Spin Embryoid Bodies as an Improved Method of Blood Cell Differentiation in Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

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Introduction

Two types of cells have been characterized as pluripotent stem cells: human embryonic stem cells (hESCs) and the more recently described induced pluripotent stem cells (iPSCs). Pluripotency means that these cells have the ability to form all the cells and tissues of the human body. hESC and iPSC differentiation into blood cells is particularly useful in studying the mechanisms of blood diseases and cell transfusion therapies.

To better understand the potential of these cells to specifically produce blood cells, it is necessary to define an efficient and reproducible system of differentiation. Previously, our and other groups have used a stromal cell co-culture method to support blood development from hESCs. In these current studies, we are employing a stromal-free and serum-free differentiation method that may facilitate clinical translation of hESC- and iPSC-derived cells. This method involves forced aggregation of defined numbers of undifferentiated hESCs or iPSCs in 96-well plates by centrifugation to form embryoid bodies (spin EBs) of a uniform size. We examine this method's potential to derive blood precursor cells and mature blood lineage cells from both hESCs and iPSCs.

Methods

Spin-EB Differentiation: Stage I and II
3,000 undifferentiated H9 (hESCs) or iPSCs were plated in each well of a round-bottom 96-well plate. The cells were forced to aggregate by centrifugation – forming "Spin EBs" of uniform size. The spin EBs were cultured for 8-12 days in a serum-free medium containing a cocktail of human cytokines (SCF, BMP4, VEGF) to induce production of blood precursor cells.

After 8 days of differentiation in Stage I, EBs were transferred to 24-well plates for a second stage of differentiation to promote generation of mature blood lineage cells. In Stage II differentiation, EBs from stage I were cultured in a serum-free medium containing a cocktail of human cytokines (SCF, VEGF, IL-3, IL-6, TPO, EPO). Cells remained Stage II for 4 weeks and were harvested regularly for phenotypic analysis.

Flow Cytometry Analysis of Differentiated hESCs and iPSCs
Stage I or II differentiated cells were harvested at regular time points for flow cytometric analysis. Cells were dissociated and stained with fluorescently-tagged antibodies to detect common markers of blood lineage development including CD34-allophycocyanin (APC), CD31-phycoerythrin (PE), CD43-PE, and CD45-PE in Stage I. Stage II cells were stained with antibodies to detect more mature blood cell phenotypes, including CD45-APC/PE, GlyA-PE, CD34-APC and CD33-PE.

Hematopoietic Colony Assay of Differentiated hESCs and iPSCs
3 x 10^4 cells harvested from day 11 Stage I Spin-EBs were plated in 35mm petri dishes in MethoCult GF+ (StemCell Technologies) semi-solid medium containing hematopoietic growth factors. Colony forming units were scored after 11 days of incubation.

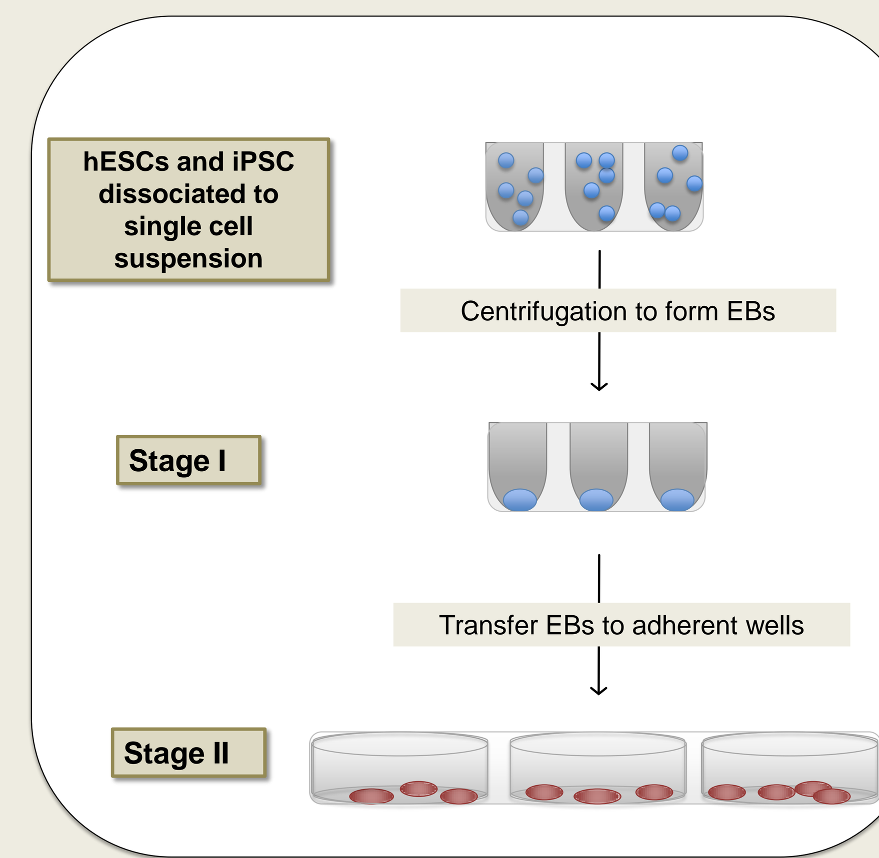

Figure 1: Spin EB differentiation scheme. hESC and iPSCs are enzymatically dissociated to a single cell suspension and plated into 96-well round bottom plates at 3,000 cells/well. In Stage I, cells are aggregated by centrifugation to induce embryoid body formation. Stage I Spin-EBs are incubated at 37°C, 5% CO_2 for 8-12 days in a serum-free media with cytokines/growth factors that promote generation of blood precursor cells. In Stage II, EBs are transferred to adherent 24-well plates with a different set of cytokines/growth factors to promote further differentiation into mature blood lineage cells.

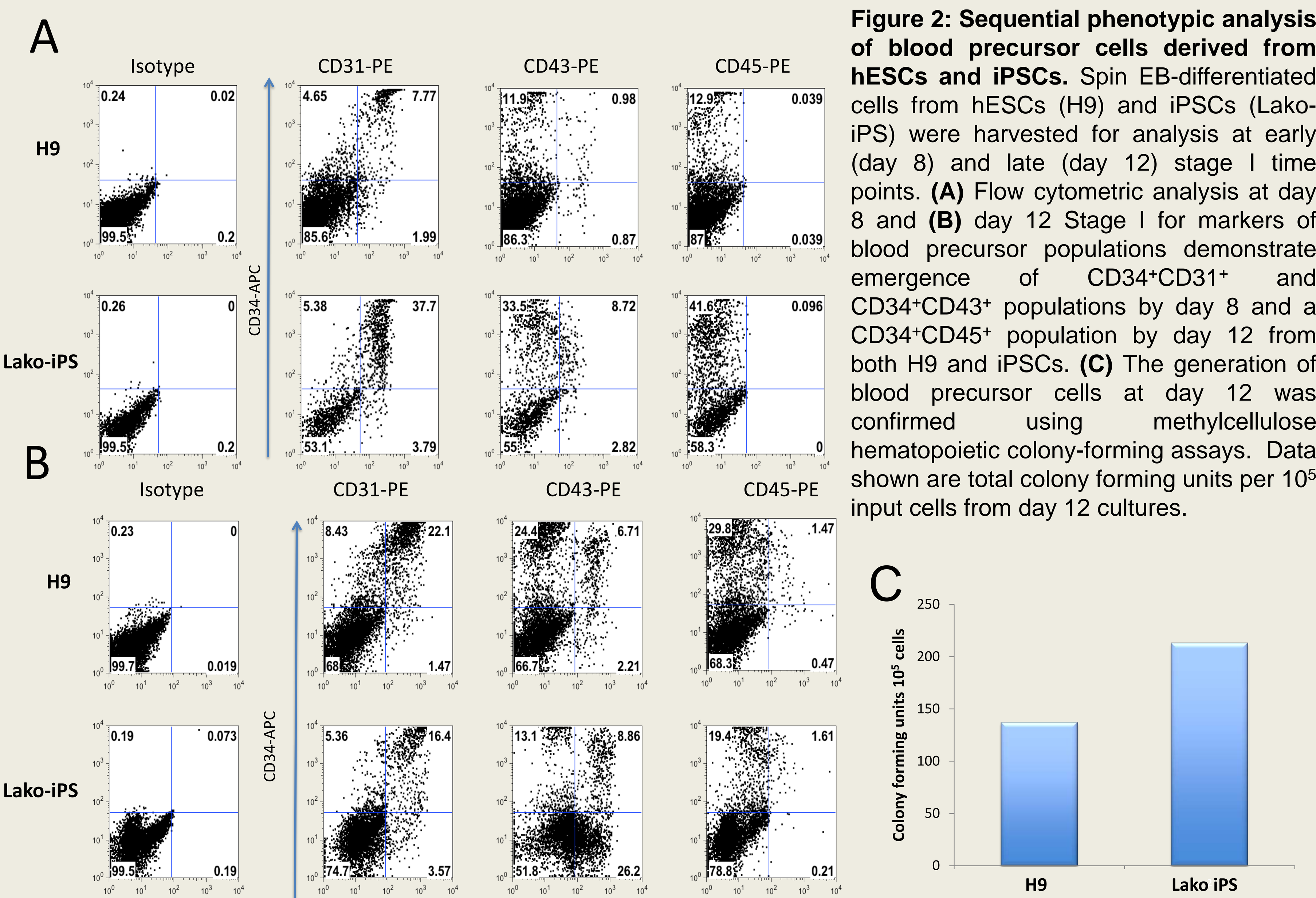

Figure 2: Sequential phenotypic analysis of blood precursor cells derived from hESCs and iPSCs. Spin EB-differentiated cells from hESCs (H9) and iPSCs (Lako-iPS) were harvested for analysis at early (day 8) and late (day 12) stage I time points. **(A)** Flow cytometric analysis at day 8 and **(B)** day 12 Stage I for markers of blood precursor populations demonstrate emergence of $CD34^+CD31^+$ and $CD34^+CD43^+$ populations by day 8 and a $CD34^+CD45^+$ population by day 12 from both H9 and iPSCs. **(C)** The generation of blood precursor cells at day 12 was confirmed using methylcellulose hematopoietic colony-forming assays. Data shown are total colony forming units per 10^5 input cells from day 12 cultures.

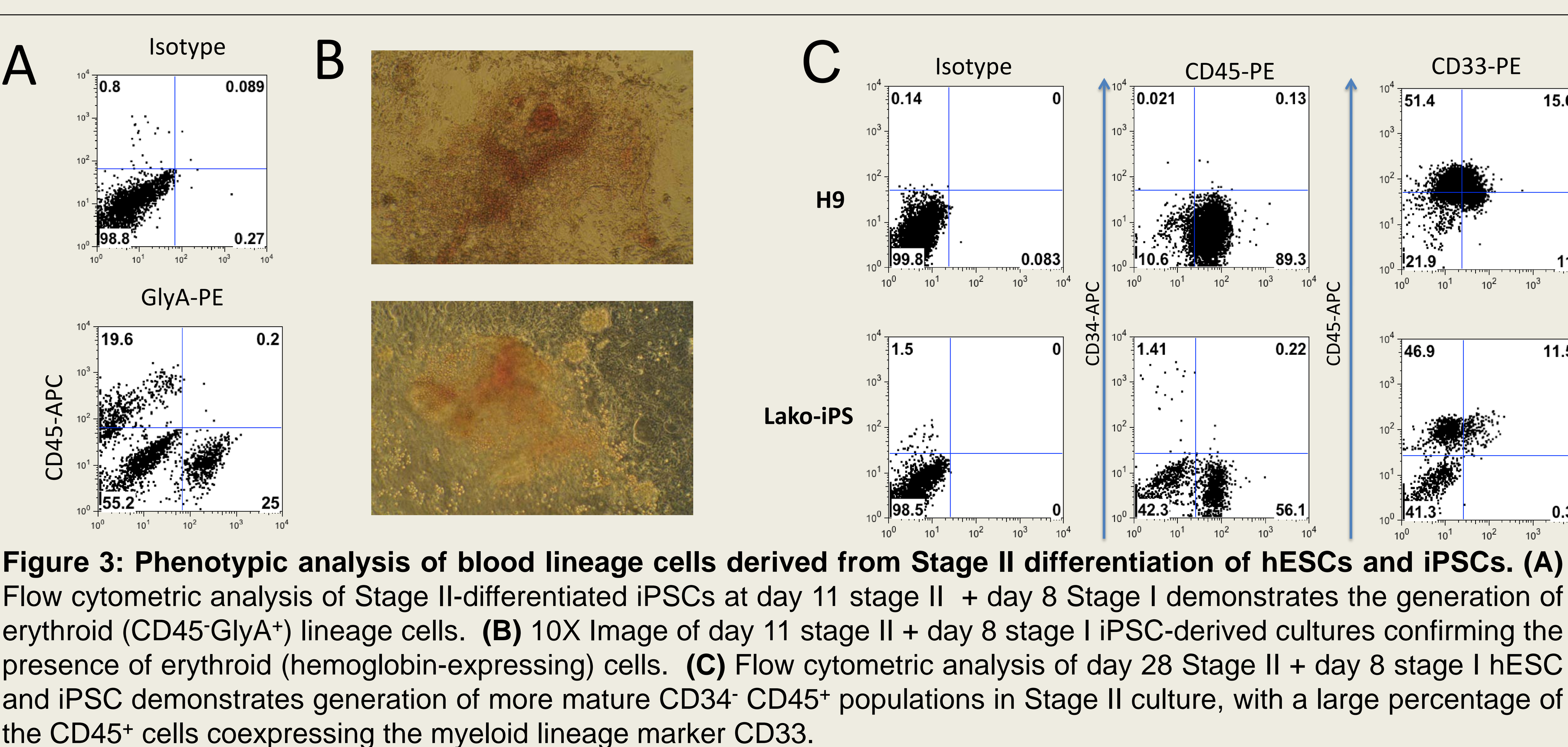

Figure 3: Phenotypic analysis of blood lineage cells derived from Stage II differentiation of hESCs and iPSCs. (A) Flow cytometric analysis of Stage II-differentiated iPSCs at day 11 stage II + day 8 Stage I demonstrates the generation of erythroid ($CD45^-GlyA^+$) lineage cells. **(B)** 10X Image of day 11 stage II + day 8 stage I iPSC-derived cultures confirming the presence of erythroid (hemoglobin-expressing) cells. **(C)** Flow cytometric analysis of day 28 Stage II + day 8 stage I hESC and iPSC demonstrates generation of more mature $CD34^- CD45^+$ populations in Stage II culture, with a large percentage of the $CD45^+$ cells coexpressing the myeloid lineage marker CD33.

Table 1: Cell surface markers for blood lineage cells

CD Antigen	Cellular Distribution
CD31	blood precursors, monocytes, platelets, granulocytes, endothelial cells
CD33	myeloid precursors, monocytes, granulocytes
CD34	blood precursors
CD43	blood precursors, leukocytes
CD45	blood cells (pan-blood lineage marker)
CD235a (GlyA)	erythrocytes

Results

- Both hESCs (H9) and iPSCs (Lako) produce $CD34^+CD31^+$ and $CD34^+CD45^+$ blood precursor cells in the serum-free and stromal-free spin EB system, as measured by flow cytometry (Fig 2 A & B).
- Generation of blood precursor cells in Stage I spin EB differentiation was confirmed by hematopoietic CFU assay for both hESCs and iPSCs (Fig 2C).
- When transferred into a second stage of differentiation, blood cell precursors generated through spin EB culture of hESCs and iPSCs further differentiate into mature blood lineage cells, including erythroid ($CD45^-GlyA^+$; Fig 3A & B) and myeloid ($CD45^+CD33^+$; Fig. 3C) lineages.

Conclusion

The results support the use of the Spin-EB protocol for efficient differentiation of hESCs and iPSCs. Cells derived from both hESCs and iPSCs expressed markers of blood precursor cells and generated mature blood lineage cells in a second stage of differentiation.

Future studies will use this spin EB method to better define the culture conditions necessary to generate lymphocyte precursors and mature lymphocytes (T, B, and NK cells) from hESCs and iPSCs .

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