A Reproducible and Simple Method to Generate Red Blood Cells From Human Pluripotent Stem Cells

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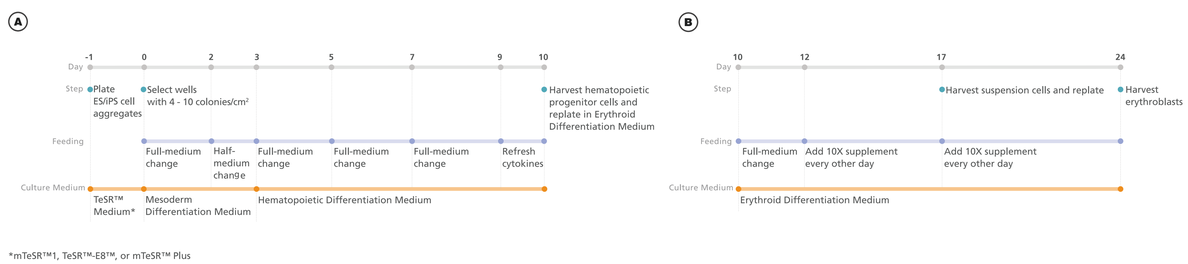
INTRODUCTION

Erythroid cells generated from human pluripotent stem cells (hPSCs) offer a potentially unlimited and safe supply of red blood cells (RBCs) for use in disease modeling, drug development, and transfusion medicine. However, obtaining sufficient quantities of RBCs for many such applications is hampered by technical challenges and the lack of reproducible and robust protocols to differentiate hPSCs into the erythroid lineage. We have developed a simple two-step, serum- and feeder cell-free culture system to generate erythroid cells from hPSCs. Using only three supplements that combine cytokines and other factors to support optimal cell expansion and differentiation efficiency, this method generates a large number of erythroid cells from multiple hPSC lines within 24 days. hPSCs are first differentiated into hematopoietic progenitor cells (HPCs) by specification to mesoderm and hemoendothelial lineage in a monolayer culture system. Next, hPSC-derived HPCs are expanded and differentiated into CD71^{high} GlyA^{+} erythroblasts using erythroid lineage-specific culture conditions. Further culture of these cells for another 7 days with human serum and EPO results in a > 90% pure population of GlyA^{+} erythroid cells and no cell loss. The cells generated in this system exhibit orthochromatic normoblast morphology, increased hemoglobinization and decreased CD71 expression, consistent with erythroid maturation. In summary, we have developed a simple, standardized and reproducible two-step, serum- and feeder cell-free method for generating large numbers of erythroid cells from multiple hPSC lines for basic and translational research.

METHODS



*mTeSR™1, TeSR™-E8™, or mTeSR™ Plus

FIGURE 1. Culture Protocol for Generating Erythoblasts from hPSCs

The protocol involves two steps: Hematopoietic specification of hPSCs **(A)**, and differentiation of hPSC-derived HPCs into erythroid cells **(B)**. **(A)** Human embryonic stem (ES) or induced pluripotent stem (iPS) cells were plated as small aggregates (100 - 200 µm diameter) at a density of 15 - 20 aggregates/cm^2 in mTeSR™1 or TeSR™-E8™ medium on Matrigel®-coated plates, and allowed to attach overnight. The next day, differentiation was initiated by changing the medium to Mesoderm Differentiation Medium. After 3 days, the medium was changed to Hematopoietic Differentiation Medium to induce hematoendothelial and hematopoietic specification. On day 10, HPCs were harvested from the suspension and counted. **(B)** Hematopoietic progenitors were replated in Erythroid Differentiation Medium at a density of 4 x 10^4 cells/mL and the cells were cultured for a further 14 days to generate erythroblasts.

Cells were harvested from the cultures at multiple timepoints, counted and analyzed by flow cytometry for expression of hematopoietic (CD43 and CD34) and erythroid (CD71 and GlyA) markers. Dead cells were excluded by light scatter profile and propidium iodide staining. The number of erythroid cells was calculated as the fraction of cells expressing GlyA on their surface. Erythroid identity of the generated cells was additionally assessed by cell morphology and hemoglobin expression. Six different hPSC lines were used in this study: the ES lines H1, H7, and H9; and the iPS lines 1C, F016, and B004. All hPSC lines were maintained in feeder-free and serum-free culture conditions prior to differentiation.

RESULTS

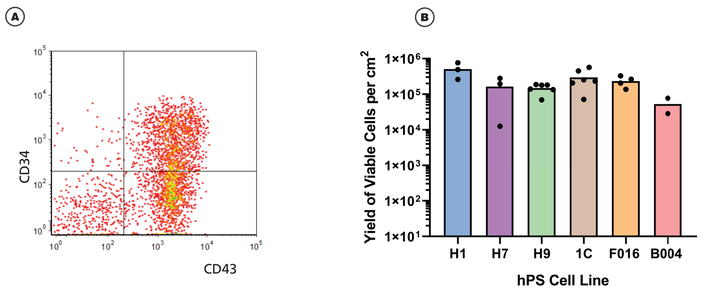


FIGURE 2. hPSCs Differentiate into Hematopoietic Progenitor Cells with High Yields

The hematopoietic specification protocol described in Figure 1A promotes the generation of large numbers of hematopoietic progenitor cells. On day 10, the supernatant cells were harvested and the phenotype and cell yields were calculated **(A & B)**. **(A)** The average frequency of cells expressing the embryonic pan-hematopoietic marker CD43 is 92% (range: 85 - 95%) and the frequency of CD34^{+} cells ranges between 24 - 55%. A representative flow cytometry plot of day 10 hematopoietic progenitors derived from F016 iPS cells is shown. **(B)** The yield of total viable cells per cm^2 from 6 hPSC lines. The overall average across all cell lines was 2.4 x 10^5 cells/cm^2. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean.

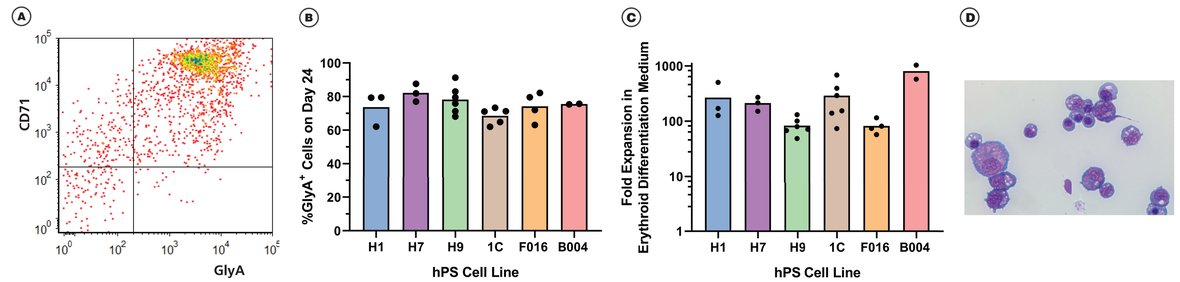


FIGURE 3. hPSC-Derived Hematopoietic Progenitors are Effectively Expanded and Differentiated into Erythroid Cells

hPSC-derived HPCs were differentiated into the erythroid lineage as described in Figure 1B. After 14 days in erythroid culture conditions, the cells were harvested, counted, and analyzed for expression of the erythroid markers CD71 and GlyA **(A-C)**. **(A)** A representative flow cytometry plot of F016 iPS-derived erythroid cells after a total of 24 days of culture. **(B)** The average frequencies of GlyA^{+} erythroid cells on day 24 across 6 hPSC lines. The overall average %GlyA^{+} cells across all cell lines was 76%. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean. **(C)** Erythroid differentiation conditions supported high expansion of hPSC-derived HPCs with overall average 300-fold expansion across all hPSC lines. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean. **(D)** Representative image of May-Grunwald Giemsa stained cells cultured for a total of 24 days (F016 iPS cell line).

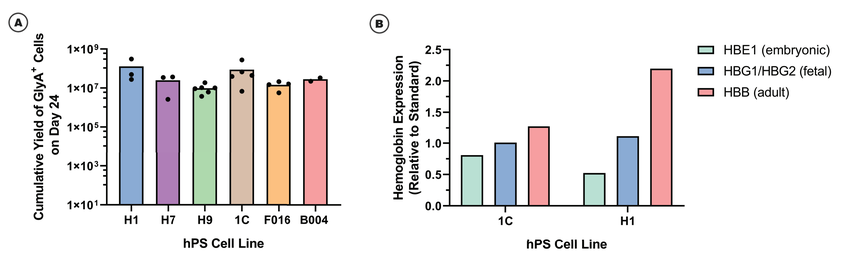


FIGURE 4. Large Numbers of Erythroid Cells Expressing a Mix of Primitive and Definitive Hemoglobin are Generated from hPSC-Derived Hematopoietic Progenitors

(A) Optimal cell differentiation and expansion during both the hematopoietic and erythroid specification steps of the protocol results in the generation of large numbers of erythroid cells after a total of 24 days of culture. The overall average number of generated erythroid cells was 5.3 x 10^7 GlyA^{+} cells/cm^2 across all hPSC lines. Shown is the cumulative yield of GlyA^{+} cells per cm^2 of each initial culture. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean. **(B)** Erythroid cells generated on day 24 of the differentiation protocol expressed a mix of 'primitive' (HBE1) and 'definitive' (HBG1/HBG2 and HBB) hemoglobin types, but with adult and fetal hemoglobin expressed at higher levels than embryonic hemoglobin. Representative RT-qPCR data are shown for two cell lines; 1C (iPS) and H1 (ES). Hemoglobin expression was normalized to expression of housekeeping genes 18S and TBP.

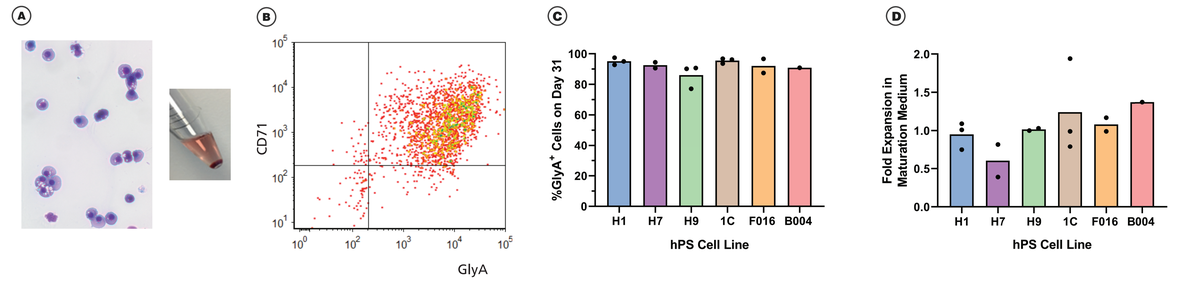


FIGURE 5. hPSC-Derived Erythroblasts Can Mature in the Culture

Erythroblasts generated from hPSC-derived HPCs were further cultured for 7 days under maturation conditions with 3 U/mL erythropoietin (EPO) + 3% human serum. After a total of 31 days of culture, the cells were harvested, counted, and assessed for expression of erythroid markers CD71 and GlyA, and changes in cell morphology**. (A)** Differentiated cells were characterized by orthochromatic normoblast morphology (left), the red color of the cell pellet (right), and **(B)** decreased CD71 expression, all consistent with erythroid maturation. **(B-C)** Maturation cultures resulted in a ≥ 90% pure population of GlyA^{+} erythroid cells. **(B)** Representative flow cytometry plot of cells derived from F016 iPS cells. **(C)** Bar graphs summarize the average frequencies of GlyA^{+} erythroid cells on day 31 across all 6 hPSC lines. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean. **(D)** Notably, no cell loss was observed during the maturation culture. Black dots depict the results of individual experiments, and each dot is an average of two technical replicates; bars show the mean. The overall average cumulative yields of GlyA^{+} cells after 31 days of culture were 9.3 x 10^7 cells per initial cm^2 across all cell lines (data not shown).

Summary

- hPSCs can be differentiated into erythroid cells in a simple two-step, feeder cell-free and serum-free culture system
- Each culture step supports optimal cell expansion and differentiation efficiency and results in high purity and yields of GlyA^{+} erythroid cells from multiple hPSC lines
- The generated erythroid cells expressed a mix of adult, fetal, and embryonic hemoglobin types
- Following maturation culture, the cells exhibited orthochromatic normoblast morphology, high GlyA and low CD71 expression and increased hemoglobinization
- Future work will aim to further increase erythroid cell maturation in vitro