Title	**Hematopoietic differentiation**
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Submitted by -	Sunita D'Souza
Adapted from -	**Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells** Kyung-Dal Choi, Maxim Vodyanik & Igor I Slukvin Nature Protocols 6, 296–313 (2011) doi:10.1038/nprot.2010.18, Published online 17 February 2011
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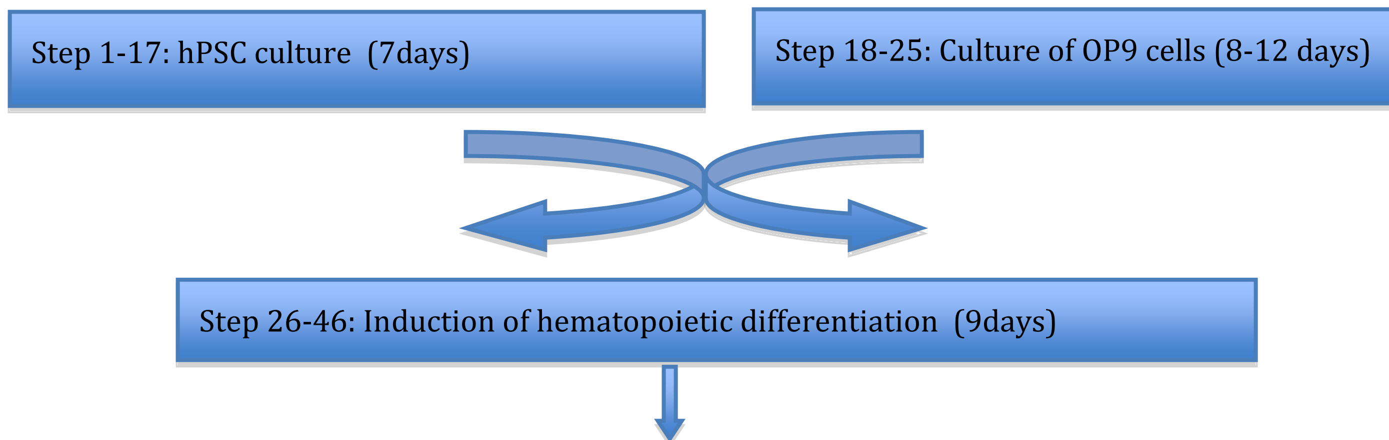
Hematopoietic differentiation adapted from Igor Slukvin Lab (Nature Protocols 2011)

INTRODUCTION

A protocol for hematopoietic differentiation of human pluripotent stem cells (hPSCs) and generation of mature myeloid cells from hPSCs through expansion and differentiation of hPSC-derived lin$^-$CD34$^+$CD43$^+$CD45$^+$ multipotent progenitors. The protocol comprises three major steps: (i) induction of hematopoietic differentiation by coculture of hPSCs with OP9 bone marrow stromal cells; (ii) short-term expansion of multipotent myeloid progenitors with a high dose of granulocyte-macrophage colony-stimulating factor; and (iii) directed differentiation of myeloid progenitors into neutrophils, eosinophils, dendritic cells, Langerhans cells, macrophages and osteoclasts. The generation of multipotent hematopoietic progenitors from hPSCs requires 9 d of culture and an additional 2 d to expand myeloid progenitors. Differentiation of myeloid progenitors into mature myeloid cells requires an additional 5–19 d of culture with cytokines, depending on the cell type.

FLOWCHART



Step 47-83: Expansion and Isolation of Lin-CD34+CD43+ CD45+ hematopoietic progenitors enriched in myeloid CFC

Generation of mature myelo monocytic cells

1. Neutrophils (8-10 days)
2. Eosinophils (12-14 days)
3. Macrophages (5-7 days)
4. Dendritic cells (7 days)
5. Langerhans cells (7 days)
6. Osteoclasts (16-19 days)
7.)

Reagents	Company	Cat #
hESC WA01 and WA09	National stem cell bank	-
Lentivirally reprogrammed iPSCs iPS (Foreskin)	National stem cell bank	-
Transgene-free iPSCs DF-19-9-7T and 4-3-7T	WiscBank	iPS-DF19-9-7T / iPS-DF4-3-7T.A
OP9 mouse bone marrow stromal cell line	ATCC	CRL-2749
Mouse Embryonic Fibroblasts	WiCell Research Institute	-
Dulbecco's modified eagle medium (DMEM), powder	GIBCO-Invitrogen	12100-046
KNOCKOUT SR (KO), serum replacement for ES cell	GIBCO-Invitrogen	10828-023
Serum-free medium, Stemline Hematopoietic stem cell expansion medium	Sigma Aldrich	S-0189
Sodium azide, NaN_3	Fisher Scientific	BP922-500
Ascorbic acid	SIGMA-ALDRICH	A4544
DMEM/nutrient mixture F-12, powder	GIBCO-Invitrogen	12400-024
α-MEM basal medium, powder	GIBCO-Invitrogen	12000-022
Iscove's modified Dulbecco's medium (IMDM), powder	GIBCO-Invitrogen	12200-036
L-glutamine	CellGro	61-030-RM
MEM non-essential amino acid, 100× solution	GIBCO-Invitrogen	11140
2-mercaptoethanol	SIGMA-ALDRICH	M7522
Sodium Bicarbonate	Fisher Scientific	S233-500
Poly (2-hydroxyethyl methacrylate) (p-HEMA)	SIGMA-ALDRICH	P3932
NaOH	Fisher Scientific	S318
Percoll, solution	SIGMA-ALDRICH	P-1644
PBS powdered, without calcium and magnesium	GIBCO-Invitrogen	21600-044
PBS, 10×	GIBCO-Invitrogen	70011-044
Gelatin from porcine skin, Type A	GIBCO-Invitrogen	G-1890
EDTA 0.5M, pH 8.0	GIBCO-Invitrogen	15575-038
MTG, monothioglycerol	SIGMA-ALDRICH	M-6145
Fetal bovine serum defined	Hyclone	SH30070.03

Trypsin 0.05%/EDTA 0.5mM	Hyclone	SH30236.02
Cell dissociation buffer, enzyme free, PBS-based	GIBCO-Invitrogen	13151-014
Collagenase Type IV	GIBCO-Invitrogen	17104-019
Ex-Cyte	Celliance-Millipore	81-129-1
Neupogen as a human G-CSF	Amgen	
Leukine as a human GM-CSF	Berlex	
Human M-CSF	Peprotech	300-25
Human FGF-basic	Peprotech	100-18B
Human Flt3-Ligand	Peprotech	100-18B
Human IL-1β	Peprotech	200-01B
Human IL-3	Peprotech	200-03
Human IL-4	Peprotech	200-04
Human IL-5	Peprotech	200-05
Human sRANKL	Peprotech	300-01
Human TGF-β_1	Peprotech	100-21
Human TNF-α	Peprotech	300-01A
1α, 25-Dihydroxyvitamin D_3, Biologically active form of vitamin D_3	SIGMA-ALDRICH	D-1530
Trypan blue solution, 0.4%	SIGMA-ALDRICH	T8154
Protocol Wright stain	Fisher Scientiific	23-264980
Protocol Phosphate buffer solution for Wright stain,pH 6.4	Fisher Scientiific	23-262234
Cytoseal 60 mounting medium	Richard-Allan Scientific-ThermoFisher	8310-4
Immersion oil	SIGMA-ALDRICH	56822
7-AAD; 7-Aminoactinomycin D	SIGMA-ALDRICH	A9400
Bovine serum albumin Fraction V	Fisher Scientific	BP1605-100
Ethanol	SIGMA-ALDRICH	E7023
Cell strainer, 40µm	BD Bioscience	352340
Cell strainer, 70µm	BD Bioscience	352350
MACS separation columns, LD	Miltenyi Biotec	130-042-303
MACS separation columns, LS	Miltenyi Biotec	130-042-401
Midi MACS separation unit	Miltenyi Biotec	130-042-302
MACS Multistand	Miltenyi Biotec	130-042-303
MACSmix Tube Rotator	Miltenyi Biotec	130-090-753
Pre-separation filters with 30µm nylon mesh	Miltenyi Biotec	130-041-407
Nalgene Disposable bottle top filter, Polyethersulfone membrane with 0.2µm pore size	Fisher Scientific	595-4520
T25 Tissue culture flask canted neck with 0.2µm vented plug seal cap, 50ml, Nonpyrogenic polystyrene	BD Bioscience	353108
T75 Tissue culture flask canted neck with 0.2µm vented plug seal cap, 250ml, Nonpyrogenic polystyrene	BD Bioscience	353136
Tissue culture dishes, polystyrene 100×20 mm	BD Bioscience	353003
Tissue culture 6well plate, Polystyrene flat bottom	BD Bioscience	353046
0.5ml microcentrifuge tube, autoclavable	Fisher Scientific	05-408-120
5ml Polystyrene round-bottom tube, 12×75mm, non-sterile	BD Bioscience	352008
5ml Polystyrene round-bottom tube with 35µm cell strainer cap, 12×75mm	BD Bioscience	352235
15ml Polypropylene Conical tubes	BD Bioscience	352097
50ml Polypropylene Conical tubes	BD Bioscience	352098
50ml Vacuum filtration system with 0.22µm pore size membrane	Millipore	SCGP00525
Serological pipet, 1ml Nonpyrogenic	Fisher Scientific	13-678-11B
Serological pipet, 5ml Nonpyrogenic	Fisher Scientific	357543

Serological pipet, 10ml Nonpyrogenic (. Cat. no. 357551)	BD Bioscience	357551
Borosilicate glass pipets, 5ml	Corning	7077-5N
Object marker, Cell dotter for inverted microscope	Nikon	MBW10020
Hemocytometer, Reichert Bright-Line counting chamber	Fisher Scientific	02-671-5
9″ Pasteur pipets, Flint glass	Fisher Scientific	13-678-6B
Sterling Nitrile-xtra powder-free exam gloves		53139

- hESC WA01 and WA09 (National KNOCKOUT SR (KO), serum replacement for ES cell (GIBCO-Invitrogen, Cat. no. 10828-028). CRITICAL: Each lot should be tested for its suitability for hPSC culture.

- Fetal bovine serum defined (Hyclone, Logan, UT, USA, Cat. no. SH30070.03). CRITICAL: FBS used in this protocol is defined FBS. Use FBS directly without the heat inactivation step for OP9 culture, hematopoietic differentiation, expansion of myeloid progenitors, and the generation of mature myelomonocytic cells from human pluripotent stem cells. Heat inactivation does not benefit culture but usually results in a higher adipogenic effect on OP9. CRITICAL: We have found that different lots of HyClone defined FBS provide relatively stable hematopoietic differentiation in hPSC/OP9 coculture and support efficient OP9 growth without significant adipogenesis. Results from other suppliers are more variable.

Antibody list

Name	**Flurophore**	**Clone**	**Company**	**Catalogue #**
Anti-human CD41a	PE	HIP8	BD Biosciences	555467
Anti-human CD45	FITC	HI30	BD Biosciences	555482
Anti-human CD235a	PE	GA-R2 (HIR2)	BD Biosciences	555570
Anti-Mouse CD29	PE	HM beta 1-1	Serotec	MCA2298PE
Anti-FITC microbeads	None	None	Miltenyi Biotec	120-000-293
Anti-PE microbeads	None	None	Miltenyi Biotec	120-000-294

Antibodies used to analyze differentiation of hematopoietic progenitors and myeloid lineages from human pluripotent stem cells

Name	**Fluorochrome**	**Clone**	**Company**	**Cat. no.**
Anti-human CD1a	PE	VIT6b	CALTAG-Invitrogen	MHCD1a04
Anti-human CD2	FITC	RPA-2.10	BD Biosciences	555326
Anti-human CD3	FITC	SK7	BD Biosciences	349201
Anti-human CD7	PE	M-T701	BD Biosciences	555361
Anti-human CD10	PE	HI10a	BD Biosciences	555375
Anti-human CD11b	FITC	VIM12	CALTAG-Invitrogen	CD11b01
Anti-human CD13	PE	TüK1	CALTAG-Invitrogen	MHCD1304
Anti-human CD14	FITC	M5E2	BD Biosciences	555397
Anti-human CD15	FITC	VIMC6	Miltenyi Biotec	130-081-101
Anti-human CD16	PE	3G8	CALTAG-Invitrogen	MHCD1604
Anti-human CD19	PE	HIB19	BD Biosciences	555413
Anti-human CD34	APC	581	BD Biosciences	555824
Anti-human CD41a	PE	HIP8	BD Biosciences	555467

Name	Fluorochrome	Clone	Company	Cat. no.
Anti-human CD43	FITC	1G10	BD Biosciences	555475
Anti-human CD45	APC	HI30	BD Biosciences	555485
Anti-human CD64	FITC	10.1	CALTAG-Invitrogen	CD6401
Anti-human CD66b	FITC	G10F5	BD Biosciences	555724
Anti-human CD90 (Thy-1)	APC	5E10	BD Sciences	559869
Anti-human CD115	PE	61708	R&D Systems	FAB329P
Anti-human CD117	APC	YB5.B8	BD Biosciences	550412
Anti-human CD123	FITC	AC145	Miltenyi Biotec	130-090-897
Anti-human CD163	PE	215927	R&D Systems	FAB1607P
Anti-human CD235a	PE	GA-R2(HIR2)	BD Biosciences	555570
DC-SIGN	FITC	DCN46	BD Biosciences	551264
HLA-DR	PE	Tü36	CALTAG-Invitrogen	MHLDR04
Lactoferrin*	PE	3C5	CALTAG-Invitrogen	GIC206
Langerin*	PE	343828	R&D Systems	FAB2088P
Major basic protein (MBP)*	None	AHE-2	BD Biosciences	550843
Myeloperoxidase (MPO)*	FITC	H-43-5	CALTAG-Invirogen	GIC205
Anti-human TRA-1-85	APC	TRA-1-85	R&D Systems	FAB3195A

REAGENTS SETUP

Collagenase solution (1mg/ml) : Add 50 mg of collagenase to 50 ml of DMEM/F-12 basal medium, and sterilize the solution by filtration using a 0.22 μm membrane filter. Keep solution at 2–8°C and use for up to one week.

0.1% Gelatin solution (w/v) : Add 500 mg of gelatin to 500 ml of endotoxin-free reagent grade distilled water. Solubilize and sterilize by autoclaving for 20 min at 121°C. Store the solution at 4°C for up to 6 months. Keep sterile.

Magnetic cells sorting (MACS) buffer : MACS buffer contains 5% FBS (v/v) and 2 mM EDTA in PBS (Ca^{2+} and Mg^{2+} free). For 500 ml, add 25 ml of FBS and 2 ml of 0.5M EDTA (pH 8.0) into Ca^{2+} and Mg^{2+} free-PBS. Sterilize MACS buffer by filtration using a 0.22 μm membrane filter and keep at 2–8°C for up to 6 months. Optional: After filtration, close lid of filter unit and keep MACS buffer under vacuum for about 10–15min for degassing.

Flow cytometry buffer : Flow cytometry buffer contains 2% FBS (v/v), 0.05% sodium azide (NaN_3, w/v) and 2 mM EDTA in PBS (Ca^{2+} and Mg^{2+} free). For 500 ml, add 10 ml of FBS, 0.25 g of NaN_3 and 2 ml of 0.5 M EDTA (pH 8.0) into Ca^{2+} and Mg^{2+} free-PBS. Filtrate the buffer using a 0.22 μm membrane filter and store at 2–8°C for up to 6 months.

10% pHEMA coating solution (w/v) : Add 4 g of pHEMA to 40 ml of 95% ethanol containing 10 mM NaOH. Dissolve completely by continuously rotating at room temperature or 37°C overnight. Store at room temperature until needed for use. CAUTION: It is very hard to dissolve pHEMA crystals completely when they aggregate. Thus, shake the solution immediately after addition of pHEMA to prevent precipitation.

5× Percoll solution: Add 5 ml of 10× PBS (Ca^{2+} and Mg^{2+} free) to 45 ml of Percoll solution (90% Percoll and 10% 10× PBS). Store at 4°C for 6 months. For 1× Percoll working solution, dilute 10 ml of 5× Percoll solution in 40 ml of 1× PBS (1/5 dilution). Use fresh.
100× (100 mM) L-glutamine/2-mercaptoethanol solution. Add 146 mg of L-glutamine and 7μl of 2-mercaptoethanol to 10 ml of PBS (Ca^{2+} and Mg^{2+} free). Sterilize the solution by filtration using a 0.22μm membrane filter and store up to 2 weeks at 2–8°C.

1000× (100 mM) MTG solution : Add 87 μl of MTG to 10 ml of endotoxin-free reagent grade distilled water. Mix well and divide into 500 μl aliquots. Store up to 6 months at −20°C. CAUTION: MTG has high viscosity, thus pipet slowly to dispense MTG accurately.

1000× (50 mg/ml) ascorbic acid solution: Add 500 mg of ascorbic acid to 10 ml of endotoxin-free reagent grade distilled water. Dissolve completely, divide into 500 μl aliquots, and store up to 6 months at −20°C.

1mM 1α,25-Dihydroxyvitamin D_3 stock solution : Dissolve 10 μg of 1α,25-Dihydroxyvitamin D_3 in 24 μl of 95% ethanol (Final concentration is 0.42 μg/μl in 95% EtOH). Store the stock solution at −20°C for up to 6 months.

50× 7-AAD solution: Dissolve 1 mg of 7-AAD in 50μl of absolute methanol, then add 950 μl of 1× PBS. Final concentration is 1 mg/ml. Store the solution in an amber glass bottle or tube at 4°C protected from light. Solution can be stored for at least up to 6 months. For working solution, the stock solution (1 mg/ml) is diluted with flow cytometry buffer to 20 μg/ml concentration. Use 10 μl for staining.

0.1% BSA/PBS solution: Dissolve 25mg of Bovine serum albumin Fraction V in 25ml of PBS (Ca^{2+} and Mg^{2+} free). Sterilize the solution by filtration using a 0.22μm membrane filter and store for up to 6 months at 2–8°C.

Reconstitution of cytokines: Centrifuge vials at maximum speed for 1 min to precipitate lyophilized pellet prior to opening vials. Reconstitute cytokines according to the product information provided by manufacturer. Dilute with 0.1% BSA/PBS solution for working concentration and store at −80°C until needed for use.

Gelatin-coated 10 cm culture dish and 6 well tissue-culture plate: Add 7–8 ml of autoclaved gelatin solution to a 10 cm culture dish or 2 ml to each well of a 6 well tissue-culture plate. Allow the gelatin solution to cover the entire plastic surface and incubate for at least 3 hrs at 37°C in an incubator. Dishes and 6-well plates containing gelatin solution can be stored for up to several days at 37°C in CO_2 incubator. Do not allow the wells to dry. Before use, aspirate the gelatin solution from the dish.

pHEMA-coated culture flask: Add 5 ml of 10% pHEMA/ethanol solution to a T75 tissue culture flask or 2 ml of the solution to a T25 tissue culture flask. Rotate the flask gently to allow pHEMA solution to cover the entire surface of the flask. Make sure that the flask is completely covered with pHEMA solution. Tip the flask to remove excess pHEMA solution and save to reuse. After coating, dry the flask overnight in a sterile biosafety cabinet, close the cap and store under sterile conditions at room temperature until needed for use. CRITICAL Treatment with pHEMA should be done quickly to avoid irregular coating due to rapid ethanol evaporation.

Reconstitution of cytokines

Cytokines	Product state	Reconstitution	Working dilution	Storage
Neupogen as a human G-CSF	Concentrated aqueous solution	-	100 μg/ml in 0.1% BSA/PBS solution	Aliquot 100 μl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Leukine as a human GM-CSF	Concentrated aqueous solution	-	100 μg/ml in 0.1% BSA/PBS solution	Aliquot 100 μl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.

Cytokines	Product state	Reconstitution	Working dilution	Storage
Human FGF-basic	Lyophilized pellet	1 mg/ml with 5mM Tris (pH7.6)	100 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human Flt3-Ligand	Lyophilized pellet	1 mg/ml with distilled water	100 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human IL-1β	Lyophilized pellet	100 µg /ml with distilled water	10 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human IL-3	Lyophilized pellet	100 µg /ml with distilled water	10 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human IL-4	Lyophilized pellet	1mg/ml with distilled water	100 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human IL-5	Lyophilized pellet	100 µg /ml with distilled water	10 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human M-CSF	Lyophilized pellet	100 µg /ml with distilled water	10 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human sRANKL	Lyophilized pellet	100 µg /ml with distilled water	10 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human TGF-β_1	Lyophilized pellet	50 µg/ml with 10 mM citric acid solution (pH 3.0)	5 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.
Human TNF-α	Lyophilized pellet	1mg/ml with distilled water	50 µg/ml in 0.1% BSA/PBS solution	Aliquot 100 µl into autoclaved 0.5 ml tubes and store at −80°C until needed for use.

CRITICAL: Perform entire procedure in a sterile biosafety cabinet. Read the product information sheet carefully before preparation of working aliquots of all cytokines.

Medium composition
All basal medium, α-MEM, DMEM, DMEM/F-12, and IMDM should be prepared freshly from powder according to the manufacturer's instructions, sterilized by filtration using a 0.22µm membrane filter and stored for up to 2 months at 2–8°C.
Figures:

MEF growth medium	Volume	Final concentration
DMEM	445 ml	
FBS	50 ml	10%
100× NEAA solution (10 mM)	5 ml	100 µM

Sterilize the medium by filtration using a 0.22 µm membrane filter and store for up to 3 weeks at 2–8°C

hESC culture medium (250 ml)	**Volume**	**Final concentration**
DMEM/F-12	195 ml	78%
KO serum replacement	50 ml	20%
100× NEAA solution (10 mM)	2.5 ml	100 μM
L-glutamine/2-mercaptoethanol solution (100 mM)	2.5 ml	1 mM
Basic FGF (100 μg/ml) for hESCs culture	10 μl	4 ng/ml

Sterilize the medium by filtration using a 0.22 μm membrane filter and store for up to 2 weeks at 2–8°C.

hiPSC culture medium	**Volume**	**Final concentration**
DMEM/F-12	195 ml	78%
KO serum replacement	50 ml	20%
100× NEAA solution (10 mM)	2.5 ml	100 μM
L-glutamine/2-mercaptoethanol solution (100 mM)	2.5 ml	1 mM
Basic FGF (100 μg/ml)	25 μl	10 ng/ml

Sterilize the medium by filtration using a 0.22 μm membrane filter and store for up to 2 weeks at 2–8°C.
CRITICAL: Note that the basic FGF concentration for hiPSC maintenance should be 2.5 times higher than for hESCs to lessen the effect of variations in MEF quality on culture of hiPSCs.

Mouse OP9 bone marrow stromal cells culture medium	**Volume**	**Final concentration**
α-MEM	200 ml	80%
FBS	50 ml	20%

Sterilize the medium by filtration using a 0.22 μm membrane filter and store for up to 2 weeks at 2–8°C.

Differentiation (hPSC/OP9 coculture) medium	**Volume**	**Final concentration**
α-MEM	450 ml	90%
FBS	50 ml	10%
1000× MTG solution (100 mM)	500 μl	100 μM

Sterilize the medium by filtration using a 0.22 μm membrane filter and store for up to 2 weeks at 2–8°C.
Optional: Ascorbic acid solution (50 mg/ml) can be added to achieve 50 μg/ml final concentration.

Multipotent myeloid progenitor expansion medium	**Volume**	**Final concentration**
Differentiation (hPSC/OP9 coculture) medium	-	
GM-CSF (100 μg/ml)	1/500 dilution	200 ng/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1 ml of medium for ~1×10^6 cocultured cells. The complete medium is prepared freshly and GM-CSF is added right before use.

Neutrophil differentiation medium	**Volume**	**Final concentration**
IMDM with 20% FBS medium	-	
G-CSF (100 μg/ml)	1/1000 dilution	100 ng/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1ml of medium for 2×10^4 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.
Eosinophil differentiation medium

Eosinophil differentiation medium	Volume	Final concentration
IMDM with 20% FBS medium	-	
IL-3 (10 μg/ml)	1/1000 dilution	10 ng/ml
IL-5 (10 μg/ml)	1/2000 dilution	5ng/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1ml of medium for 2×10^4 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

DC differentiation medium	Volume	Final concentration
Serum free medium (StemLine, SIGMA-Aldrich)	-	
GM-CSF (100 μg/ml)	1/5000 dilution	20 ng/ml
IL-4 (100 μg/ml)	1/5000 dilution	20 ng/ml
TNF-α (50 μg/ml)	1/20000 dilution	2.5 ng/ml
Ex-Cyte	1/500 dilution	2 μl/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1 ml of medium for 10^5 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

LC differentiation medium	Volume	Final concentration
Serum free medium (StemLine, SIGMA-Aldrich)	-	
GM-CSF (100μg/ml)	1/5000 dilution	20ng/ml
TGF-β_1 (5μg/ml)	1/2000 dilution	2.5ng/ml
TNF-α (50μg/ml)	1/50000 dilution	1ng/ml
Ex-Cyte	1/500 dilution	2μl/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1ml of medium for 10^5 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

IMDM medium containing 10% FBS	Volume	Final concentration
IMDM	225ml	90%
FBS	25ml	10%

Sterilize the medium by filtration with 0.22 μm membrane filter and keep sterile at 2–8°C for up to 2 weeks.

Macrophage differentiation medium	Volume	Final concentration
IMDM with 10% FBS medium	-	
M-CSF (10 μg/ml)	1/500 dilution	20 ng/ml

Macrophage differentiation medium	Volume	Final concentration
IL-1β (10 μg/ml)	1/1000 dilution	10 ng/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1ml of medium for 10^5 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

Osteoclast progenitor expansion medium	Volume	Final concentration
Differentiation (hPSC/OP9 coculture) medium	-	
GM-CSF (100 μg/ml)	1/2000 dilution	50 ng/ml
1α, 25-Dihydroxyvitamin D_3 (1mM)	1/5000 dilution	200nM

Volume of the medium will vary depending on input cell number. In this protocol, we use 1 ml of medium for 2×10^4 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

Osteoclast maturation medium	Volume	Final concentration
Differentiation (hPSC/OP9 coculture) medium	-	
GM-CSF (100 μg/ml)	1/2000 dilution	50 ng/ml
1α, 25-Dihydroxyvitamin D_3 (1 mM)	1/5000 dilution	200 nM
RANKL (10 μg/ml)	1/1000 dilution	10 ng/ml

Volume of the medium will vary depending on input cell number. In this protocol, we use 1ml of medium for 5×10^4 cells. The complete medium is prepared freshly and all cytokines and supplements are added right before use.

IMDM medium containing 20% FBS	Volume	Final concentration
α-MEM	200 ml	80%
FBS	50 ml	20%

Sterilize the medium by filtration using a 0.22μm membrane filter and keep sterile at 2–8°C for up to 2 weeks.
Diluted Buffer for Wright Stain Procedure
Mix 30 ml of Protocol phosphate buffer pH 6.4 with 100 ml of deionized water. Store at room temperature for up to 6 months.

MEFs preparation for Human ES/iPSC culture TIMING 24 hours

1 Prepare MEFs according to WiCell protocol (http://www.wicell.org/index.php?option=com_content&task=category§ionid=7&id=246&Itemid=248).
2 Inactivate MEFs with gamma irradiation at 8,000 rad
3 Resuspend MEFs at 2×10^5 cells/ml in prewarmed MEF growth.
4 Add 2 mL/well of prewarmed MEF growth medium and then dispense MEF suspension on gelatin- coated 6-well plate (1ml/well). CRITICAL STEP Distribute MEFs evenly with a back/forth and right/left movement twice. Irregular distribution of MEFs may cause death and unwanted differentiation of hPSC colonies during culture.
5 Incubate MEF plates in a CO_2 incubator at 37°C for at least 24 hrs before adding hPSCs. CRITICAL STEP MEFs should be used for hPSC passage within one week. Before plating hPSCs, aspirate MEF medium, add 2ml of PBS, swirl once and aspirate PBS. Add 2 ml of prewarmed hPSC medium and place plate into CO_2 incubator at 37°C. Now MEF feeders are ready for hPSC plating (step 15).

hES/iPSC culture TIMING 7 days

6 Aspirate hPSC growth medium from one well of the 6-well plate of hESCs or iPSCs. CRITICAL STEP: Note that cells will need to be split every 6–7 days.
7 Wash cells with 2 ml/well of PBS (Ca^{2+} and Mg^{2+} free) stored at room temperature.
8 Add 1 ml/well of collagenase IV solution (1 mg/ml) and incubate at 37°C in a CO_2 incubator until the edges of the hPSC colonies begin to curl (approximately 7–10 minutes).
9 Add 1 ml of hESC or hiPSC growth medium and break up the colonies into small cell aggregates by gently pipetting. CRITICAL STEP: After collagenase treatment, hPSC colonies are loosely attached and can be collected by gentle pipetting. Do not use excessive mechanical force or scraping which can provoke spontaneous differentiation.
10 Transfer cells to a 15 ml conical tube.
11 Centrifuge at 200×g at room temperature for 3–5 min.
12 Aspirate the medium gently without disturbing the pellet.
13 Resuspend cells in 3 ml of hESC or hiPSC growth medium, and wash cells by repeating steps 11 and 12.
14 Resuspend cell pellet in 3 ml of hESC or hiPSC growth medium.
15 Plate 0.5 ml/well of cell suspension onto MEF-grown 6-well plate from Step 5.

TROUBLESHOOTING

16 Feed hPSCs daily by replacing the old medium with 3ml of prewarmed hESC or hiPSC medium.
17 Passage undifferentiated hES/iPSCs (Fig. 1a) weekly at $1.2–1.5×10^6$ cells/well density on MEFs. CRITICAL STEP: If spontaneous differentiation of hESCs or hiPSCs occurs, differentiated hPSC colonies should be eliminated during the maintenance; observe hPSCs every day before changing medium. Mark differentiated colonies with an objective marker under the inverted microscope and aspirate marked areas using a glass Pasteur pipette while feeding hPSC with fresh medium. CRITICAL STEP: Alternatively, hPSCs can be maintained under feeder-free conditions[32]. In OP9 coculture system, we did not observe significant differences in the efficiency of hematopoietic differentiation of hPSCs maintained on MEFs or in feeder-free cultures.

Culture of mouse OP9 cells TIMING 8–12 days

18 Aspirate OP9 growth medium and wash cells twice with 10 ml of PBS. CRITICAL STEP: Note that cells will need to be split every 4 days.
19 Add 5 ml of trypsin/EDTA(0.05%/0.5 mM) solution and incubate for 5 min at 37°C in a CO_2 incubator. CRITICAL STEP: OP9 feeders consist of heterogeneous cell populations which include cells with at least adipogenic and osteogenic potential. To maintain a proper balance of cells following passage, OP9 feeders should be digested and detached completely by trypsin treatment. Inadequate washing of cells with PBS or using an old trypsin may results in partial detachment and enrichment in adipogenic cells.
20 Add 5 ml of OP9 growth medium and collect cells by pipetting.
21 Transfer cell suspension into a 15 ml conical tube and centrifuge for 5 min at 300×g at room temperature.
22 Aspirate supernatant and resuspend cells in 1 ml of OP9 growth medium.
23 Add 100 μl of cell suspension to 10 ml of OP9 growth medium and plate cells onto 10 cm gelatin-coated culture dishes. CRITICAL STEP: It is essential to culture OP9 on gelatin-coated plates to prevent spontaneous adipogenesis.

TROUBLESHOOTING

24 When cultures are confluent, split 1 dish for maintenance. This should occur after approximately 4 days of growth.
25 To prepare overgrown OP9 for coculture with hPSCs, change half of the medium after 4 days of culture on gelatin-coated plates and incubate for an additional 4–8 days to achieve a dense OP9 monolayer. CRITICAL STEP: OP9 should be split every 4 days for maintenance/expansion. OP9 used for hPSC differentiation should be fed with fresh media at confluence (day 4) and incubated for an additional 4–8 days to form a dense monolayer embedded in extracellular matrix.

Hematopoietic differentiation on OP9 TIMING 9 days

26 Remove overgrown OP9 dishes prepared for coculture from the CO_2 incubator.
27 Aspirate OP9 growth medium.
28 Add 10 ml of differentiation medium and keep at 37°C in a CO_2 incubator.
29 From one well of a 6-well hPSC plate from Step 17, aspirate hES/hiPSC growth medium. Add 1 ml of collagenase IV solution (1 mg/ml), and incubate cells for 10 min at 37°C.

30 Add 1 ml/well of differentiation medium directly to the well and break up colonies into small cell aggregates by gentle pipetting. Transfer cells into a 15 ml conical tube. CRITICAL STEP: hPSCs for differentiation studies should be prepared as small aggregates. Single hPSCs will not survive on OP9.
31 Centrifuge cells at 200×g for 3–5 min at room temperature.
32 Aspirate the medium gently without disturbing the pellet.
33 Resuspend the cell pellet with 1mL of differentiation medium.
34 Add 1mL of hES/hiPSC suspension to 1 OP9 dish prepared in steps 26–28. CRITICAL STEP: Efficiency of hematopoietic differentiation is significantly affected by the density of hPSCs plated on OP9. In our experience, the optimal plating density of hPSCs is 1.0–1.5 × 10^6 cells per 10 cm dish of OP9. To estimate the number of cells in a suspension consisting of small hPSC aggregates, one well of a 6-well plate can be used to prepare a single cell suspension by treatment with trypsin for cell counting. Alternatively, aliquots of hPSC aggregates can be collected, treated with trypsin, and counted.
35 Distribute cells evenly with a back/forth and right/left movement twice.
36 The following day (day 1), aspirate all of the media to waste and replace with 20 ml of prewarmed differentiation medium.
37 On day 4, change half of the medium.
38 On day 6, change half of the medium
39 To collect cells on day 9, aspirate the supernatant and add 5 ml of prewarmed collagenase solution (1mg/ml) to each dish of hPSC/OP9 coculture and incubate for 30 minutes at 37°C in a CO_2 incubator.
40 Remove the collagenase solution and keep it on ice in a 15 ml conical tube for subsequent collection of trypsin digested cells (cell collection tube).CRITICAL STEP: hPSC/OP9 coculture produces collagen-rich matrix and pretreatment with collagenase is essential to achieve efficient digestion of cells with trypsin. Because some cultures might form an excessive amount of extracellular matrix, the time of treatment with collagenase can be extended up to 40–50 minutes to achieve complete dissociation and maximize cell recovery.
41 Add 5 ml of prewarmed Trypsin/EDTA solution (0.05%/0.5mM) to the dish from Step 39 and incubate for 15–20 minutes at 37°C CO_2 incubator.
42 Add 2 ml/dish of MACS buffer, suspend coculture cells by pipetting and transfer to the collection tube from Step 40.
43 Add an additional 5 ml/dish of MACS buffer to the coculture dish and collect the remaining cells into the collection tube.
44 Centrifuge cell suspension at 300×g for 5min at room temperature.
45 Wash cells once by adding 5 ml of MACS buffer to cell pellet followed by pipetting and centrifugation at 300×g for 5 min at room temperature.
46 Cells are ready to use in further applications such as flow cytometry, CFC assay, and further differentiation. CRITICAL STEP: The success of subsequent steps in this differentiation protocol largely depends on effective induction of hematopoietic differentiation and lin-$CD34^+CD43^+CD45^+$ progenitors in coculture with OP9. Therefore analysis of CD43 expression and simultaneous detection of CD235a/$CD41a^+$ and $CD45^+$ cells within $CD43^+$ population can be performed to confirm myeloid commitment[28] (see Fig.1). CRITICAL STEP: Because we observed a decline in the hematopoietic differentiation capacity of hESCs after passage 50, we do not recommend the use of hESC lines beyond this passage. OP9 cells should not be used beyond passage 60, because of significant decrease in hematopoiesis-inductive potential.

TROUBLESHOOTING

Short-term expansion of multipotent myeloid progenitors TIMING 2 days

47 Wash out pHEMA-coated flasks with 20 ml (T75 flask) PBS.
48 Resuspend differentiated hPSCs (from Step 46) in multipotent myeloid progenitor expansion medium at a concentration of ~ 1×10^6 cells/ml. CRITICAL STEP: Note that typically, 1.5–2×10^7 cells are recovered from one 10 cm dish of hPSC/OP9 coculture. We usually culture cells collected from 2 dishes in one T75 flask. GM-CSF is a single key factor required for expansion of hPSC-derived myeloid progenitors. The addition of SCF and/or FLT3L to expansion cultures has little effect on the growth of myeloid precursors, but significantly increases the proportion of $CD235a^+$ erythroid cells.
49 Incubate 2 days at 37°C in a CO_2 incubator. CRITICAL STEP: Differentiated hPSCs cultured in non-adherent conditions spontaneously reaggregate and form large floating cellular conglomerates with myeloid progenitors proliferating in suspension as single cells.

Purification of multipotent myeloid progenitors TIMING 3 hrs

50 Collect myeloid cultures (from Step 49) and filter through a 70 μm cell strainer into a 50 ml tube to remove cell aggregates.
51 Pellet the cells by centrifugation at 250×g for 5 min at room temperature
52 Resuspend cells with 5 ml of MACS buffer in a 15 ml tube.
53 Underlay cell suspension with 1.0–1.5 ml of Percoll solution. Place a 1 ml plastic serological pipet filled with Percoll solution into the tube with cell suspension, so the pipet tip touches the bottom of the tube. Very carefully dispense Percoll solution to underlay cell suspension avoiding mixing.
54 Centrifuge tube at 300×g for 10–15 min at room temperature.
55 Aspirate supernatant and interface containing dead cells and debris.
56 Resuspend cells with 5 ml of MACS buffer and centrifuge at 250×g for 5 min at room temperature. Take an aliquot of the resuspended cells before centrifugation to count the number of isolated cells.
57 Aspirate supernatant and add 0.2 ml of MACS buffer to cell pellet.
58 Add 1 μl of anti-human CD235a-PE, and 5 μl of anti-human CD41a-PE antibodies per 10^6 cells.CRITICAL STEP: Usually cells collected after Percoll separation are free of residual OP9 cells. If significant contamination of human cells with OP9 cells occurs, 10 μl of anti-mouse CD29-PE Ab can optionally be added to the cell pellet to deplete the mouse cells. The presence of contaminating OP9 cells in suspension can be evaluated by flow cytometry using mouse-specific CD29 antibodies.[28]
59 Set up tube on the MACS mixer and incubate at the lowest rotation speed at 4°C for 15–20 minutes.
60 Wash cells with ice-cold MACS buffer by adding 5 ml of MACS buffer to cell pellet followed by pipetting and centrifugation at 300×g for 5 min at 4°C, resuspend in 0.4 ml of MACS buffer, and add 10 μl of anti-PE magnetic beads.
61 Repeat step 59.
62 Wash cells with ice-cold MACS buffer as described in step 60 and resuspend in 1 ml of MACS buffer.
63 Filter cells through a 30 μm pre-separation filter. CRITICAL STEP: It is important to filter cells before magnetic separation to remove large cell aggregates which may block the magnetic column.
64 Assemble the MACS-LD separation column according to the manufacturer's instructions.
65 Wash column with 2 ml of MACS buffer.
66 Apply the cell suspension from Step 63 to the LD column allowing cells to pass completely through the column into 15 ml collection tube.
67 Wash column with 2 ml of MACS buffer and collect in same collection tube.
68 Recap and remove collection tube with unlabeled cells (CD235a-CD41a- human cells).
69 Centrifuge cells at 300×g for 5 min at 4°C.
70 Aspirate supernatant and add 80 μl of MACS buffer and 20 μl of anti-human CD45-FITC Ab.
71 Place the tube on the MACS mixer and incubate at the lowest rotation speed at 4°C for 15–20 minutes.
72 Wash cells with ice-cold MACS buffer as described in step 60, resuspend in 80 μl of MACS buffer, and add 20 μl of anti-FITC magnetic beads.
73 Repeat step 69.
74 Wash cells with ice-cold MACS buffer as described in step 60 and resuspend in 1ml of MACS buffer.
75 Filter cells through a 30 μm pre-separation filter.
76 Assemble MACS-LS separation unit according to the manufacturer's instructions.
77 Rinse column with 2 ml of MACS buffer.
78 To purify CD235a/CD41a^{-}CD45^{+} multipotent myeloid progenitors, apply the cell suspension from Step 75 to the LS column allowing cells to pass completely through the column into collection tube.
79 Wash column with 2 ml of MACS buffer and collect in same collection tube then discard.
80 Remove the column from the magnet and place in an empty 15 ml tube.
81 Wash out CD45^{+} cells with 5 ml MACS buffer using the plunger supplied with column.
82 Centrifuge cells at 300×g for 5 min at 4°C.
83 Resuspend cells in 0.2 ml of MACS buffer and keep on ice. Cells are ready to use for further differentiation.

TROUBLESHOOTING

Differentiation of hPSC-derived myelomonocytic cells
84 Differentiate hPSC-derived lin^{-}CD34^{+}CD43^{+}CD45^{+} progenitors using option A for neutrophils, option B for eosinophils, option C for macrophages, option D for DCs, option E for LCs, and option F for osteoclasts. Protocol for cytospin preparation and staining of differentiated cells is provided as option G.

(A) Neutrophil differentiation TIMING 8–10 days

i. Prepare 6-well plates with semiconfluent OP9 monolayer.
ii. Add 5×10^4 purified $CD45^+$ cells in 2.5 ml of neutrophil differentiation medium per well of the 6-well plate.
CRITICAL STEP: To obtain a pure population of neutrophils, it is essential to limit cytokine addition to G-CSF alone. Although IL-3, IL-6, and GM-CSF have been used to increase the output of neutrophils from somatic $CD34^+$ cells, we found that addition of these cytokines to the neutrophil differentiation cultures resulted in production of a mixture of eosinophils and neutrophils, with eosinophils often predominating.
iii. On day 3, add 2.5 ml of neutrophil differentiation medium.
iv. On day 6, change half of the neutrophil differentiation medium.
v. On day 8–10, collect differentiated cells from OP9 by gentle pipetting.
CRITICAL STEP: The presence of mature neutrophils in cultures can be quickly evaluated using Wright-stained cytospins (see protocol G below). The mature neutrophils have segmented nucleus (usually 2–5 segments joined by thin filaments) and pale pink cytoplasm (see Fig. 2). Because the viability and functionality of neutrophils are decreased with the extension of culture time, the optimal time for cell collection should be determined using vital dye staining and functional analysis of collected cells.

(B) Eosinophil differentiation TIMING 12–14 days

i. Prepare 6-well plates with semiconfluent OP9 monolayer.
ii. Add 5×10^4 purified $CD45^+$ cells in 2.5 ml of eosinophil differentiation medium per well of the 6-well plate.
CRITICAL STEP: IL5 alone is sufficient to differentiate hPSC-derived myeloid progenitors into eosinophils, however the addition of IL3 significantly increases the total cell output.
iii. On day 3, add 2.5ml of eosinophil differentiation medium.
iv. Change half of the eosinophil differentiation medium on days 6 and 9.
v. On day 12–14, collect differentiated cells by gentle pipetting.
CRITICAL STEP: The presence of mature eosinophils in cultures can be quickly evaluated using Wright-stained cytospins (see protocol G below). The mature eosinophils have classic bilobed nucleus and abundant eosinophilic cytoplasm filled by numerous coarse, orange-red granules of uniform size (see Fig. 2). In contrast, immature myeloid cells have round nucleus and less abundant basophilic or amphophilc cytoplasm. The optimal collection time for eosinophils should be determined based on predominance of viable mature cells in cultures.

(C) Macrophage differentiation TIMING 5–7 days

i. Wash a pHEMA-coated T25 tissue culture flask with 10 ml of PBS.
ii. Add $1–5\times10^5$ purified $CD45^+$ cells in 5mL of macrophage differentiation medium to the T25 flask.
CRITICAL STEP: M-CSF and IL-1β are two critical cytokines for the differentiation of myeloid precursors into macrophages. We do not recommend using GM-CSF in macrophage differentiation cultures, because cells continue to proliferate and do not mature into macrophages in presence of GM-CSF.
iii. At day 3, add an additional 5ml of macrophage differentiation medium.
iv. At day 5–7, collect differentiated cells.
CRITICAL STEP: The presence of mature macrophages in cultures can be quickly evaluated using Wright-stained cytospins (see protocol G below).The macrophages have round nucleus and abundant foamy cytoplasm (see Fig. 2). Because the viability and functionality of macrophages are decreased with the extension of culture time, the optimal time for cell collection should be determined using vital dye staining and functional analysis of collected cells.

(D) Dendritic cell differentiation TIMING 7 days

i. Wash a pHEMA-coated T25 tissue culture flask with 10 ml of PBS.
ii. Add 5×10^5 isolated $CD45^+$ cells in 5ml of DC differentiation medium without TNF-α to the T25 flask.
CRITICAL STEP: It is essential to avoid adding TNF-α during the first day of culture. We noted a significant decrease in cell viability if TNF-α was added to the freshly isolated cells.
iii. On day 2–3, add 5ml of DC differentiation medium with TNF-α (2.5 ng/ml).
iv. On day 7, collect differentiated cells.

CRITICAL STEP: On Wright-stained cytospins, cells of dendritic lineage can be recognized by the presence of cytoplasmic veils and dendritic projections (see Fig.2). However, flow cytometric analysis[20,28] is essential to identify myeloid DCs which have $CD1a^{+}HLA\text{-}DR^{+}DC\text{-}SIGN^{+}CD11b^{+}Langerin^{-}$ phenotype.

(E) Langerhans cell differentiation TIMING 7 days

i. Wash a pHEMA-coated T25 tissue culture flask with 10 ml of PBS.
ii. Add 5×10^{5} cells in 5 ml LC differentiation medium without TNF-α and TGF-β1 to the T25 flask.
CRITICAL STEP: It is essential to avoid adding TNF-α and TGF-β1 during the first day of culture. We noted a significant decrease in cell viability if these cytokines were added to the freshly isolated cells.
iii. On day 2–3, add 5ml of complete LC differentiation medium with TNF-α and TGF-β1 (2.5 ng/ml).
iv. On day 7, collect differentiated cells.
CRITICAL STEP: On Wright-stained cytospins, cells of dendritic lineage can be recognized by the presence of cytoplasmic veils and dendritic projections (see Fig.2). However, flow cytometric analysis[20,28] is essential to identify LCs which have $CD1a^{high}HLA\text{-}DR^{+}DC\text{-}SIGN^{-}CD11b^{low}Langerin^{+}$ phenotype.

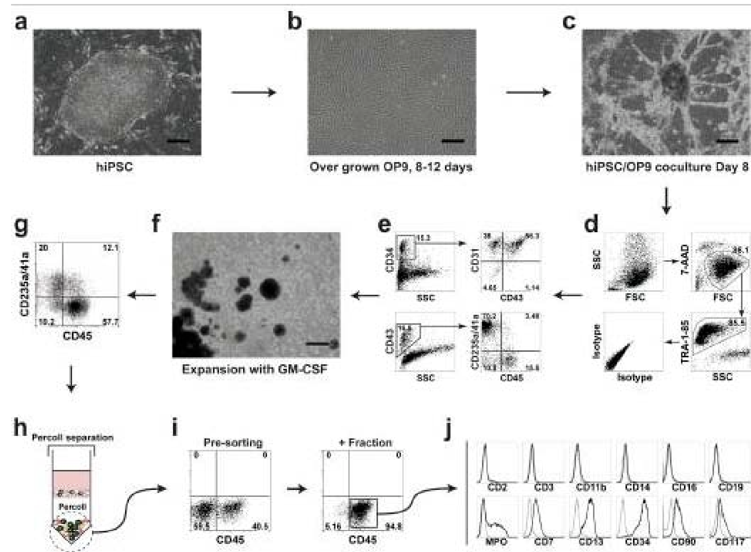
(F) Osteoclast differentiation TIMING 16–19 days

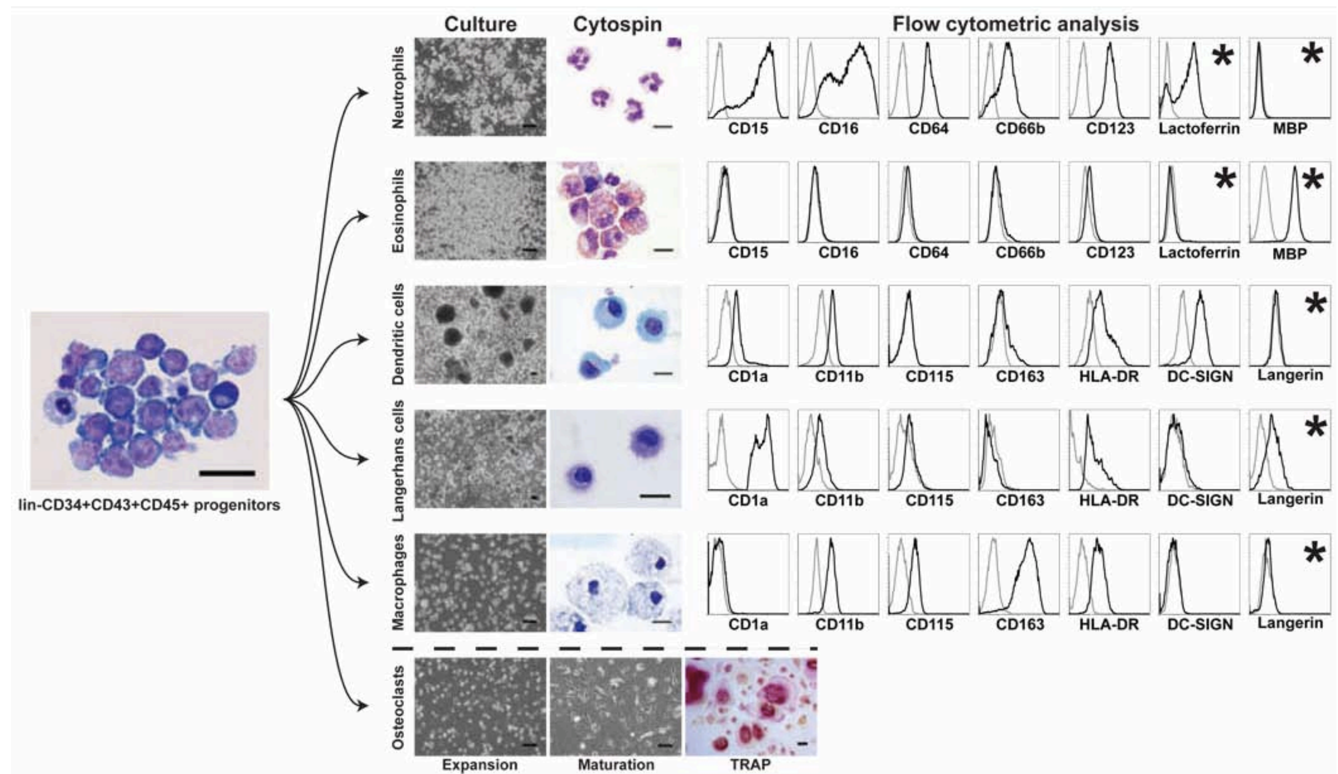
i. *Expansion step for osteoclast precursors*. Wash a pHEMA-coated T25 tissue culture flask with 10 ml of PBS.
ii. Add 5×10^{4} to 1×10^{5} cells in 5–10 ml of osteoclast progenitor expansion medium to the T25 flask.
iii. Change half of the medium on day 2–3.
iv. On day 4–5, collect cells; during the expansion step, osteoclast precursors adhere to the surface of the pHEMA coated-culture flask. Therefore, treat with 3 ml PBS (Ca^{2+} and Mg^{2+} free) or enzyme free cell dissociation buffer for 5–10min and collect cells by gentle pipetting.
v. *Maturation step for osteoclasts*. Plate 1–2×10^{5} cells/well of a 6-well plate in 2.5 ml of osteoclast maturation medium.
vi. Change half of the osteoclast maturation media every 3 days until the osteoclasts mature (12–14 days of culture). CRITICAL STEP: Large, adhesive and multinucleated cells are markers for mature osteoclasts. However, immature myeloid cells (small round and non-adherent cells) keep proliferating due to the presence of GM-CSF and these proliferating cells interfere with maturation of the osteoclasts. Therefore, eliminate non-adherent cells by aspiration during regular medium changes. We found that the addition of M-CSF had no effect on development of osteoclasts in our differentiation system. Moreover, the addition of M-CSF to osteoclast cultures shifted differentiation hiPSC-derived myeloid progenitors toward macrophages.

(G) Cytospin prepartion and Wright staining

i. Use 2.5–3×10^{4} cells per slide for Cytospin preparation and Wright staining
ii. *Cytospin preparation*. Collect cells in 1.5 ml microcentrifuge tube and centrifuge at 400×g for 4 min.
iii. Wash cells once by adding ice cold 0.5 ml of flow cytometry buffer followed centrifugation at 400×g for 4 minutes. Discard supernate and resuspend cells with 250μl of flow flow cytometry buffer.
iv. During centrifugation, label glass slide and assemble with Cytofuse filter concentrator unit according to manufacturer's instruction.
v. Transfer cell suspension into sample chamber of assembled glass slide-Cytofuse filter concentrator unit.
vi. Spin samples at 700 rpm (27 ×g) for 4 min at room temperature.
vii. Dry slide completely at room temperature.
viii. Fix slide with absolute methanol for 30–60 seconds at room temperature.
ix. *Wright staining*. Cover cytospin area with 300μl of Protocol Wright Stain and allow to stain for 3 minutes at room temperature. Make sure that stain covers the entire area with cells to ensure uniform staining of cells on cytospin.
x. Add 450 μl of pH 6.4 Diluted Buffer to the Wright stain-covered slide.
xi. Mix the stain and buffer together by gently rocking slide for 1 minute and incubate the mixture for additional 4 minutes at room temperature.
xii. Rinse the slide with deionized water and dry completely in the air.
xiii. Drop 2–3 droplets of Cytoseal 60 mounting medium on the stained cell area and cover with cover glass.
xiv. Observe under the microscope.

IMAGES





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