsomorphin at stage 2.

H21 (Gibco# 12800) with high D-glucose (4,500mg/L).

(D) Day 6,8 Stage 3 MEDIUM

Reagent	Stock conc.	Working conc.	Per ml
H21 (high glucose)	1X	1X	1ml
Glutamine	100X	1%	10ul
B27	100X	1%	10ul
Ascorbic acid	5mg/ml	50ug/ml	10ul
Cyclopamine	0.1mM	0.25uM	2.5ul
RA	10mM	2uM	0.2ul
Noggin	100ug/ml	50ng/ml	0.5ul
FGF10	100ug/mL	50ng/mL	0.5ul

E) Day 9,11 Stage 4 MEDIUM

Reagent	Stock conc.	Working conc.	Per ml
H21 (high glucose)	1X	1X	1ml
Glutamine	100X	1%	10ul
B27	100X	1%	10ul
Ascorbic acid	5mg/ml	50ug/ml	10ul
SB431542	20mM	6uM	0.3ul
Noggin	100ug/ml	50ng/ml	0.5ul

(F) Day 12-20 Stage 5 MEDIUM

Reagent	Stock conc.	Working conc.	Per ml
SFD	1X	1X	1ml
Glutamine	100X	1%	10ul
Glucose	26λ/2mLs	3ul/ml	3ul
Ascorbic acid	5mg/ml	50ug/ml	10ul
SB431542	20mM	6uM	0.3ul
Noggin	100ug/ml	50ng/ml	0.5ul
℘ Secretase Inhibitor (L685,458)**	10mM	1uM	1ul

Including Glucose present in SFD, the final Glucose concentration is 40mM.
**** We find that the concentration of γsecretase Inhibitor (L-685,458) can be dropped to 0.25uM.**

METHOD

Methods

This protocol is designed for H1 and H9 human embryonic stem cell lines. Other lines may not require Dorsomorphin at stage 2 of differentiation.

hESC maintenance

Our lab routinely adapts hESCs to trypsin passage as this allows for easy passage and t he maintenance and production of large numbers of cells. Successful maintenance of healthy undifferentiated hESCs is dependent on the appropriate concentrations of feeder cells and hESCs.

Mouse Embryonic Feeder Cells (MEFs). should be approximately 80% confluent and fresh, ideally cultured for only 24 hours prior to use. If the density of the MEFs is too high (confluent) the hESCs do not form discrete colonies but rather grow as disperse groups of cells, forming a monolayer. MEFs that are too sparce (<50%) do not provide adequate support for hESC maintenance. We routinely freeze irradiated MEFs at 2x10^6 cells per vial. Each vial contains enough cells for 18-24 wells of a twelve-well plate. The plating efficiency of each batch of MEFs needs to be tested.

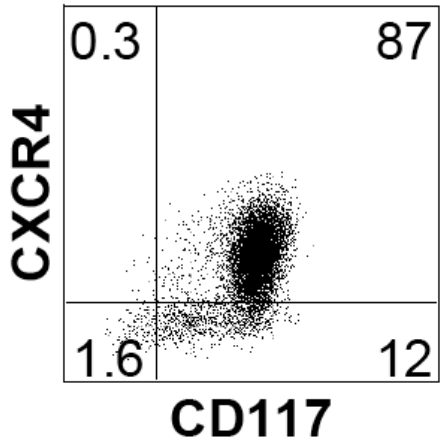
hESCs. hESCs should be cultured at a density that allows the growth of distinct colonies with sharp borders within 4-5 days of culture. If the cells are too dense, the developing colonies grow into each other and form a monolayer. When too few cells are cultured, they can differentiate and tend to grow slower. Our stock of hESCs are frozen at 2x10^6 cells per vial. This concentration can be used for 6-24 wells of a twelve-well plate. The number of wells that can be cultured is dependent on the hESC line as well as the extent to which they are adapted to trypsin passage. Under optimal conditions with well adapted hESCs, you should be able to reach 70% confluency 4-5 days after plating, at this stage cells are ready to be differentiated.
Note: The protocol described below is designed to be carried out in a 12-well plate format.

Day 0: Stage 1 Endoderm Progenitors
1. Remove the medium from hESCs and wash once with RPMI.
2. To each well, add 1mL of ***MEDIA (A)***. Incubate for 24 hours at 37°C in a 5%CO2 incubator.

Day 1-2: Stage 1 Endoderm Progenitors
1. There will be some debris in the cultures after 24 hours. Remove MEDIA A and wash once with RPMI.
2. To each well, add 1mL of ***MEDIA (B)***. Incubate for 24 hours at 37°C in a 5%CO2 incubator.
3. Repeat steps 1-2 at day 2.
Note: Endoderm induction should be evaluated by flow cytometric analysis, monitoring the cells for expression of CXCR4 (CD184) and CD117 (c-KIT). As each hESC line has its own unique kinetics, it is best to define the endoderm stage based on the CXCR4/CD117 profile rather than by time in culture. The endoderm stage is defined by the appearance of a population that co-expresses CXCR4 and CD117. Using this protocol H1 gives rise to an average of 85% CXCR4+/CD117+ cells at day 3.

Day 3: Harvest for Flow Cytometry
1. Aspirate the medium and add 1mL of *TRYPSIN-EDTA*. Incubate in a 37°C incubator for 2-3 minutes and then stop the reaction with 1mL of *STOP MEDIUM+DNase* .
2. Spin for 5min at 1000 RPM, aspirate and resuspend in *PBS (-Ca^{2+}-Mg^{2+})+10%FCS* (usually 500uL per well harvested). Pass the cells through a 70 um filter to remove any clumps that are still remaining.
3. Stain with the desired antibodies (CXCR4, CD117) according to product datasheets and perform flow cytometric analysis.

Day 3, 5 : Stage 2 Foregut/Midgut Endoderm
1. There will be some debris in the cultures after 24 hours. Remove MEDIA B and wash once with RPMI.
2. To each well, add 1mL of ***MEDIA (C)***. Incubate for 48 hours at 37°C in a 5%CO2 incubator.
3. On day 5, remove MEDIA C.
4. To each well, add 1mL of ***MEDIA (C)***. Incubate for 24 hours at 37°C in a 5%CO2 incubator.



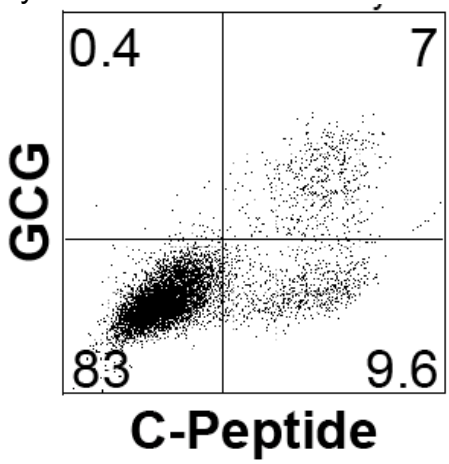
Day 6-8: Stage 3 Pancreatic Endoderm
1. Remove Media C.
2. To each well, add 1mL of ***MEDIA (D)***. Incubate for 24 hours at 37°C in a 5%CO2 incubator.
3. Repeat steps 1-2 on day 7 and 8.

Note: Due to the high instability of RA, we tend to feed in the dark and as fast as possible during stage 3.

Day 9, 11: Stage 4 Endocrine Progenitors
1. Remove Media D.
2. To each well, add 1mL of ***MEDIA (E)***. Incubate for 48 hours at 37°C in a 5%CO2 incubator.
3. On day 11, remove MEDIA E.
4. To each well, add 1mL of ***MEDIA (E)***. Incubate for 48 hours at 37°C in a 5%CO2 incubator.

Day 13-20: Stage 5 Endocrine Cells
1. Remove Media E.
2. To each well, add 1mL of ***MEDIA (F)***. Incubate for 72 hours at 37°C in a 5%CO2 incubator.
3. Feed every three days. During the course of this time hormone-expressing cells aggregate with each other and form clusters visible by eye.
4. Harvest at day 20.

Note: The percentage of endocrine cells should be evaluated by flow cytometric analysis, monitoring the cells for expression of C-Peptide and GCG. Below a typical profile for H1-differentiated cells at day 20.



REFERENCE:

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