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Directed differentiation of definitive hemogenic endothelium and hematopoietic progenitors from human pluripotent stem cells

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ABSTRACT

The generation of hematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs) remains a major goal for regenerative medicine and disease modeling. However, hPSC differentiation cultures produce mostly hematopoietic progenitors belonging to the embryonic HSC-independent hematopoietic program, which may not be relevant or accurate for modeling normal and disease-state adult hematopoietic processes. Through a stage-specific directed differentiation approach, it is now possible to generate exclusively definitive hematopoietic progenitors from hPSCs showing characteristics of the more developmentally advanced fetal hematopoiesis. Here, we summarize recent efforts at generating hPSC-derived definitive hematopoiesis through embryoid body differentiation under defined conditions. Embryoid bodies are generated through enzymatic dissociation of hPSCs from matrigel-coated plasticware, followed by recombinant BMP4, driving mesoderm specification. Definitive hematopoiesis is specified by a GSK3β-inhibitor, followed by recombinant VEGF and supportive hematopoietic cytokines. The CD34+ cells obtained using this method are then suitable for hematopoietic assays for definitive hematopoietic potential.

1. Introduction

Human pluripotent stem cells (hPSCs) have the potential to differentiate into every adult cell type found in the body, making them an enormously powerful tool for the *in vitro* study of human development and disease modeling. Additionally, as they can be theoretically expanded indefinitely in the tissue culture dish, hPSCs are a potential unique source of cellular material for cell-based therapies and regenerative medicine [1]. While great strides towards this goal have been made with many different cell types, some even reaching clinical trials, the generation of adult-like hPSC-derived hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) has posed a significant challenge.

To succeed in this endeavor, we have approached this challenge by developing a method for the directed differentiation of HSCs/HPCs which aims to recapitulate the ordered sequence of signal events that occurs *in vivo*. As a first consideration, we must remain cognizant of the existence of different hematopoietic programs within the developing embryo. The extraembryonic primitive hematopoietic program proceeds transiently in an HSC-independent manner, giving rise to a limited subset of erythro-myeloid hematopoietic lineages. Conversely, the definitive hematopoietic program gives rise to the full-suite of hematopoietic lineages found within the fetus and adult, including the HSC (reviewed in [2]). It is the definitive program that is of predominant interest to developmental and regenerative medicine biologists due to its therapeutic potential, and as such hPSC differentiation approaches and assays must be able to distinguish between the two programs.

Many developmental studies have indicated that each program develops from distinct progenitors (reviewed in [2]). We recently developed a serum-free, stromal-free directed differentiation approach to generate mesodermal progenitors of either primitive or definitive hematopoiesis [3]. Mesodermal progenitors of the primitive hematopoietic program can be identified by the expression of the VEGF receptor 2 (KDR) and Glycophorin A (CD235a). This population ultimately gives rise to the hematopoietic lineages associated with the primitive hematopoietic program, and lacks definitive hematopoietic potential. Conversely, mesoderm with definitive hematopoietic potential is identified by the expression of KDR and the absence of CD235a expression [4]. As a result, it is now possible to derive the ontological precursor to the definitive hematopoietic program, the hemogenic endothelium (HE) from the differentiation cultures (reviewed in [5]). These unique cells can be isolated by fluorescence-activated cell sorting (FACS) through the

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combined use of CD34 expression, and an absence of expression of CD73, CD184 and hematopoietic cell-surface markers. Isolated hPSC-derived HE is capable of giving rise to erythro-myelo-lymphoid multilineage definitive hematopoiesis, including hallmark lineages such as T-lymphocytes and gamma globin-expressing BFU-E [6]. As such, it is now possible to perform highly-detailed studies on hPSC-derived definitive hematopoietic progenitors for any of the goals discussed above.

Herein, we outline a detailed approach to the directed differentiation of hPSCs towards mesoderm, HE and its derivative primitive and definitive HPCs, which can then be isolated by the investigator for their specific downstream application(s).

2. Materials

2.1. Cell lines

- Human embryonic stem cells or induced pluripotent stem cells
- Mouse embryonic fibroblasts (MEFs) [7]

2.2. Reagents

- Iscove's Modified Dulbecco's Medium (IMDM; Corning Cat #10-016)
- Fetal Bovine Serum (FBS), ES cell-rated (Gemini Bioproducts, Cat #100-500)
- L-Glutamine, 200 mM solution (Life Technologies, Cat #25030-081)
- Penicillin–streptomycin, 100X (Life Technologies, Cat #15070-063)
- 0.25% Trypsin–EDTA (Life Technologies, Cat # 25200056)
- 0.05% Trypsin–EDTA (Life Technologies, Cat # 25300054)
- Gelatin, porcine skin Type A (Sigma–Aldrich, Cat #G1890)
- DMEM-F12 (Corning, Cat # 10-092-CV)
- Knock-out serum replacement (KOSR; Life Technologies, Cat #10828028)
- Non-essential amino acids (NEAA; Life Technologies, Cat #11140050)
- β-Mercaptoethanol, 55 mM solution (Life Technologies, Cat #21985023)
- Hydrochloric acid (HCl; Sigma–Aldrich, Cat #H1758)
- Fraction V Bovine Serum Albumin (BSA; Fisher Scientific, Cat #BP1605)
- Ham's F-12 (Corning, Cat # 10-080)
- N2 supplement (Life Technologies, Cat #17502048)
- B27 supplement, no vitamin A (Life Technologies, Cat #12587010)
- Stempro-34 (SP34; Life Technologies, Cat #10639011)
- Growth-factor reduced matrigel (Corning, Cat # 354230)
- L-ascorbic acid (Sigma–Aldrich, Cat #A4403)
- Human serum transferrin (Roche Diagnostics, Cat #10652202001)
- Monothioglycerol (MTG, Sigma–Aldrich, Cat #M6145)
- Collagenase B (Roche Diagnostics, Cat #11088831001)
- Collagenase II (Life Technologies, Cat #17101015)
- DNAse I Bovine Pancreas (Calbiochem, Cat #260913)
- Phosphate Buffered Saline (PBS; Life Technologies Cat #14190144)
- Recombinant cytokines and small molecule inhibitors – see Table 1
- anti-human antibodies for flow cytometry/FACS – see Table 2

2.3. Equipment

- Milli-Q water purification system (EMD Millipore) or equivalent

Table 1
Cytokines and small molecules used for directed differentiation of hPSCs.

Cytokine	Supplier	Catalog #	Stock concentration	Final concentration
bFGF	R&D Systems	233-FB	10 μg/mL	10 ng/mL
BMP4	R&D Systems	314-BP	10 μg/mL	10 ng/mL
Activin A	R&D Systems	338-AC	10 μg/mL	1 ng/mL
VEGF	R&D Systems	293-VE	10 μg/mL	15 ng/mL
SCF	R&D Systems	255-SC	50 μg/mL	100 ng/mL
IGF-1	R&D Systems	291-G1	25 μg/mL	25 ng/mL
IL-3	R&D Systems	203-IL	10 μg/mL	30 ng/mL
IL-6	R&D Systems	206-IL	10 μg/mL	5 ng/mL
IL-11	R&D Systems	218-IL	10 μg/mL	5 ng/mL
TPO	R&D Systems	288-TP	10 μg/mL	30 ng/mL
EPO	Peprotech	100-64	2000 IU	2 IU
CHIR99021	Tocris	4423	3 mM	3 μM
IWP2	Tocris	3533	10 mM	3 μM
Angiotensin II	Sigma–Aldrich	A9525	10 mg/mL	10 μg/mL
Losartan potassium	Tocris	3798	100 mM	100 μM

Table 2
Antibodies used for analysis of differentiation cultures.

Antigen	Clone	Supplier	Conjugate	Dilution
CD34	8G12	BD Biosciences	APC	1:100
CD34	4H11	BD Biosciences	PE-Cy7	1:100
CD43	1G10	BD Biosciences	PE	1:20
CD43	1G10	BD Biosciences	FITC	1:10
CD45	J.33	Beckman Coulter	Krome Orange	1:50
CD45	2D1	BD Biosciences	APC-Cy7	1:50
CD45	2D1	BD Biosciences	eFluor450	1:50
CD73	AD2	BD Biosciences	APC	1:50
CD184	12G5	BioLegend	Brilliant-Violet 421	1:50
CD235a	HIR2	BD Biosciences	APC	1:100
KDR	89106	R&D Systems	PE	1:7

- 5% CO_2 incubator set at 37 °C
- Multi-gas incubator set at 5% CO_2 5% O_2, 37 °C
- 6-Well and 24-well tissue culture plate (Corning)
- 6-Well and 96-well low-adherence tissue culture plates (Corning)
- 5, 15, 50 mL sterile centrifuge tubes (Corning)
- 2, 5, 10, 25 mL sterile serological pipets (Corning)
- Cell scrapers (Corning)
- 2.0 mL cryovials (Corning)
- 40 μm cell strainers (Corning)
- Cell culture centrifuge
- Biosafety hood
- Water bath set at 37 °C
- FACS AriaII or equivalent
- LSRii or equivalent
- FlowJo software (TreeStar Inc.)
- 0.22 μm filtration system (Corning)
- Autoclave
- 4 °C refrigerator
- −20 °C freezer
- −80 °C freezer or liquid N_2 cryostorage

2.4. Reagent preparation

2.4.1. 0.1% gelatin solution

Prepare a sterile solution 0.1% w/v of gelatin in PBS by autoclaving. Once cooled, aliquot 250 mL plastic bottles and store at 4 °C.

2.4.1.1. Gelatin-coated plasticware. Apply 1.5 mL of 0.1% gelatin to each well of a 6-well tissue culture plate. Incubate at room