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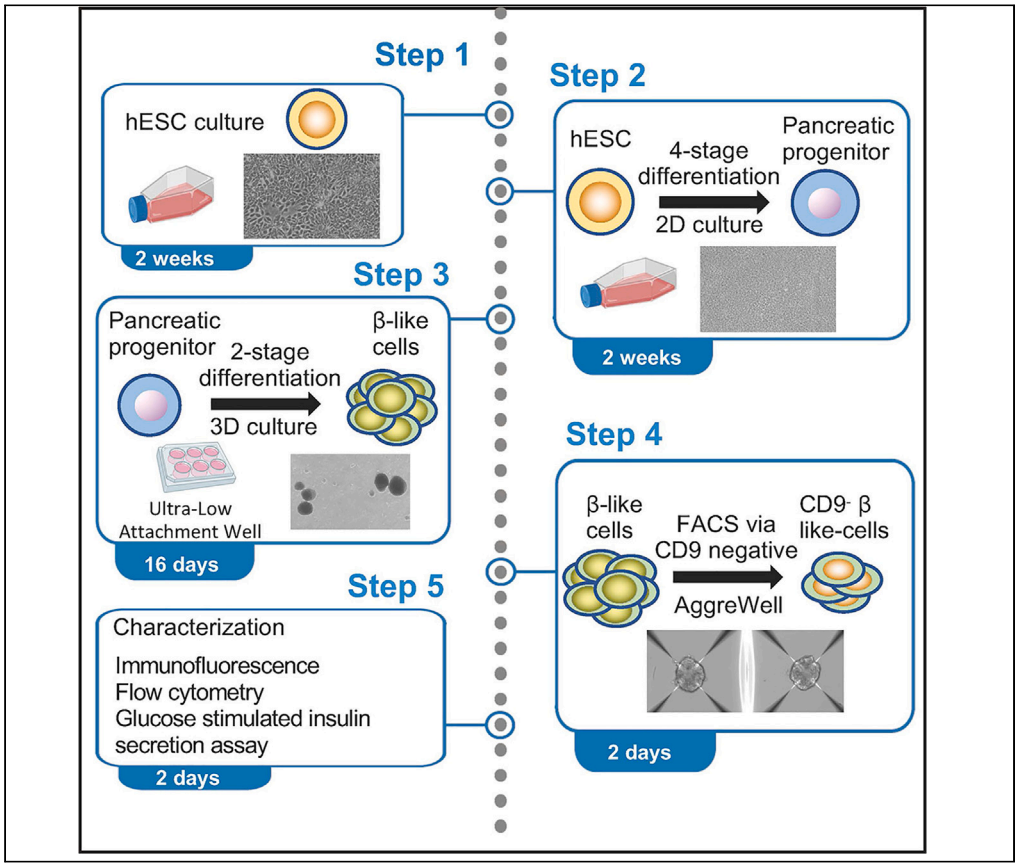
A differentiation protocol for the generation of pancreatic beta-like cells from human embryonic stem cells



By virtue of their capability to replicate and regenerate, human stem-cell-derived beta-like cells could be a valuable resource for cellular therapy targeting insulin-dependent diabetes. Here, we present a protocol to generate beta-like cells from human embryonic stem cells (hESCs). We first describe steps for differentiation of beta-like cells from hESCs and CD9-negative beta-like cell enrichment through fluorescence-activated cell sorting. We then detail immunofluorescence, flow cytometry, and glucose-stimulated insulin secretion assay for characterization of human beta-like cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Human beta-like cells are differentiated from hESCs by a six-step procedure

Combined 2- and 3-dimensional cultures are used for beta-like cell differentiation

Human beta-like cells are purified via CD9-based fluorescence-activated cell sorting

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A differentiation protocol for the generation of pancreatic beta-like cells from human embryonic stem cells

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SUMMARY

By virtue of their capability to replicate and regenerate, human stem-cell-derived beta-like cells could be a valuable resource for cellular therapy targeting insulin-dependent diabetes. Here, we present a protocol to generate beta-like cells from human embryonic stem cells (hESCs). We first describe steps for differentiation of beta-like cells from hESCs and CD9-negative beta-like cell enrichment through fluorescence-activated cell sorting. We then detail immunofluorescence, flow cytometry, and glucose-stimulated insulin secretion assay for characterization of human beta-like cells.

For complete details on the use and execution of this protocol, please refer to Li et al. (2020).[1]

BEFORE YOU BEGIN

Preparation of Matrigel coating solution

Timing: 1 day

1. Preparation of hESC-qualified Matrigel coating solution.
   a. Thaw the hESC-qualified Matrigel on ice overnight (18–24 h).
   b. Dilute the hESC-qualified Matrigel in cold DMEM/F12 at the ratio of 1:100. The coating solution can be stable at 4°C for 1 month.

   Note: To avoid the multiple freeze-thaws, it is recommended to aliquot the hESC-qualified Matrigel into 1.5 mL micro-centrifuge tubes and store at −80°C until use.

2. Preparation of growth factor reduced (GFR)-Matrigel coating solution.
   a. Thaw the GFR-Matrigel on ice overnight (18–24 h).
   b. Dilute the GFR-Matrigel in cold DMEM/F12 at the ratio of 1:30. The coating solution can be stable at 4°C for 1 month.

   Note: To avoid the multiple freeze-thaws, it is recommended to aliquot the GFR-Matrigel into 1.5 mL micro-centrifuge tubes and store at −80°C until use.

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Table 1. Coating volumes for different cell culture ware

Culture ware	Growth area (cm^2)	Volume of coating solution
12 well plate	3.8	0.5 mL
6 well plate	9.6	1 mL
T25 flask	25	2 mL
T75 flask	75	5 mL

Preparation of tissue culture plates or flasks coated with Matrigel

Timing: 2 h

3. hESCs are cultured on hESC-qualified Matrigel coated plates or flasks for cell culture maintenance and beta cell differentiation starts on GFR-Matrigel coated plates or flasks.
   a. Add the hESC-qualified Matrigel coating solution or GFR-Matrigel coating solution to the culture plates or flasks according to Table 1.
   b. Gently shake the flasks or plates and incubate them in a cell culture incubator at 37°C and 5% CO_2 for 1 h.
   c. After 1 h, aspirate the coating solution and wash twice with PBS. The plates or flasks are ready to use.

Note: Avoid air bubbles in Matrigel coating solution during plate or flask coating. Make sure the coating is even. If not used immediately, add enough PBS and seal the plates or flasks to prevent evaporation; stored at 4°C for up to 1 week.

Preparation of media

4. Preparation of mTeSR™1 medium.
   a. Thaw the 100 mL mTeSR™1 with 5 × Supplement at 4°C overnight (18–24 h) until it is fully dissolved.
   b. Mix 100 mL 5 × Supplement to 400 mL mTeSR™1 Basal Medium.

Note: Do not thaw or prewarm the mTeSR™1 supplement or complete medium at a 37°C water bath. The complete medium should be protected from light and stored at 4°C for up to 2 weeks.

5. Preparation of beta cell differentiation media.
   a. Prepare B27-RPMI according to Table 2.

Note: B27-RPMI medium should be protected from light and stored at 4°C for up to one month.

   b. Prepare B27-DMEM according to Table 3.

Note: B27-DMEM medium should be protected from light and stored at 4°C for up to one month.

   c. Prepare of MCDB131 differentiation medium according to Table 4.

Table 2. Composition of B27-RPMI

Reagent	Final concentration	Amount
RPMI1640	N/A	490 mL
B27 supplement (minus vitamin A, 50 ×)	2% (1 ×)	10 mL
Total	**N/A**	**500 mL**

Table 3. Composition of B27-DMEM

Reagent	Final concentration	Amount
DMEM	N/A	490 mL
B27 supplement (minus vitamin A, 50 ×)	2% (1 ×)	10 mL
Total	N/A	500 mL

Note: MCDB131 differentiation medium should be filtered through 0.22 μm filter. The medium should be protected from light and stored at 4°C for up to one month.

Preparation of stock solutions of growth factors and small molecules

Note: Most growth factors and small molecules are unstable after reconstitution. To reduce repeated freeze-thawing, the reconstituted growth factors and small molecules should be aliquoted and stored at −80°C until use. As most growth factors and small molecules are sterile, reconstitution and aliquoting should be performed in a BSL 2 hood using aseptic technique.

6. Preparation of 0.1% BSA in PBS used to reconstitute growth factors.
   a. Dissolve 0.1 g BSA in 100 mL 1×PBS.
   b. Filter through 0.22 μm syringe filter and store buffer at 4°C.
7. Preparation of hActivin A at a stock concentration of 100 μg/mL.
   a. Add 1 mL PBS containing 0.1% BSA into 1 mg hActivin A until it is fully dissolved. Do not vortex.
   b. Further dilute this solution with PBS containing 0.1% BSA at a concentration of 100 μg/mL and aliquot into 50 μL and store at −80°C until use.
8. Preparation of hFGF-2 at a stock concentration of 10 μg/mL.
   a. Add 500 μL PBS containing 0.1% BSA into 50 μg hFGF-2 until it is fully dissolved. Do not vortex.
   b. Further dilute this solution 10 times with PBS containing 0.1% BSA at a concentration of 10 μg/mL and aliquot into 50 μL and store at −80°C until use.
9. Preparation of hKGF at a stock concentration of 100 μg/mL.
   a. Add 1 mL PBS containing 0.1% BSA into 100 μg hKGF until it is fully dissolved. Do not vortex.
   b. Aliquot into 50 μL and store at −80°C until use.
10. Preparation of hNoggin at a stock concentration of 100 μg/mL.
   a. Add 1 mL PBS containing 0.1% BSA into 100 μg hNoggin until it is fully dissolved. Do not vortex.
   b. Aliquot into 50 μL and store at −80°C until use.
11. Preparation of hEGF at a stock concentration of 200 μg/mL.
   a. Add 500 μL PBS containing 0.1% BSA into 500 μg hEGF until it is fully dissolved. Do not vortex.
   b. Further dilute this solution 5 times with PBS containing 0.1% BSA at a concentration of 200 μg/mL and aliquot into 50 μL and store at −80°C until use.

Table 4. Composition of MCDB131 differentiation medium

Reagent	Final concentration	Amount
MCDB131	N/A	494.75 mL
$NaHCO_3$	1.5 g/L	0.75 g
D-glucose	4.5 g/L	2.25 g
Fat acid free-BSA	2%	10 g
L-GlutaMAX	1%	5 mL
ITS-X	0.05%	0.25 mL
Total	N/A	500 mL

12. Preparation of Vitamin C at a stock concentration of 50 mg/mL.
    a. Dissolve 0.5 g Vitamin C in 10 mL 1×PBS to obtain 50 mg/mL Vitamin C.
    b. Filter through 0.22 μm syringe filter and aliquot into 50 μL. Protect from light and store at −80°C until use.
13. Preparation of SANT-1 at a stock concentration of 0.5 mM.
    a. Add 2.677 mL DMSO into 5 mg SANT-1 and vortex until it is fully dissolved to obtain 5 mM SANT-1.
    b. Further dilute 10 times into 0.5 mM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
14. Preparation of Retinoic acid at a stock concentration of 4 mM and 200 μM.
    a. Add 2.08 mL DMSO into 50 mg Retinoic acid and vortex until it is fully dissolved to obtain 80 mM Retinoic acid.
    b. Further dilute into 4 mM and 200 μM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
15. Preparation of Chir99021 at a stock concentration of 5 mM.
    a. Add 2.15 mL DMSO into 5 mg Chir99021 and vortex until it is fully dissolved to obtain 5 mM Chir99021.
    b. Protect from light and store at −80°C until use.
16. Preparation of Nicotinamide at a stock concentration of 1 M.
    a. Dissolve 12.212 g Nicotinamide in 100 mL 1×PBS and filter through 0.22 μm syringe filter.
    b. Aliquot into 5 mL and store at -80°C until use.
17. Preparation of LDN193189 at a stock concentration of 1 mM.
    a. Add 1.2301 mL DMSO into 5 mg LDN193189 and vortex until it is fully dissolved to obtain 10 mM LDN193189.
    b. Further dilute into 1 mM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
18. Preparation of Compound E at a stock concentration of 1 mM.
    a. Add 407.75 μL DMSO into 1 mg Compound E and vortex until it is fully dissolved to obtain 5 mM Compound E.
    b. Further dilute into 1 mM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
19. Preparation of RepSox at a stock concentration of 50 mM.
    a. Add 1.74 mL DMSO into 25 mg RepSox and vortex until it is fully dissolved to obtain 50 mM RepSox.
    b. Aliquot into 50 μL. Protect from light and store at −80°C until use.
20. Preparation of GC1 at a stock concentration of 5 mM.
    a. Add 609 μL DMSO into 10 mg GC1 and vortex until it is fully dissolved to obtain 50 mM GC1.
    b. Further dilute into 5 mM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
21. Preparation of R428 at a stock concentration of 10 mM.
    a. Add 987 μL DMSO into 10 mg R428 and vortex until it is fully dissolved to obtain 20 mM R428.
    b. Further dilute into 10 mM by DMSO and aliquot into 50 μL. Protect from light and store at −80°C until use.
22. Preparation of Trolox at a stock concentration of 50 mM.
    a. Add 799 μL DMSO into 10 mg Trolox and vortex until it is fully dissolved to obtain 50 mM Trolox.
    b. Aliquot into 50 μL. Protect from light and store at −80°C until use.
23. Preparation of N-acetyl cysteine at a stock concentration of 500 mM.
    a. Dissolve 4.08 g N-acetyl cysteine in 50 mL PBS. Incubate in a 37°C water bath until it is fully dissolved to obtain 500 mM N-acetyl cysteine.
    b. Filter through 0.22 μm syringe filter. Aliquot into 5 mL and store at −80°C until use.
24. Preparation of $ZnSO_4$ at a stock concentration of 10 mM.

a. Dissolve 0.807 g $ZnSO_4$ in 50 mL PBS to obtain 100 mM $ZnSO_4$ and filter through 0.22 μm syringe filter.
b. Further dilute the 10 mL 100 mM $ZnSO_4$ by adding 90 mL PBS to obtain 10 mM $ZnSO_4$ and store the buffer at 4°C for up to 3 months.

25. Preparation of Heparin at a stock concentration of 10 mg/mL.
    a. Dissolve 0.1 g Heparin in 10 mL PBS to obtain 10 mg/mL Heparin and filter through 0.22 μm syringe filter.
    b. Store the buffer at 4°C for up to 3 months.
26. Preparation of Rock (Rho kinase) inhibitor (Y-27632) at a stock concentration of 10 mM.
    a. Add 3.1225 mL sterile ddH_2O into 10 mg Rock inhibitor until it is fully dissolved to obtain 100 mM stock.
    b. Further dilute this solution with PBS at a concentration of 10 mM and aliquot into 500 μL and stored at −80°C until use. It can be stored at 4°C for up to 3 months.

Preparation of Dispase solution

27. Preparation of Dispase solution at a concentration of 1 U/mL.
    a. Dissolve 100 U of Dispase in 100 mL DMEM at a concentration of 1 U/mL.
    b. Filter through 0.22 μm membrane filter, and aliquot into 5 mL for storage at −80°C until use.

Note: The enzyme unit of different batches of Dispase may vary. Calculate and add accordingly.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD9-PE(Dilution 1:20 for FACS)	BioLegend	Cat#312105
Mouse IgG1-PE (Dilution 1:20 for FACS)	BioLegend	Cat#400111
Anti-NKX6.1 (Dilution 1:400 for immunofluorescence; 1:200 for flow cytometry)	R&D System	AF5857
Anti-PDX1 (Dilution 1:200)	R&D System	AF2419
Anti-PDX1 (Dilution 1:200)	R&D System	Ab47267
Anti-C-peptide (Dilution 1:1000 for immunofluorescence; 1:500 for flow cytometry)	DSHB	GN-ID4
Donkey anti goat 546 (Dilution 1:600 for immunofluorescence)	Life Technologies	A11056
Donkey anti rat 488 (Dilution 1:600 for immunofluorescence; 1:1000 for flow cytometry)	Life Technologies	A21208
Donkey anti goat 647 (Dilution 1:1000 for flow cytometry)	Life Technologies	A21447
Donkey anti rabbit 488 (Dilution 1:600 for immunofluorescence)	Life Technologies	A21206
Chemicals, peptides, and recombinant proteins		
mTeSR™1	Stem Cell Technologies	Cat# 85850
RPMI1640	Thermo Fisher	Cat# 11875119
DMEM with high glucose	Thermo Fisher	Cat# 11995073
MCDB131	Corning	Cat# 15-100-CV
DMEM/F12	Thermo Fisher	Cat# 11330057
B27 Supplement (Minus Vitamin A)	Life Technologies	Cat# 12587010
L-GlutaMAX	Life Technologies	Cat# 35050061
Fatty acid free-BSA	Proliant	Cat# 68700
PBS without calcium and magnesium	Life Technologies	Cat# 10010072
Accutase	Life Technologies	Cat# A11105-01
Dispase	Thermo Fisher	Cat# 17105041
Penicillin-streptomycin	Life Technologies	Cat# 15140122

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
hESC-qualified Matrigel	Corning	Cat# 354277
Growth Factor Reduced (GFR) Matrigel	Corning	Cat# 356321
ITS-X	Life Technologies	Cat# 51500056
$NaHCO_3$	Sigma-Aldrich	Cat# S5761
D-Glucose	Sigma-Aldrich	Cat# G8270
FBS	HyClone	Cat# SH30070.03E
TrypLE™ Express Enzyme	Thermo Fisher	Cat# 12604021
Rock inhibitor (Y-27632)	Selleckchem	Cat# S1049
Human Activin A	Peprotech	Cat# 120-14E
Chir99021	Selleckchem	Cat# S2924
Vitamin C	Sigma-Aldrich	Cat# A92902
Human FGF-2	PepreTech	Cat# 100-18B
Human KGF	PepreTech	Cat# AF-100-19
SANT-1	Sigma-Aldrich	Cat# S4572
Retinoic acid	Sigma-Aldrich	Cat# R2625
Human Noggin	PeproTech	Cat# 120-10C
Human EGF	PeproTech	Cat# AF-100-15
Nicotinamide	Sigma-Aldrich	Cat# N3376
LDN193189	Sigma-Aldrich	Cat# SML0559
Compound E	Stem Cell Technologies	Cat# 73954
RepSox	Selleckchem	Cat# S7223
GC1	R&D Systems	Cat# 4554/10
R428	Cayman	Cat# 21523
Trolox	Sigma-Aldrich	Cat# 238813
N-Acetyl cysteine	Sigma-Aldrich	Cat# A9165
Heparin	Sigma-Aldrich	Cat# H3149
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat #D2650
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich	Cat# Z0251
Tween-20	Sigma-Aldrich	Cat# P1379
Triton X-100	Sigma-Aldrich	Cat# X100
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat# 158127
NaCl	Sigma-Aldrich	Cat# S9888
KCl	Sigma-Aldrich	Cat# P3911
$MgCl_2$	Sigma-Aldrich	Cat# M8266
$CaCl_2$	Sigma-Aldrich	Cat# C4901
Na_2HPO_4	Sigma-Aldrich	Cat# S9763
KH_2PO_4	Sigma-Aldrich	Cat# P0662
Sucrose	Sigma-Aldrich	Cat# S0389
Hoechst 33342 (10 mg/mL)	Thermo Fisher	Cat# H3570
Dapi solution	BD Pharmingen	Cat# 564907
HEPES (1 M)	Life Technologies	Cat# 15630130
Critical commercial assays		
Human insulin ELISA kit	ALPO Diagnostics	Cat# 80-INSHU-E01.1
Ultra-Low Attachment 6 Well Plate	Corning	Cat# 3471
AggreWell™400 starter kit	Stem Cell Technologies	Cat# 34450
Experimental models: Cell lines		
H9 Human Embryonic Stem Cells (hESCs) (NIH Approval number NIHhESC-10-0062)	WiCell	Cat# WA09
Software and algorithms		
LAS Advanced Fluorescence Software	Leica Microsystems	https://www.leica-microsystems.com
FlowJo 10.4	BD Biosciences	https://www.flowjo.com/
Other		
5 mL serological pipette	Genetimes ExCell	Cat# 91005
10 mL serological pipette	Genetimes ExCell	Cat# 91010
Syringe filter 0.22 μm	Genesee Scientific	Cat# 25-243

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell lifter	Corning	Cat# 3008
Aqua-Poly/Mount medium	Polysciences	Cat# 18606
Superfrost plus slides	Fisher Scientific	Cat# 22-037-246
PAP pen	Abcam	Cat# Ab2601
Leica DM4 M fluorescence microscope	Leica	https://www.leica-microsystems.com/products/light-microscopes/p/leica-dm6-m/
BD FACSAria II Cell Sorter	BD Biosciences	https://www.bdbiosciences.com/en-us/products/instruments/flow-cytometers/research-cell-sorters
CytoFLEX	Beckman Coulter	https://www.beckman.com/flow-cytometry/research-flow-cytometers/cytoflex
Cryostat microtome	Fisher Scientific	Cat# 956640EC

MATERIALS AND EQUIPMENT

FACS buffer

Reagent	Final concentration	Amount
PBS	N/A	97 mL
Penicillin-Streptomycin	1%	1 mL
FBS	2%	2 mL
Total	**N/A**	**100 mL**

Store at 4°C for up to 1 month.

FACS Collection buffer

Reagent	Final concentration	Amount
MCDB131	N/A	45 mL
FBS	10%	5 mL
Rock inhibitor (Y-27632)	10 μM	50 μL of 10 mM stock
Total	**N/A**	**50 mL**

Store at 4°C for up to 1 month.

4% PFA fixation solution

Reagent	Final concentration	Amount
PFA	4%	40 g
PBS	N/A	1 L
Total	**N/A**	**1 L**

Add 40 g PFA powder into 1 L heated PBS solution. Add NaOH and stir gently until PFA is dissolved. Adjust the pH to 7.4 with 1 N HCl, the solution was filtered and aliquoted and frozen at −80 C until use.

30% sucrose

Reagent	Final concentration	Amount
sucrose	30 (w/v) %	30 g
PBS	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Store at 4°C for up to 3 months.

PBST

Reagent	Final concentration	Amount
Tween-20	0.1 (v/v) %	1 mL
PBS	N/A	999 mL
Total	**N/A**	**1 L**

Store at room temperature (20°C–25°C) for up to 6 months.

0.5% Tween-20 PBST		
Reagent	Final concentration	Amount
Tween-20	0.5 (v/v) %	5 mL
PBS	N/A	995 mL
Total	**N/A**	**1 L**
Store at room temperature (20°C–25°C) for up to 6 months.		

10% donkey serum PBST		
Reagent	Final concentration	Amount
Donkey serum	10 (v/v) %	1 mL
PBST	N/A	9 mL
Total	**N/A**	**10 mL**
Store at 4°C for up to 1 week.		

1% BSA PBST		
Reagent	Final concentration	Amount
BSA	1(w/v) %	0.1 g
PBST	N/A	10 mL
Total	**N/A**	**10 mL**
Store at 4°C for up to 1 week.		

Hoechst 33342 working solution		
Reagent	Final concentration	Amount
Hoechst 33342 (10 mg/mL)	1 μg/mL	1 μL
deionized water	N/A	10 mL
Total	**N/A**	**10 mL**
Prepare freshly and protect from light.		

Perm/Blocking buffer		
Reagent	Final concentration	Amount
FBS	5 (v/v) %	5 mL
Triton X-100	0.1 (v/v) %	0.1 mL
PBS	N/A	94.9 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to 1 week.		

Krebs buffer		
Reagent	Final concentration	Amount
NaCl	128 mM	3.74 g
KCl	5 mM	0.1864 g
$MgCl_2$	1.2 mM	0.0572 g
$CaCl_2$	2.7 mM	0.14985 g
Na_2HPO_4	1 mM	0.071 g
KH_2PO_4	1.2 mM	0.08165 g
$NaHCO_3$	5 mM	0.21 g
HEPES	10 mM	5 mL
Fatty acid free-BSA	0.1 (w/w) %	0.5 g
deionized water	N/A	Up to 500 mL
Total	**N/A**	**500 mL**

Note: $MgCl_2$ and $CaCl_2$ are added into deionized water firstly with magnetic stirrer. And other components are added until $MgCl_2$ and $CaCl_2$ are dissolved.

Low Glucose Krebs buffer		
Reagent	Final concentration	Amount
Glucose	2 mM	0.03603 g
Krebs buffer	N/A	100 mL
Total	**N/A**	**100 mL**

High Glucose Krebs buffer		
Reagent	Final concentration	Amount
Glucose	20 mM	0.36031 g
Krebs buffer	N/A	100 mL
Total	**N/A**	**100 mL**

KCl Krebs buffer		
Reagent	Final concentration	Amount
Glucose	2 mM	0.03603 g
KCl	30 mM	0.22365 g
Krebs buffer	N/A	100 mL
Total	**N/A**	**100 mL**

All Krebs buffers are sterilized using 0.22 μm filter and stored at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

This paper contains protocols for hESCs cultures, 2D planar culture differentiation of hESCs to pancreatic progenitor, 3D culture differentiation of pancreatic progenitor to beta-like cells and negative enrichment of beta-like cells through CD9. The schematic of the protocol and representative images of cells at different stages are shown in Figure 1. It also contains protocols for immunostaining of beta-like cells, flow cytometry and *in vitro* glucose-stimulated insulin secretion assay (GSIS).

Thaw and culture hESCs

Timing: 2 weeks

These steps describe how hESCs are thawed and how to culture hESCs on a hESC-qualified Matrigel coated T75 flask for maintenance culture.

1. Thaw hESCs.
   a. Coat one T75 flask with 5 mL hESC-qualified Matrigel coating solution.
   b. Incubate the flask in a cell culture incubator at 37°C and 5% CO_2 for 1 h.
   c. Prepare 25 mL mTeSR™1 medium with 10 μM Rock inhibitor.
   d. Equilibrate the medium in a biological safety hood to room temperature (20°C–25°C).

Note: Rock inhibitor will be used overnight (18–24 h) to enhance the survival of hESCs after the cells are cryopreserved or dissociated into single cells. Therefore, it is of importance to add Rock inhibitor when hESCs are thawed or passaged when hESCs are dissociated ito single cells.[2]

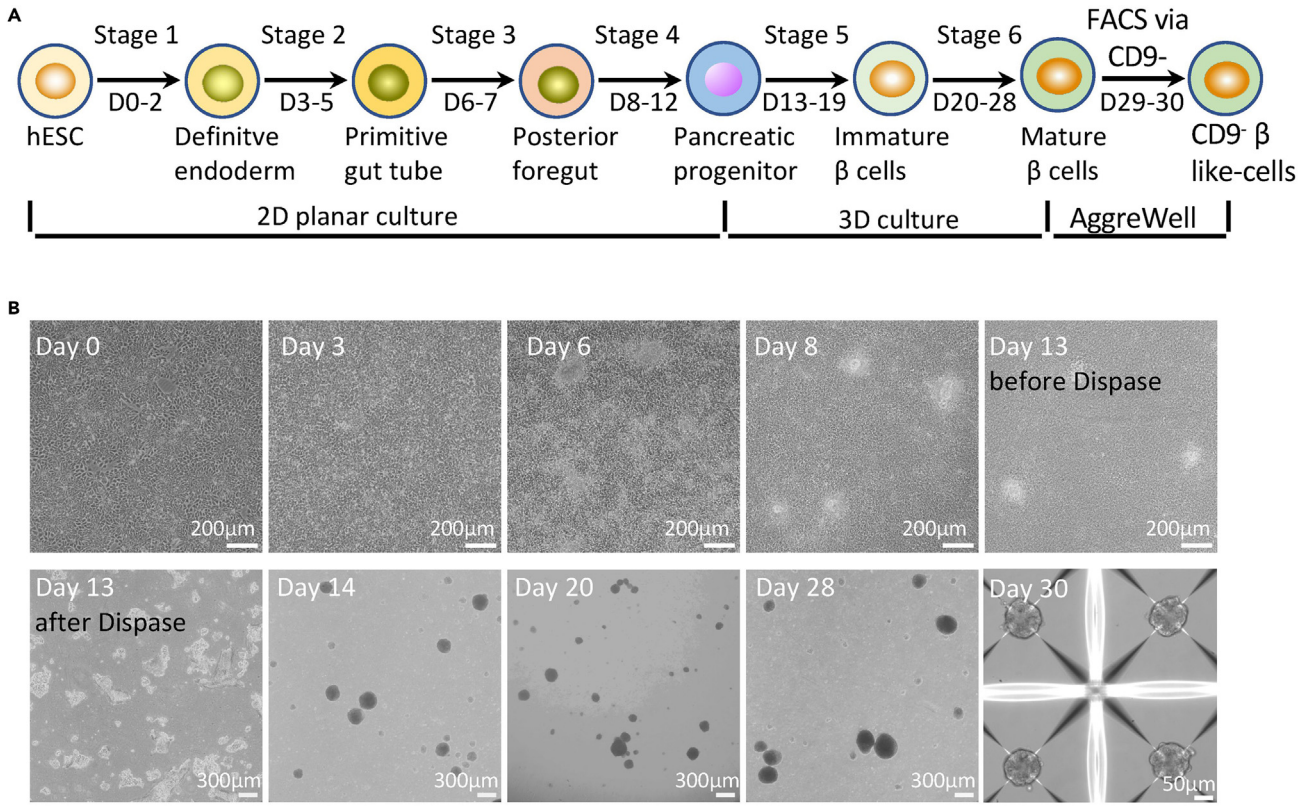

Figure 1. Overview of differentiation protocol for hESC derived beta-like cells

(A) Schematic of the protocol used to differentiate hESCs toward beta-like cells. Summary of 6-stage differentiation including 2D planar culture and 3D culture. At the final of stage 6, CD9 negative cells were sorted by FACS and re-aggregated with AggreWell 400.

(B) Representative brightfield images of cells at different stages. On day 0, hESC; Day 3, stage 1, definitive endoderm; Day 6, stage 2, primitive gut tube; Day 8, stage 3, posterior foregut; Day 13, stage 4, pancreatic progenitor. Cells were treated with Dispase and broke into cell aggregates on day 13 and transferred to ultra-low wells for 3D culture. On Day 14, cell aggregates formed 3D spheroids spontaneously. Day 20, stage 5, immature beta-like cells; Day 28, stage 6, mature beta-like cells. On Day 29, cells were dissociated into single cells and sorted for CD9 negative cells. CD9- cells self-organized into spheroids on day 30.

e. Remove one cryogenic vial of hESCs from liquid nitrogen tank.
f. Quickly thaw cells in a 37°C water bath by gently shaking the vial until only a small frozen cell pellet remains.
g. Transfer the cells to 15 mL conical tube.
h. Add 9 mL mTeSR™1 medium with 10 μM Rock inhibitor dropwise to the 15 mL tube and mix gently.
i. Centrifuge the cells at 300 × *g* for 5 min to pellet the cells.
j. Aspirate the supernatant and resuspend the pellet in 1 mL mTesR™1 medium with 10 μM Rock inhibitor.
k. Count the cell number by hemocytometer.
l. Prepare 1,125,000 cells (15,000 cells / cm^2) in 15 mL mTeSR™1 with 10 μM Rock inhibitor
m. Seed the cells in one T75 flask precoated with hESC-qualified Matrigel.

Note: For the H9 hESC line, our experience shows that the flask will reach 90% confluency in 5 days at this seeding density. One T75 can generate 20 million hESCs approximately when it reaches 90% confluency. For different cell lines, the doubling times may be different, and the seeding density should be optimized.

n. Distribute the cells by moving the flask back and forth, left and right for several times.
o. Place the flask in a humidified incubator at 37°C and 5% CO_2.

2. On the next day, refresh the medium with 15 mL fresh mTeSR™1 medium without Rock inhibitor.
3. Change medium every day until hESCs reach 90% confluency.
4. Enzymatic Passaging.
   a. Before passaging hESCs, coat one T75 flask with 5 mL hESC-qualified Matrigel coating solution at 37°C for 1 h.
   b. Prepare mTeSR™1 medium with 10 μM Rock inhibitor and equilibrate in a biological safety hood to room temperature (20°C–25°C).
   c. Aspirate the hESC medium and rinse the cells with 5 mL PBS.
   d. Aspirate the PBS and add 5 mL Accutase.
   e. Incubate the cells for 6 min in a cell culture incubator at 37°C and 5% CO_2.
   f. Observe under microscope to make sure all the cells are detached.
   g. Add 3-fold volume of DMEM/F12 to quench the Accutase.
   h. Transfer the cells to conical tube and centrifuge at 250 × *g* for 5 min.
   i. Aspirate the supernatant and resuspend the pellet with 5 mL mTeSR™1 with 10 μM Rock inhibitor.
   j. Count the cell number with hemocytometer.
   k. Seed the cells on hESC qualified Matrigel-coated culture ware for maintenance or GFR-Matrigel coated culture ware for differentiation.

Note: Before differentiation, hESCs are adapted to grow for at least two passages after thawing.

Pancreatic progenitor differentiation

Timing: 2 weeks

Pancreatic progenitor differentiation comprises 4 stages: from day 0 to day 2, stage 1 for definitive endoderm; day 3 to day 5, stage 2 for primitive gut tube; day 6 to day 7, stage 3 for posterior foregut; day 8 to day 12, stage 4 for pancreatic progenitor. This part is modified from.[3]

Here describes the setting of pancreatic beta-like cell differentiation from H9 on a T75 flask.

Note: before changing the medium every day, warm the differentiation medium in a 37°C water bath and keep the growth factors on ice and chemicals at room temperature (20°C–25°C).

5. On day -1, seed hESC for differentiation.
   a. Prepare 20 mL mTeSR™1 with 10 μM Rock inhibitor and prewarm in a biological safety hood to room temperature (20°C–25°C).
   b. Coat one T75 flask with 5 mL GFR-Matrigel coating solution and incubate it in a cell culture incubator at 37°C and 5% CO_2 for 1 h.
   c. Prepare 20 mL mTeSR™1 with 10 μM Rock inhibitor and prewarm in a biological safety hood to room temperature (20°C–25°C).
   d. Dissociate 90% confluent hESC cultures into single cell suspension using Accutase (Refer to enzymatic passaging).
   e. After centrifugation, resuspend cell pellet in 5 mL mTeSR™1 supplemented with 10 μM Rock inhibitor.
   f. Count the cell number by hemocytometer.
   g. Seed 11,250,000 hESCs (150,000 cells / cm^2) in 15 mL mTeSR™1 supplemented with 10 μM Rock inhibitor on a T75 flask coated with GFR-Matrigel.

Note: Scale the media volumes and cell densities when different cultureware is used based on the surface area.

6. On day 0, aspirate the hESC culture medium and wash the flask with 5 mL PBS twice.
7. Add 15 mL RPMI medium supplemented with 15 μL of 100 μg/mL hActivin A and 3 μL of 10 mM Chir99021.
8. On day 1, refresh the medium with 15 mL RPMI medium supplemented with 15 μL of 100 μg/mL hActivin A, 15 μL of 50 mg/mL Vitamin C and 7.5 μL of 10 μg/mL hFGF-2.
9. On day 2, refresh the medium with 15 mL RPMI medium supplemented with 15 μL of 100 μg/mL hActivin A, 15 μL of 50 mg/mL Vitamin C and 7.5 μL of 10 μg/mL hFGF-2.
10. On day 3, refresh the medium with 15 mL B27-RPMI medium supplemented with 7.5 μL of 100 μg/mL hKGF and 15 μL of 50 mg/mL Vitamin C.
11. On day 4 and 5, replace the medium with the same medium as day 3.
12. On day 6, refresh the medium with 15 mL B27-DMEM medium supplemented with 7.5 μL of 0.5 mM SANT-1, 7.5 μL of 4 mM Retinoic acid, 7.5 μL of 100 μg/mL hNoggin, 7.5 μL of 100 μg/mL hKGF and 15 μL of 50 mg/mL Vitamin C.
13. On day 7, refresh the medium with the same medium as day 6.
14. On day 8, refresh the medium with 15 mL B27-DMEM medium supplemented with 7.5 μL of 100 μg/mL hNoggin, 7.5 μL of 100 μg/mL hEGF, 150 μL of 1 M Nicotinamide and 15 μL of 50 mg/mL Vitamin C.
15. On day 9–12, refresh the medium with the same medium as day 8.
16. On day 13, the pancreatic progenitor cells are ready for harvesting.

Pancreatic beta-like cells differentiation

Timing: 16 days

Differentiation of pancreatic beta-like cells from progenitor cells contains 2 stages: day 13 to day 19, stage 5 for immature beta-like cells; and day 20 to day 28, stage 6 for mature beta-like cells. This part is modified based on.[4,5]

17. On day 13, change to 3D culture.
    a. Prewarm 5 mL 1 U/mL Dispase in a 37°C water bath.
    b. Aspirate the medium and rinse the cells with 5 mL PBS twice.
    c. Add 5 mL prewarmed Dispase into the culture and place the culture ware in a 37°C and 5% CO_2 humidified incubator for 7 min.
    d. Gently aspirate the Dispase and use 5 mL DMEM to wash the culture once.
    e. Add 10 mL DMEM and gently detach the cells by a cell lifter.

    Note: Take care to minimize the breakup of colonies.

    f. Transfer the detached cell aggregates into a 15 mL conical tube.
    g. Gently use pipette to break the cell patches into cell aggregates (100–200 μm).

    ⚠ CRITICAL: This process must be very gentle. Too small aggregates may die during differentiation, and too big aggregates may cause lower differentiation efficiency.

    h. Centrifuge the cell aggregates at 80 × *g* for 2 min and aspirate the supernatant.
    i. Add 12 mL MCDB131 differentiation medium supplemented with 6 μL of 0.5 mM SANT-1, 6 μL of 200 μM Retinoic acid, 2.4 μL of 50 mM RepSox, 2.4 μL of 5 mM GC1, 12 μL of 1 mM Compound E, 1.2 μL of 1 mM LDN193189, 12 μL of 50 mg/mL Vitamin C and 12 μL of 10 mM Rock inhibitor into the pellet.
    j. Gently pipette the cell aggregates up and down.

k. Aliquot 2 mL into each well of one Ultra-Low Attachment 6 Well plate.

Note: One Ultra-Low Attachment 6 Well plate is enough for one T75 flask of cell aggregates.

18. On day 14, the cell aggregates will form 3-dimensional (3D) spheroids spontaneously.
    a. Check the 3D culture.

Note: The diameter of most aggregates should be between 100 to 200 μm.

    b. Refresh medium in 3D culture.
        i. Transfer the culture into a 15 mL conical tube.
        ii. Let the cell spheroids settle down for 10 min with no disturbance.

Note: Cell spheroids will gather at the bottom of the tube.

        iii. Remove the supernatant and replace with the same differentiation medium as day 13.
        iv. Plate the cell spheroids back to the same Ultra-Low Attachment 6 Well plate.
19. On day 15, check the cell spheroids without changing medium.

Note: If big clumps formed, disrupt the clumps by triturating with a P200 pipettor gently when necessary.

20. On day 16 to day 19, refresh the medium with 12 mL MCDB131 differentiation medium supplemented with 2.4 μL of 50 mM RepSox, 2.4 μL of 5 mM GC1, 12 μL of 1 mM Compound E, 1.2 μL of 1 mM LDN193189, 12 μL of 50 mg/mL Vitamin C and 12 μL of 10 mM Rock inhibitor every other day.
21. On day 20 to day 28, refresh the medium with 12 mL MCDB131 differentiation medium supplemented with 2.4 μL of 50 mM RepSox, 2.4 μL of 5 mM GC1, 2.4 μL of 50 mM Trolox, 2.4 μL of 10 mM R428, 24 μL of 500 mM N-acetyl cysteine and 12 μL of 50 mg/mL Vitamin C every other day.
22. On day 29, the human beta-like cells are ready to harvest for quality examination including immunofluorescence assay or downstream functional assays.

Negative enrichment of human beta-like cells through CD9

Timing: 2 days (for steps 23 to 25)

This part describes steps for negatively enriching beta-like cells through targeting the cell surface marker CD9 based on fluorescence activated cell sorter, followed by re-aggregation of the CD9 negative cells by AggreWell 400.

23. On day 29, collect the beta-like cells.
    a. Transfer the beta-like cell clusters into a 50 mL conical tube.
    b. Let the cell clusters settle down for 10 min without disturbance.

Note: Cell clusters will gather at the bottom of the tube.

    c. Prewarm 10 mL TrypLE Express in a 37°C water bath in the meantime.
    d. Aspirate the supernatant and add 5 mL PBS to wash the cell clusters.
    e. Let the clusters settle down again for another 10 min without disturbance and aspirate the supernatant.
    f. Add 10 mL prewarmed TrypLE Express.
    g. Gently pipette up and down by P1000 for 12 min.

Note: Pipetting must be very gentle to reduce the damage to the cells during dissociation.

h. Add 20 mL MCDB131 differentiation medium to quench the enzymatic dissociation.
i. Centrifuge the cells at 500 × *g* for 5 min.
j. Aspirate the supernatant.
k. Wash the cells once with 5 mL FACS buffer.

24. Staining of CD9 for sorting.
    a. Count the cell numbers with a hemocytometer.
    b. Add 5 μL conjugated anti-human CD9-PE antibody per 1 million cells in 100 μL FACS buffer.
    c. Prepare isotype control staining at the same time.
    d. Incubate on ice for 30 min in the dark.
    e. Cells are then washed twice with FACS buffer and resuspended in 1 mL FACS buffer.
    f. Perform fluorescence activated cell sorting.

Note: Dead cells are excluded by Dapi positive staining for live cell analysis. Dapi is diluted freshly in PBS (1:10,000). Use isotype control for gating out the CD9 negative population.

g. Use FACS Collection buffer to collect cells and sort 600,000 CD9 negative cells in each collection tube.

Note: Generally, one Ultra-Low Attachment 6 Well Plate of beta-like cell culture can generate 3 million cells.

25. Re-aggregate the CD9 negative cells.
    a. Prepare one AggreWell™400 24-well plate according to the product instructions.
    b. After sorting, centrifuge the collection tubes at 500 × *g* for 5 min.
    c. Prepare 5 mL MCDB131 differentiation medium supplemented with 1 μL of 50 mM RepSox, 1 μL of 5 mM GC1, 1 μL of 50 mM Trolox, 1 μL of 10 mM R428, 10 μL of 500 mM N-acetyl cysteine, 1 μL of 50 mg/mL Vitamin C and 1 μL of 10 mM Rock inhibitor.
    d. Resuspend each pellet by 1 mL of the above medium and seed the cells into one well of one AggreWell™400 24-well plate.
    e. After overnight (18–24 h) culture, sorted CD9 negative cells will spontaneously self-organize into spherical 3D organoids.

Note: Cells can then be harvested for downstream functional assay, such as *in vitro* glucose-stimulated insulin secretion.

Final concentration of growth factors and chemicals for beta cell differentiation

Day No.	Medium	Final concentration of growth factors and chemicals
D-1	mTeSR™1	10 μM Rock inhibitor
D0	RPMI1640	100 ng/mL hActivin A, 2 μM Chir99021
D1,2	RPMI1640	100 ng/mL hActivin A, 5 ng/mL hFGF-2, 50 μg/mL Vitamin C
D3-5	RPMI1640, 2% B27	50 ng/mL hKGF, 50 μg/mL Vitamin C
D6-7	DMEM, 2% B27	50 ng/mL hKGF, 50 μg/mL Vitamin C, 0.25 μM SANT-1, 2 μM Retinoic acid, 50 μg/mL hNoggin
D8-12	DMEM, 2% B27	50 μg/mL Vitamin C, 50 ng/mL hNoggin, 50 ng/mL hEGF, 10 mM Nicotinamide
D13-15	MCDB131 differentiation medium	0.25 μM SANT-1, 100 nM Retinoic acid, 10 μM RepSox, 1 μM GC1, 100 nM LDN193189, 1 μM Compound E, 10 μM Rock inhibitor, 50 μg/mL Vitamin C

(*Continued on next page*)

Continued

Day No.	Medium	Final concentration of growth factors and chemicals
D16-19	MCDB131 differentiation medium	10 μM RepSox, 1 μM GC1, 100 nM LDN193189, 1 μM Compound E, 10 μM Rock inhibitor and 50 μg/mL Vitamin C
D20-28	MCDB131 differentiation medium	10 μM RepSox, 1 μM GC1, 10 μM Trolox, 2 μM R428, 1 mM N-acetyl cysteine, 50 μg/mL Vitamin C
D29-30	MCDB131 differentiation medium	10 μM RepSox, 1 μM GC1, 10 μM Trolox, 2 μM R428, 1 mM N-acetyl cysteine, 50 μg/mL Vitamin C, 10 μM Rock inhibitor

Immunofluorescence assay for frozen section of beta cells

Timing: 2 days

This part describes steps for preparation of frozen section of pancreatic progenitors and beta-like cells clusters and immunofluorescence staining assay.

26. Preparation for OCT frozen section.
    a. Transfer the progenitor or beta-like cell clusters into 1.5 mL centrifuge tube.
    b. Centrifuge the tube at 80 × *g* for 2 min.

    Note: The cell clusters will be pelleted, and dead cells will be in the supernatant.

    c. Aspirate the supernatant and add 1 mL PBS to wash the cell clusters.
    d. Centrifuge the tube at 80 × *g* for 2 min and remove the supernatant.
    e. Fix the cell clusters with 1 mL 4% PFA for 1 h at room temperature (20°C–25°C).
    f. Centrifuge the tube at 80 × *g* for 3 min to pellet the cell clusters.
    g. Remove the supernatant PFA and add 1 mL PBS to wash the cell clusters for 3 times.
    h. Equilibrate the cell clusters with 30% sucrose at 4°C overnight (18–24 h).

    Note: Cell clusters will be settled down at the bottom after overnight (18–24 h) equilibration.

    i. Remove the sucrose as much as possible.
    j. Add one drop of OCT compound to the cell clusters and transfer the cell cluster with OCT to a disposable base mold (7 × 7 mm). Store at −80°C.
    k. Cut 5 μm sections by a cryostat and place on Superfrost plus slides.

    Pause point: The slides can be used for immunostaining directly or stored at −80°C.

27. Immunofluorescence staining assay
    a. Put the slides at room temperature (20°C–25°C) for 1 h prior to immunostaining.
    b. Wash the slides in PBS once to remove the OCT.

    Note: All washing step is performed in coplin staining jar at room temperature (20°C–25°C).

    c. Permeabilize the cells by incubating the slides with 0.5% Tween-20 in PBS for 10 min.
    d. Use Pap pen to draw a liquid repellent barrier around the section of cell clusters.
    e. Add 200 μL 10% donkey serum in PBST to the middle of the section for blocking for 30 min.
    f. Tap off the blocking buffer.
    g. Incubate the section with 100–200 μL primary antibodies diluted in 1% BSA PBST (PDX1 1:200, NKX6.1, 1:400; C-peptide, 1:1000) at 4°C overnight (18–24 h).

h. The second day, remove the primary antibody and wash the slides with PBST for 3 times.
i. Incubate the sections with 200 μL secondary antibodies (Alexa-Donkey anti rat 488 or Alexa-Donkey anti rabbit 488, Alexa-Donkey anti goat 594, 1:600 dilution) diluted in 1% BSA PBST for 1 h at room temperature (20°C–25°C).

Note: Prepare the secondary antibodies freshly and protect from light for staining.

j. Remove the secondary antibodies.
k. Add Dapi or Hoechst 33342 (1:10,000 diluted in distilled water) for 10 min.

Note: Prepare the Dapi or Hoechst 33342 freshly and protect from light.

l. Wash the slides with PBST for 3 times.
m. Add one drop of mounting medium to the center of section.
n. Place the coverslip on top of the mounting media.
o. The slides can be imaged using a fluorescence microscope.

Intracellular flow cytometry for C-peptide and NKX6.1 of beta cells

Timing: 2 days

28. Intracellular staining for flow cytometry.
    a. Transfer one well of 6-well ultra-low attachment plate to 15 mL conical tube.
    b. Let the cell clusters settle down for 10 min without disturbance.

    Note: Cell clusters will gather at the bottom of the tube.

    c. Prewarm 2 mL TrypLE Express in a 37°C water bath in the meantime.
    d. Aspirate the supernatant and add 2 mL PBS to wash the cell clusters.
    e. Let the clusters settle down again for another 10 min without disturbance.
    f. Aspirate the supernatant.
    g. Add 2 mL prewarmed TrypLE Express and pipette gently up and down by P1000 for 12 min.

    Note: Pipetting must be very gentle to reduce the damage to the cells during dissociation.

    h. Add 2 mL MCDB131 differentiation medium to quench the enzymatic dissociation.
    i. Centrifuge the cells at 500 × *g* for 5 min.
    j. Aspirate the supernatant and wash the cells once with PBS.
    k. Add 1 mL cold 4% PFA to fix the cells on ice for 1 h.
    l. Centrifuge the cells at 500 × *g* for 5 min and remove the PFA.

    Note: Swinging bucket rotor is recommended for centrifuge to avoid the loss of cells.

    m. Wash the cells with PBS twice.
    n. Incubate the cells with 300 μL Perm/Blocking buffer on ice for 1 h.
    o. Centrifuge the cells at 500 × *g* for 5 min.
    p. Resuspend the pellet in 200 μL Perm/blocking buffer with goat anti-NKX6.1 antibody (1:200 dilution) and rat anti-C-peptide antibody (1:500 dilution).
    q. Incubate at 4°C overnight (18–24 h).
    r. Wash the cells with Perm/Blocking buffer twice.
    s. Incubate the cells with 200 μL Perm/Blocking buffer with Alexa-Donkey anti rat 488, Alexa-Donkey anti goat 647 (1:1000 dilution) at room temperature (20°C–25°C) for 1 h.

Note: Prepare the secondary antibodies freshly and protect from light for staining.

t. Wash the cells with Perm/Blocking buffer for 3 times.
u. Resuspend the cells in 500 μL FACS buffer for flow acquisition.
v. Acquire flow cytometry data with a CytoFLEX Platform (can be replaced with other flow cytometry) using excitation lines at 488 nm and 635 nm.

Note: Intact cells are identified based on forward and side light scatter. Cells stained with secondary antibody alone are used for background estimation and gating strategy.

Glucose stimulated insulin secretion assay

Timing: 1 day (for step 29)

This part describes steps for glucose-stimulated insulin secretion assay for beta-like cell clusters from Aggrewell400. It can also be used for beta cell clusters from ultra-low attachment plates. All buffers are equilibrated to 37°C before use.

29. Glucose stimulated insulin secretion assay for CD9- beta-like cells.
    a. Collect the beta cell clusters from Aggrewell400 to 1.5 mL micro-centrifuge tubes.
    b. Centrifuge the tube at 80 × *g* for 2 min
    c. Wash the cell pellets with Krebs buffer twice.
    d. Incubate the cell clusters with 1 mL low glucose Krebs for 1 h in a 5% CO_2/37°C incubator.
    e. Wash the cell cluster with Krebs buffer twice.
    f. Incubate in 1 mL low glucose Krebs for 40 min.
    g. Centrifuge the tube at 80 × *g* for 2 min and collect the supernatant.
    h. Wash the cell cluster with Krebs buffer twice.
    i. Incubate in 1 mL high glucose Krebs for 40 min.
    j. Centrifuge the tube at 80 × *g* for 2 min and collect the supernatant.
    k. Wash the cell cluster with Krebs buffer twice.
    l. Incubate in 1 mL KCl Krebs buffer for 40 min.
    m. Centrifuge the tube at 80 × *g* for 2 min and collect the supernatant.
    n. Dissociate the cell pellets with TrypLE Express Enzyme.
    o. Count the cell number by hemocytometer.
    p. Filter all the supernatant is through 0.22 μm syringe filter.

    Pause point: Filtered supernatant can be used for determination of insulin directly or stored at −80°C until use.

    q. Quantify the human insulin with human ultrasensitive insulin ELISA kit and normalize the data by cell number.

    Note: The incubation time for each step can be adjusted from 30 min to 2 h depending on the cell number. The supernatant can be diluted to make sure the insulin measurements are within the range of the standard.

EXPECTED OUTCOMES

Pancreatic progenitor differentiation (S4, day 13)

On day 13, the cells at the end of stage 4 will transit from two-dimensional (2D) to three-dimensional (3D) cell differentiation (Figure 1B, Day 13). Generally, cells number can reach 30–40 million per T75 flask at Stage 4. To verify the cell lineage, we can check the expression of PDX1 and NKX6.1 in

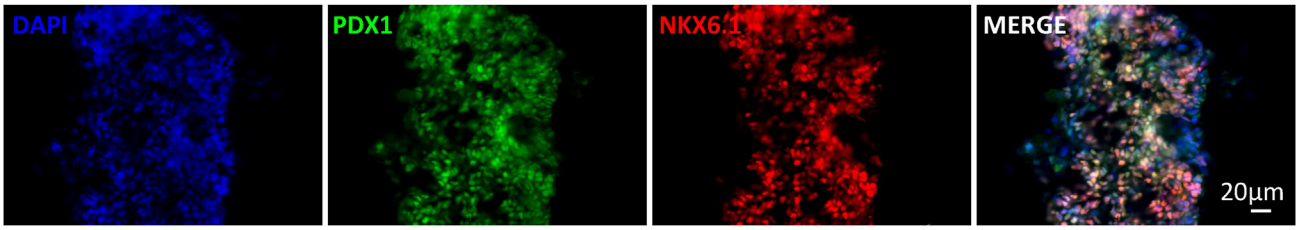

Figure 2. Representative immunofluorescent staining of S4 cells for PDX1 and NKX6.1
DAPI (blue), PDX1 (green) and NKX6.1 (red) of day 13 cells. Scale bar represents 20 μm.

pancreatic progenitor cells by immunofluorescence. Figure 2 shows the expected differentiation efficiency as determined by marker expression. PDX1 and NKX6.1 double positive pancreatic progenitor cells will be regarded as pancreatic progenitors. After counting PDX1+NKX6.1+ cells among all cells, percent of PDX1+NKX6.1+ cells should reach approximately 50% at Stage 4.

Pancreatic beta-like cells differentiation (S6, day 29)

On day 29, one T75 can generate 10–15 million 3D beta-like cells in one ultra-low plate. Cells can be harvested for immunofluorescence assay. Figure 3 shows the expected differentiation efficiency of H9 into NKX6.1 and C-PEPTIDE double positive beta-like cells. NKX6.1+ and C-PEPTIDE+ cells should reach around 30% at Stage 6.

Negative enrichment of human beta-like cells through CD9 (day 29–30)

Figure 4A shows the gating of CD9 negatively sorted cells by flow cytometry. The percentage of CD9 negative cells accounts for about 50%. After an overnight culture (18–24 h), 500 sorted CD9 negative cells can spontaneously self-organize into spherical 3D organoids in AggreWell (Figure 1B, Day 30). Around 3 million CD9 negative cells can be purified from one Ultra-low Attachment plate. Figure 4B shows immunofluorescence staining for NKX6.1 and C-PEPTIDE on CD9 negative organoids. After enrichment, the percentage of NKX6.1 and C-PEPTIDE double positive cells among CD9 negative beta-like cells should be increased by at least 10% compared with cells before enrichment. The CD9 negative beta-like spheroid can perform the glucose stimulated insulin secretion, refer to.[1]

QUANTIFICATION AND STATISTICAL ANALYSIS

We use the percentage of NKX6.1 and C-PEPTIDE double positive cells in total cells to determine the differentiation efficiency of mature beta-like cells. By quantification using immunofluorescence, 20 beta-like clusters per group should be counted and repeated for three times with a total of 60 beta-like clusters per group. Flow cytometry analysis can be used for quantification as well.

LIMITATIONS

We have confirmed this protocol with other hESC lines such as H9, H1 and H7 hESCs.[1] We did not try differentiation of beta-like cells from human induced pluripotent stem cells (hiPSCs) using this protocol. The cell seed density, concentration of growth factors and inhibitors used in this protocol may need to be optimized again for other cell lines.

TROUBLESHOOTING

Problem 1

On day 1, there are a lot of dead cells floating in the culture ware.

Potential solution

It is normal to observe dead cells on day 1 since the differentiation medium does not contain serum or cell culture supplement. On day 0, hESCs should reach more than 80% confluency and on

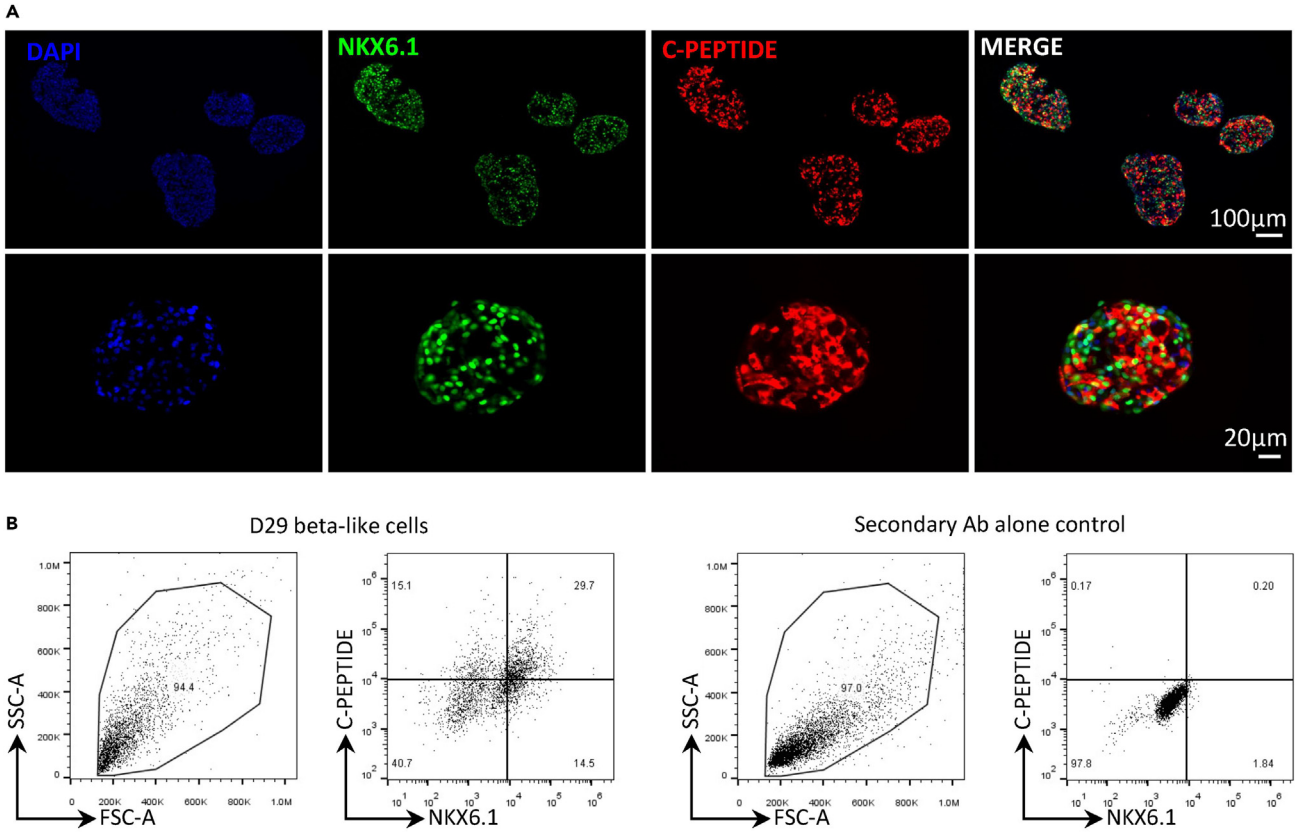

Figure 3. The expected differentiation efficiency of hESCs into NKX6.1+ C-PEPTIDE+ human pancreatic beta-like cells

(A) Immunofluorescence for DAPI (blue), NKX6.1 (green) and C-PEPTIDE (red) of day 28 cells.

(B) Flow cytometry analysis of S6 beta-like cells for expression of NKX6.1 and C-PEPTIDE. Cells staining with secondary antibody alone were used for background estimation.

day 1, the differentiated cells should reach more than 50% confluency. If most cells are dead, the seeding density of hESCs on day −1 should be adjusted.

Problem 2

The cells are not easily detached after Dispase incubation.

Potential solution

Prolong the incubation time or increase the Dispase concentration.

Problem 3

On day 14, cell aggregates cannot form spheroids spontaneously after the overnight culture in Ultra-Low Attachment 6 Well Plate.

Potential solution

The process of breaking cell aggregates should be very gentle. Make sure the diameter of most aggregates is between 100 to 200 μm. Aggregates that are too small will die easily during differentiation. It is important to add 10 μM Rock inhibitor in day 14 differentiation medium to enhance the cell viability.

Problem 4

On day 28, the %NKX6.1+C-peptide is low than 20%.

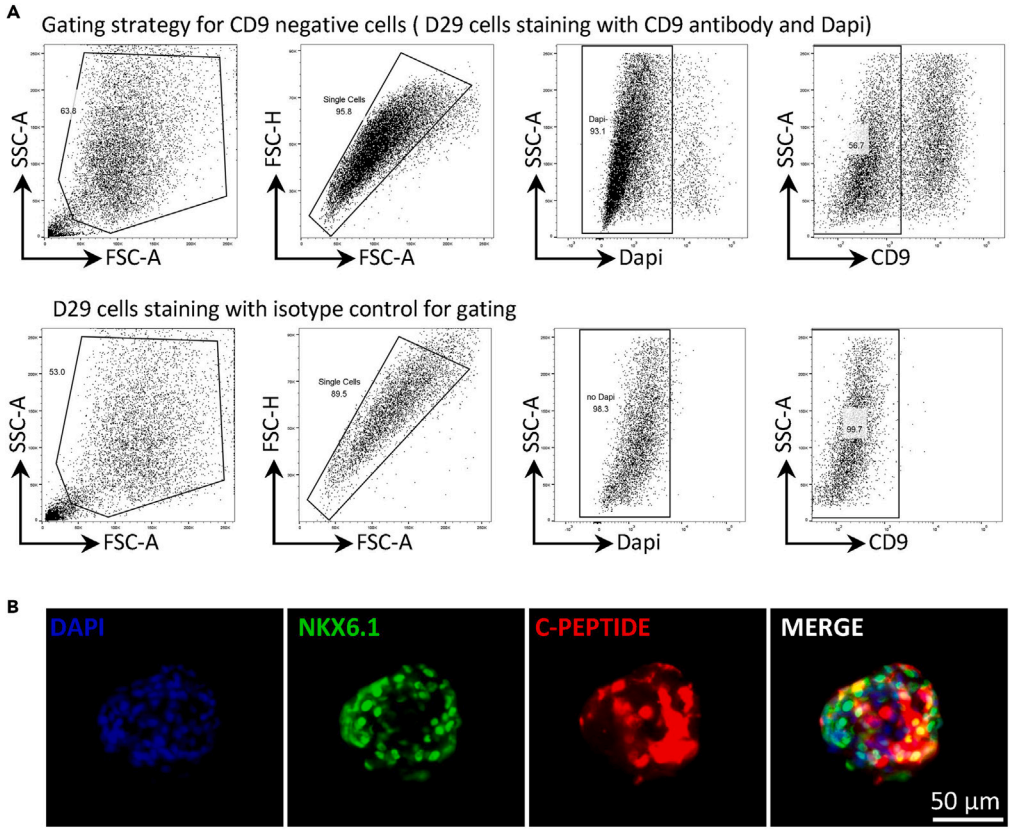

Figure 4. Enrichment of CD9 negative human pancreatic organoids with flow cytometry followed by reaggregation in AggreWell

(A) FACS-sorting CD9 negative cells at S6 (day 29). Upper panel, CD9-PE and Dapi staining; lower panel, isotype control without Dapi staining.

(B) Immunofluorescence for C-PEPTIDE (red) and NKX6.1 (green) of CD9- organoids. Scale bar represents 50 µm.

Potential solution

Make sure the differentiation media are freshly prepared and add the recombinant proteins and/or small molecules at the correct concentrations respectively. The pluripotency of hESCs is compromised. Check the specific stem cell markers such as TRA-1-60, OCT 4 by flow cytometry to determine the pluripotency.

Problem 5

After sorting, CD9 negative cells cannot form spheroids spontaneously in AggreWell™400 wells.

Potential solution

Add 10 µM Rock inhibitor in day 29 differentiation medium to enhance the cell viability. Treat the AggreWell 400 wells according to the product instructions before seeding the cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Kathy O. Lui (kathyolui@cuhk.edu.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate and/or analyze any new dataset.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.L., K.O.L.; methodology, X.L., Z.M.; writing – original draft: X.L.; writing – review & editing: K.O.L.; supervision, project administration, and funding acquisition: K.O.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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