

Generation of Megakaryocytes and Platelets from Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSC) have the potential to produce any tissue type in the body and thus represent a source of cells for regenerative medicine. Here we have shown that human platelets can be produced from embryonic or induced pluripotent stem cells in a defined culture system. We describe a serum- and feeder-free culture system that enabled the generation of megakaryocyte (Mk) progenitors and functional platelets from hPSCs. After 13 days the differentiated population included precursor cells that formed colonies containing differentiated Mks, and after 20 days these Mks were able to fragment into platelet-like particles that were functional. This protocol represents an important step towards the generation of human platelets for therapeutic use.

Keywords: Embryonic stem cells, Megakaryocytes, Platelets, Feeder-free, Differentiation, Hematopoiesis

1 Introduction

Pancytopenia and thrombocytopenia remain significant clinical problems for patients suffering from a range of medical conditions. Therefore, generating platelets from human pluripotent stem cells could prove to be a useful alternative source to single donor platelets obtained from healthy individuals for transfusion. Defined conditions are critical for the reproducibility of protocols and the removal of materials containing animal products is highly desirable. We have established an animal product-free and stromal cell-free differentiation protocol for differentiating cells towards CD41-positive Mks that then releases platelet-like particles that display functionality. Our laboratory has established protocols based on the generation of homogenous “spin embryoid bodies (EBs)” from hPSCs (1) in a defined medium that is supplemented with cytokines to bias differentiation towards desired outcomes (2, 3). This protocol allowed for the robust generation of CD41⁺ and/or CD34⁺ hematopoietic cells that contain Mks progenitors. Furthermore, quantitative PCR

analyses showed that the CD41⁺ cells express high levels of Mk-associated genes and that the cells display polyploidy characteristic of developing Mks. In addition, the supernatant of day 20 cultures yielded CD41⁺ CD42b⁺ platelet-like particles that could be activated by ADP and thus upregulate CD62P.

2 Materials

2.1 Mouse Embryonic Fibroblast (MEF) Medium (notes 1 and 2)

500 ml DMEM medium high glucose (4.5 g/l) (Invitrogen) supplemented with 5 ml Penicillin–Streptomycin (Pen-Strep) Solution (Biological industries) and 5 ml L-Glutamine (Biological Industries) and 10 % fetal bovine serum (Invitrogen).

2.2 Human Pluripotent Stem Cell (hPSC) Medium (notes 1 and 2)

500 ml DMEM/Hams F12 medium (Invitrogen) supplemented with 15 % knockout serum replacer (Invitrogen), 5 ml Non-essential amino acids (Invitrogen) 5 ml Pen-Strep Solution, 5 ml L-Glutamine, 50 mM 2-β Mercaptoethanol (Sigma) and 4 ng/ml recombinant human fibroblast growth factor (rhFGF2) (Peprotech).

2.3 Chemically Defined Medium (notes 1 and 2)

1. IMDM (Invitrogen) and F12 Medium (Invitrogen) are added at a 1:1 ratio—212.4 ml each.
2. 25 ml (5 mg/ml) of recombinant human serum albumin (Novozymes).
3. 5 ml (100×) Lipids (Invitrogen).
4. 5 ml (100×) insulin, transferrin, selenium solution (Invitrogen).
5. 50 mM mono-thioglycerol (Sigma).
6. 25 ml protein free hybridoma medium (Invitrogen).
7. 2 mM glutamax (Invitrogen).
8. 2.5 ml Pen-Strep.

3 Methods

3.1 Preparation of Mouse Embryonic Fibroblasts (MEF) Feeder Layer (note 3)

1. Cells are isolated from 12.5 to 13.5 day mouse embryos into single cells suspension.
2. Cells are grown in 14 mm diameter tissue culture plates (Nunc) until confluent with MEF medium.
3. Once confluent (2–4 days), cells were trypsinized (Trypsin EDTA Solution A, Biological Industries) and split 1–3. This is P1.
4. Once confluent the cells were passaged again with trypsin and split 1–3. This is P2.

5. Once P2 is confluent cells were treated for 2 h with 1 mg/ml of Mitomycin C (Fermentek) to stop cell division.
6. Cells are then trypsinized and counted.

3.2 Human Embryonic or Induced Pluripotent Stem Cells (hPSC) Maintenance (note 4)

1. MEFs were thawed and placed on gelatin (0.1 %, Sigma)-coated tissue culture plates at $2.0\text{--}6.0 \times 10^4$ cells/cm² and incubated for at least 4 h before adding hPSC cells.
2. Human pluripotent stem cells were maintained in pluripotent state on mitomycin-treated MEF feeder cells in hPSC medium.
3. When confluent, every 3–4 days, cells were enzymatic passaged with trypsin solution A (Invitrogen) as previously described (2, 4).

3.3 Differentiation of hPSCs

1. hPSCs were harvested with TrypLE Select (Invitrogen).
2. Single cells were resuspended in serum-free CDM medium (5) supplemented with 5–15 ng/ml rh Bone Morphogenic Protein (BMP4) (Peprotech), 10–15 ng/ml rh Vascular Endothelial Growth Factor (VEGF) (Peprotech), 10 ng/ml rhFGF2 (Peprotech) and 25 ng/ml rh Stem Cell Factor (SCF) (Peprotech) as described previously (2) (note 5).
3. One hundred microlitres of cell suspension containing 5,000 hPSCs were placed into a well of round-bottom low-adherent 96-well plates (Nunc).
4. Within 24 h, single embryoid bodies (EBs) formed in each well.
5. At 10 days, 72 EBs were transferred to each well of a 6-well flat-bottom tissue culture plate (Nunc) containing 7.2 ml of CDM supplemented with 20–30 ng/ml rh thrombopoietin (TPO) (Peprotech), 25 ng/ml rhSCF and 25–50 ng/ml rh interleukin (IL)-3 (Peprotech).
6. EBs were further cultured for a total of 20 days.
7. At any point after 13 days of differentiation the cells generated in differentiation cultures can be removed and with the use of tryple select dissociated into a single cell suspension for immunophenotyping, colony assays, or ploidy assessment.
8. At any point after 13 days of differentiation the EBs generated in differentiation cultures can be removed and RB buffer directly added to the pellet for RNA extraction.

3.4 Flow Cytometric Analysis of Differentiated hPSCs (note 6)

1. After 13 days of differentiation cultures were assessed for the presence of hematopoietic cells and megakaryocytes using monoclonal antibodies such as anti-CD34 (BD Biosciences), anti-CD41 (BD Biosciences) and CD45-PE (BD Biosciences).
2. Incubation of the antibodies with cells should be for a minimum of 20 min at 40 °C.

3. Cells were washed in phosphate buffered saline (PBS) to remove unbound antibody.
4. Centrifugation of cells was at $480 \times g$ for 5 min at 40 °C.
5. Before acquisition on the flow cytometer differentiated cells pellets were resuspended in 200 μ l of PBS containing propidium iodide (PI) to exclude dead cells.
6. Analysis of differentiation cells was performed on a FACSCalibur using CellQuest Pro software (BD Biosciences).

3.5 Methylcellulose-Based Myeloid Colony Forming Assay

1. Triplicate assays were completed in 24-well tissue culture treated plates (Nunc) with 10,000 cells added per well in 0.5 ml of Methocult™ (Stem Cell Technologies, Canada).
2. Methocult was supplemented with 20 ng/ml rh granulocyte-macrophage-colony stimulating factor (GM-CSF), 50 ng/ml rh SCF, 20 ng/ml rh IL-3, 3 U/ml rh erythropoietin (EPO) and 20 ng/ml rh IL-6 (all from Peprotech).
3. Cells were incubated at 37 °C in 5 % CO₂ for 14 days.
4. Colonies were scored according to their morphology either as GEMM-, GM-, G- or M-CFU.
5. To determine presence of Mks in GEMM-CFUs, colonies were picked from the methylcellulose, dried on a slide and stained with May-Grunwald/Giemsa to detect typical Mk morphology (Fig. 1).

3.6 Collagen-Based Megakaryocyte Colony Forming Assay

1. Duplicate assays were performed in 24-well tissue culture treated plates into which 10,000 cells were mixed with a collagen-based medium (MegaCult™, Stem Cell Technologies).
2. Megaocult was supplemented with 50 ng/ml rhTPO, 25 ng/ml rhSCF and 10 ng/ml rh IL-3 to stimulate colony formation.
3. After 14 days plates were dried and stained with anti-CD41 antibody and an alkaline phosphatase colorimetric assay according to the kits instructions (Stem Cell Technologies) (Fig. 1a).

3.7 Quantitative Real-Time PCR

1. RNA was extracted from hPSCs at various time points using an RNA extraction kit (GeneAid).
2. Superscript III (Invitrogen) was used to reverse transcribed RNA to cDNA with random hexamer primers.
3. Real-time PCR was performed using Taqman gene expression probes (Applied Bioscience) and the one-step PCR system absolute thermal cycler and software (Applied Bioscience).
4. The comparative cycle threshold (CT) method was used to analyze data, with gene expression levels compared to GAPDH expression as previously described (2) and ddCT used for quantitation (Fig. 2).

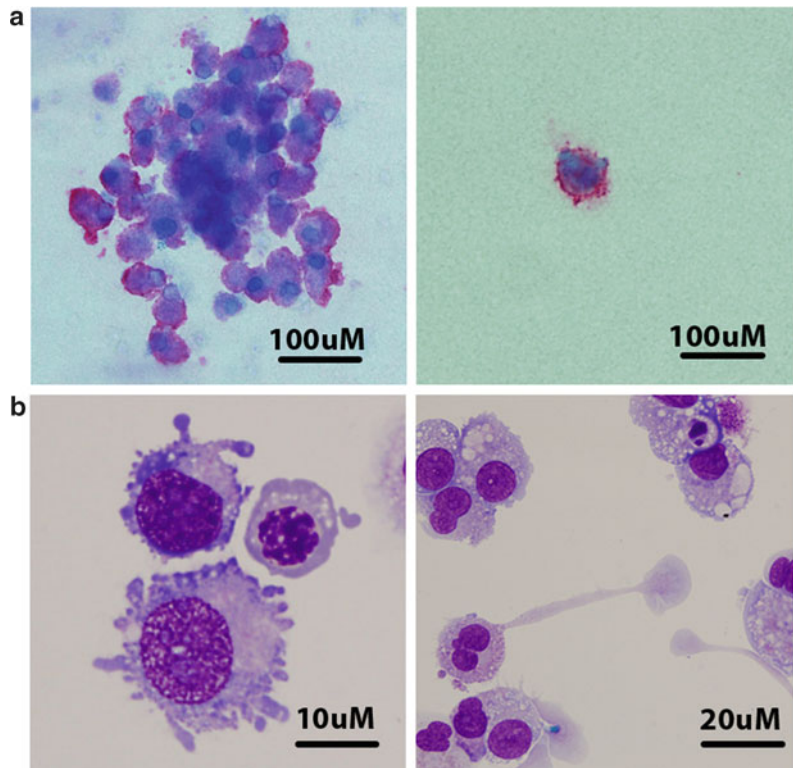


Fig. 1 (a) Images of megakaryocytic colonies expressing CD41 cultured from differentiated human ES cells. (b) Images of megakaryocytes stained with May-Grunwald/Giemsa picked from colonies generated from hPSCs differentiation cultures

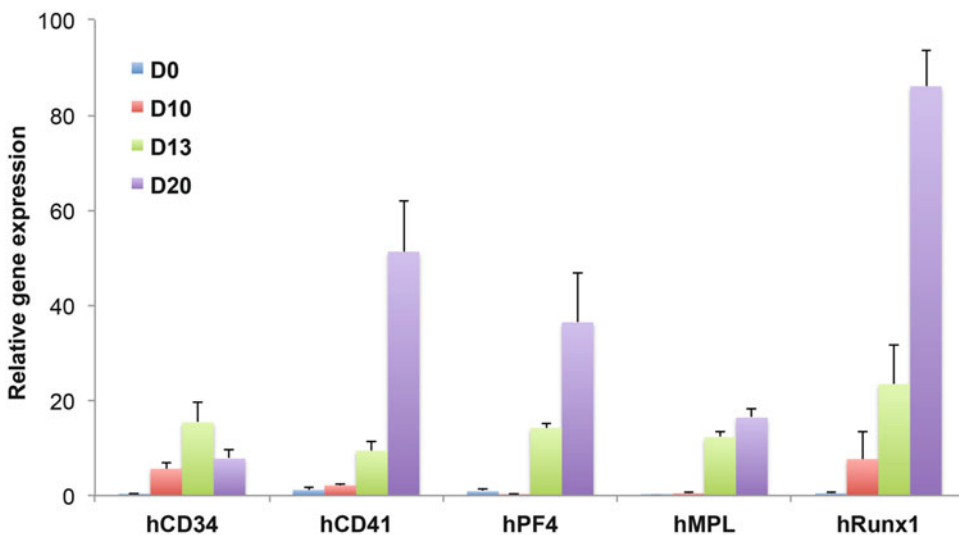


Fig. 2 Hematopoietic gene expression in differentiated hPSCs. cDNA was generated from EBs. Gene expression was quantified by real-time PCR analysis for the indicated genes. Histograms show relative gene expression expressed as mean \pm sSEM of five individual experiments

3.8 Analysis of Cell Ploidy (note 7)

1. Megakaryocytes have a distinct characteristic that allows the DNA to double without the cell dividing—polyploidization.
2. This polyploidization or cell ploidy can be analyzed by staining cellular DNA with PI.
3. Single cell suspensions of cells were fixed in cold ethanol at 40 °C overnight.
4. Cells were centrifuged at $850 \times g$ for 5 min.
5. To the resuspended pellet PBS is added.
6. RNase A (2 mg/ml) (Sigma) was added to the resuspended cells and incubated for 30 min at 370 °C.
7. 15 min before cells are ready to be acquired in the flow cytometer, PI (Sigma) was added.
8. At least 50,000 events were collected to allow enough cells in the >8 N peaks to be seen.

3.9 Flow Cytometric Analysis of Platelet-Like Particles (note 8)

1. To harvest platelet-like particles from hPSCs cultures, the supernatants were collected.
2. Centrifugation at $480 \times g$ for 5 min at 4 °C was performed to collect the particles.
3. Immunophenotyping of the particles were performed using monoclonal antibodies. CD41a-FITC and CD42b-PE and stained similarly to previous protocol.
4. Acquisition of the flow cytometry data used log amplification of forward and side scatter to include the small platelet-like particles.
5. Isotype controls for the antibody stains must be included and depends on the supplier of the antibodies.
6. At least 15,000 events were acquired.

3.10 Platelet Activation

1. Platelet-like particles were harvested as described above, pelleted by centrifugation and resuspended in PBS.
2. The pelleted particles were incubated in the presence or absence of 20nM ADP at room temperature for 20 min and then stained for the expression of CD41a-FITC and CD62P-PE (6).
3. Cord blood platelets were included as a positive control (6) (note 8).
4. Isotype controls for the antibody stains are essential.
5. 15,000 events were acquired on the flow cytometer.
6. Platelet-like particles that were activated by ADP will express higher levels of the CD62P marker and can be seen as an increase in intensity or shift in the histogram (3, 6).

4 Notes

1. All components of the medium should be mixed together and filtered with an 0.2 μm size media filter (Nunc) to improve sterility.
2. Complete medium should be stored at 4 °C for up to 1 month.
3. To store large numbers of MEFs for long term $1\text{--}2 \times 10^6$ of them can be placed into a cryopreservation tube (Nunc) together with MEF medium and 10 % DMSO and aliquots of MEFs were kept in liquid nitrogen for storage until use. The MEFs can be then thawed and used according to need.
4. HPSCs should be checked for chromosomal abnormalities via karyotyping every 6 months and stemness of hPSCs must be checked with the use of antibody expression of Tra-1—60 and Oct4 every 12 weeks.
5. Cytokines should be reconstituted with a carrier protein, such as bovine serum albumin, to maintain stability and stored at -80 °C in high concentrations. Aliquots can then be defrosted prepared in working concentration and either stored short term at -20 °C for maximum 4 weeks or at 4 °C for up to a week.
6. All antibodies used are titrated to give optimal staining and usually the quantity needed is much lower than the amount suggested by the supplier. This allows for extended usage of the antibody.
7. For cell cycle analysis and ploidy the use of peripheral blood as a control for the 2 N peak to configure the flow cytometer for the first time. Also allows easier identification of the ploidy Mks.
8. Platelets generated from umbilical cord blood can be used as a positive control for all experiments testing the platelets produced in culture (6). This makes it much easier to configure the flow cytometer and allow better identification of the cells.

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