Generation of Large Numbers of Human Megakaryocytes from Pluripotent Stem Cells

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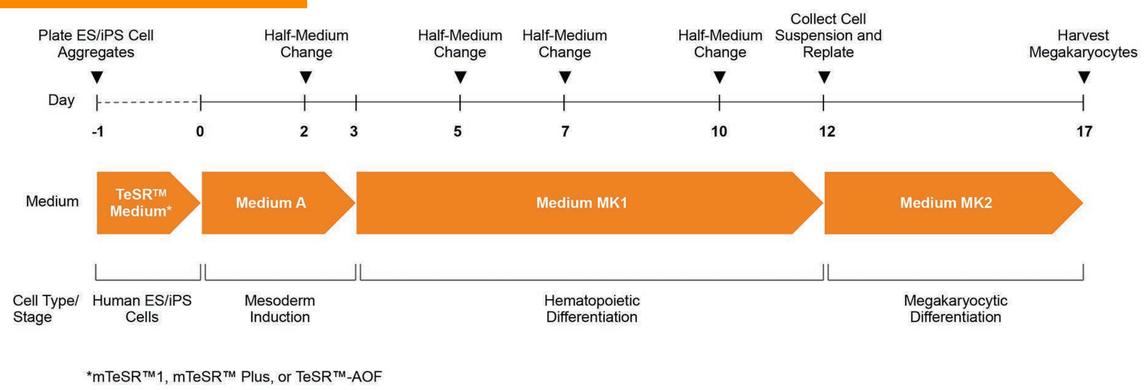
INTRODUCTION

Platelets (PLTs, also known as thrombocytes) are generated by the proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) into megakaryocytes (MKs), a rare subset of large polyploid bone marrow cells. Culture methods that promote the differentiation of human pluripotent stem cells (hPSCs) into MKs and PLTs could potentially be used to generate large numbers of PLTs that can help alleviate chronic PLT shortages and reduce transfusion-related complications. We have developed a simple and robust feeder- and serum-free method for generating MKs and platelet-like particles (PLPs) from hPSCs. This method uses three supplements in a monolayer culture system to generate high yields of MKs from various hPSC lines within 17 days (Figure 1). hPSCs are first differentiated through mesoderm induction and endothelial-to-hematopoietic transition (EHT) into HSPCs favouring megakaryocyte-erythroid differentiation. Next, the megakaryocyte-erythroid-biased HSPCs are cultured in megakaryocyte maturation medium, where they further expand and differentiate into mature MKs that co-express CD41 and CD42, but not erythroid marker glycophorin A (GlyA). The MKs produced by this system are large, polyploid, and capable of shedding PLPs.

METHODS



*mTeSR™1, mTeSR™ Plus, or TeSR™-AOF

FIGURE 1. Protocol for Generating Megakaryocytes from hPSCs

The 17-day protocol includes two main stages: a 12-day stage to differentiate human embryonic stem (ES) or induced pluripotent stem (iPS) cells into megakaryocyte-biased hematopoietic progenitor cells (HPCs), and a 5-day stage to further differentiate human ES or iPS cell-derived HPCs into mature megakaryocytes (MKs). On Day -1, human ES or iPS cells are plated as aggregates (100 - 200 µm diameter, ~100 cells per aggregate) at a density of 10 - 20 aggregates/cm^2 in mTeSR™1, mTeSR™ Plus, or TeSR™-AOF on Corning® Matrigel®-coated plates. After attaching overnight and confirming the number of adhered colonies is within 4 - 10 colonies/cm^2, mesoderm induction is initiated by replacing TeSR™ medium with Medium A (STEMdiff™ Hematopoietic Basal Medium + STEMdiff™ Hematopoietic Supplement A). On Day 3, the medium is changed to Medium MK1 (STEMdiff™ Hematopoietic Basal Medium + STEMdiff™ Megakaryocyte Supplement MK1) for endothelial-to-hematopoietic transition (EHT) and hematopoietic specification. During this phase, human ES or iPS cell-derived HPCs emerge from an adherent layer of endothelial cells and are released into suspension. On Day 12, HPCs in suspension are harvested and replated in Medium MK2 (StemSpan™ SFEM II + STEMdiff™ Megakaryocyte Supplement MK2) at a density of 1 - 3.5 x 10^5 cells/mL and cultured for 5 days to generate mature MKs. The cells were assessed for hematopoietic (CD34 and CD45), MK (CD41 and CD42), and erythroid (GlyA) markers on Day 12 and Day 17. Additionally, the cells were assessed for polyploidy and PLP production by flow cytometry and immunofluorescence microscopy. Four hPSC lines were used in these experiments: human ES cell lines H1 and H9, and human iPS cell lines WLS-1C and STiPS-R038.

RESULTS

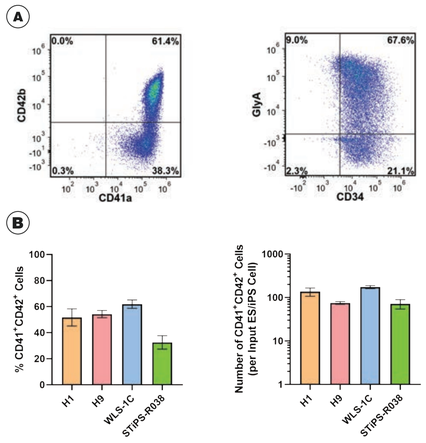


FIGURE 2. hPSCs Differentiate into Hematopoietic Stem and Progenitor Cells in the H-phase on Day 12

Human ES and iPS cells were induced to differentiate to megakaryocyte-erythroid biased HPCs following the protocol described in Figure 1. On Day 12, cells were harvested from the supernatant and analyzed for expression of CD41a, CD42b, CD34, and GlyA by flow cytometry. Dead cells were excluded by light scatter profile and propidium iodide (PI) staining. **(A)** Representative flow cytometry plots for human ES-derived (H9) cells on Day 12. The cells show high levels of CD41a and CD42b as well as of CD34 and GlyA expression, indicating that the protocol supports differentiation of hPSCs to megakaryocyte-erythroid progenitors by Day 12. **(B)** Frequencies and numbers of CD41a+CD42b+ cells per input cell for two human ES cell lines (H1 and H9) and two human iPS cell lines (WLS-1C and STiPS-R038). The average frequency of viable CD41a+CD42b+ cells on Day 12 ranged between 33% and 62%. The average yield of CD41a+CD42b+ cells generated per input cell ranged between 72 and 174. Data are shown as mean ± SEM (n = 7 for H1, n = 20 for H9, n = 19 for WLS-1C, n = 7 for STiPS-R038).

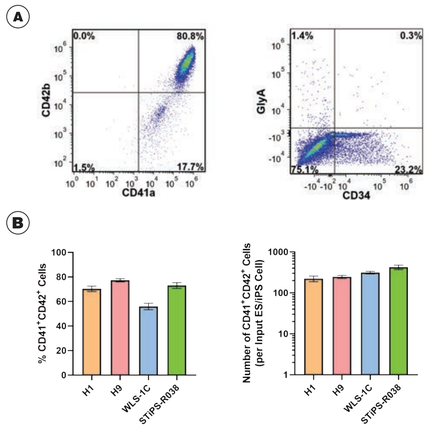


FIGURE 3. hPSC-Derived HSPCs Efficiently Expand and Differentiate into Megakaryocytes on Day 17

On Day 12, hPSC-derived HPCs were cultured for 5 additional days in Medium MK2 to promote differentiation into mature MKs following the protocol described in Figure 1. Cells were harvested and analyzed for expression of CD41a, CD42b, CD34, and GlyA by flow cytometry. Dead cells were excluded by light scatter profile and PI staining. **(A)** Representative flow cytometry plots for human ES-derived (H9) cells on Day 17. The cells expressed high levels of CD41a and CD42b and low levels of GlyA and CD34 markers, indicating differentiation to megakaryocytes. **(B)** Frequencies and numbers of CD41a+CD42b+ MKs per input cell for two human ES cell lines (H1 and H9) and two human iPS cell lines (WLS-1C and STiPS-R038). On Day 17, the average frequency of viable CD41a+CD42b+ cells ranged between 56% and 77%. The average yield of CD41a+CD42b+ MKs generated per input cell ranged between 223 and 425. Data are shown as mean ± SEM (n = 12 for H1, n = 29 for H9, n = 27 for WLS-1C, n = 12 for STiPS-R038).

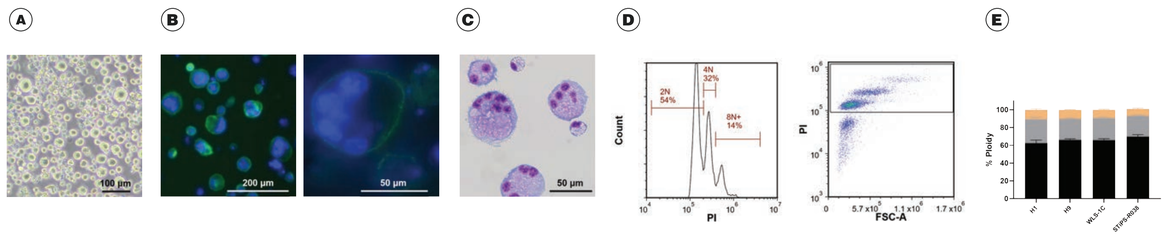


FIGURE 4. hPSC-Derived Megakaryocytes are Polyploid

hPSC-derived MKs display mature and adult-like features: cellular enlargement and polyploidization. **(A)** A representative bright-field image taken on Day 17 showing large MKs derived from H1 cells (10X magnification). **(B)** Representative immunofluorescence images taken on Day 17 showing that CD41a+ MKs derived from H1 and WLS-1C cells are polyploid (20X and 63X magnification, respectively). The cells were formaldehyde-fixed and stained with a fluorescein-conjugated antibody against surface marker CD41a (green), and DAPI (blue). **(C)** A representative cytospin of MKs derived from H9 cells on Day 17 showing high ploidy (40X magnification, May-Grunwald Giemsa stain). **(D)** Representative flow cytometry histogram and scatter plot showing the DNA ploidy profile of ethanol-fixed MKs derived from H9 cells on Day 17. The DNA content was determined by the quantity of PI staining, with different peaks on the histogram representing 2N, 4N, and 8N+ cells. Ploidy analysis was done on gated CD41a+ cells. **(E)** Ploidy distribution of MKs generated from two human ES cell lines (H1 and H9) and two human iPS cell lines (WLS-1C and STiPS-R038). The average ploidy distributions of CD41a+ cells on Day 17 were 66%, 24.5%, and 9.5% for 2N, 4N, and 8N+, respectively. Data are shown as mean ± SEM (n = 6 for H1, n = 28 for H9, n = 19 for WLS-1C, n = 10 for STiPS-R038).

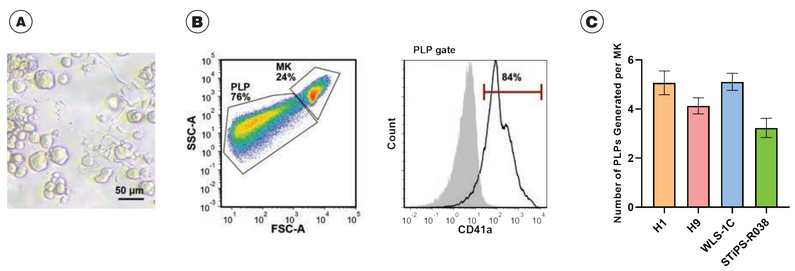


FIGURE 5. hPSC-Derived Megakaryocytes Generate Platelet-Like Particles

hPSC-derived MKs obtained using STEMdiff™ Megakaryocyte Kit are capable of proplatelet formation to yield functional PLPs. **(A)** A representative bright-field image taken on Day 17 showing MKs derived from H1 cells formed proplatelets with long cytoplasmic protrusions (10X magnification). **(B)** Representative flow cytometry forward/side scatter profile of MKs and PLPs and histogram of PLPs derived from H9 cells on Day 17. The PLP gate is based on control platelets (PLTs) prepared from fresh blood. Cells were also stained with antibodies against CD41a, CD45, and GlyA for PLP characterization and enumeration. PLPs showed a high level of CD41a expression (and no CD45 and GlyA expression, data not shown) as in control PLTs. Grey filled histogram represents CD41a Fluorescence Minus One (FMO) control. **(C)** Numbers of PLPs generated per MK on Day 17 for two human ES cell lines (H1 and H9) and two human iPS cell lines (WLS-1C and STiPS-R038). PLPs and MKs were enumerated based on the number of CD41a+CD45-GlyA- cells in the PLP gate and viable CD41a+ cells in the MK gate, respectively. The average yield of PLPs generated per MK ranged between 3.2 and 5.1. Data are shown as mean ± SEM (n = 12 for H1, n = 28 for H9, n = 27 for WLS-1C, n = 12 for STiPS-R038).

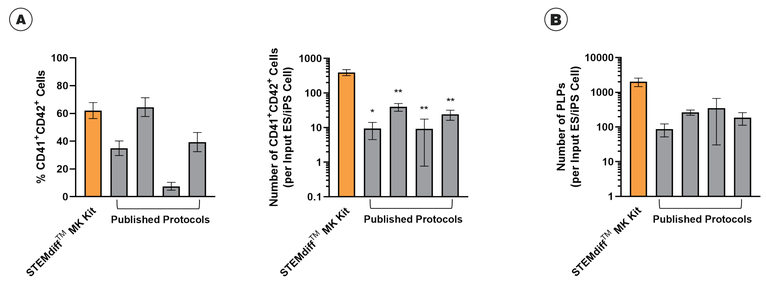


FIGURE 6. STEMdiff™ Megakaryocyte Kit Produces More Megakaryocytes and Platelet-Like Particles than Other Published Protocols

Human ES and human iPS cells were differentiated into MKs using STEMdiff™ Megakaryocyte Kit and four different published protocols from the literature with modifications. **(A)** Frequencies and numbers of CD41a+CD42b+ MKs per input cell for two human ES cell lines (H1 and H9) and two human iPS cell lines (WLS-1C and STiPS-R038) were analyzed by flow cytometry as shown in Figure 2. **(B)** Numbers of PLPs generated per input cell were enumerated as described in Figure 5. Compared to the published protocols, STEMdiff™ Megakaryocyte Kit produced 10- to 40-fold more CD41a+CD42b+ MKs and 6- to 23-fold more PLPs per input cell. P values were calculated using a one-way ANOVA followed by Dunnett's post hoc test (*$p < 0.05$, **$p < 0.01$). Data are shown as mean ± SEM (n = 7 - 8).

Summary

- We developed a simple, reproducible, two-step serum- and feeder-free culture system for generating large numbers of human megakaryocytes from hPSCs
- The first culture phase (H-phase, days 0 - 12) produces large numbers of CD34+CD41+GlyA+ HSPCs from human ES and iPS cells
- The second phase of culture (MK-phase, days 12 - 17) further matures the HSPCs into CD41+CD42+ megakaryocytes that are large, polyploid, and capable of producing platelet-like particles
- Our culture system produces more megakaryocytes and platelet-like particles than other published protocols
- Future work will focus on assessing functionality of PLPs and increasing production to levels that are closer to those obtainable for primary bone marrow-derived MKs