Video Article

Megakaryocyte Differentiation and Platelet Formation from Human Cord Blood-derived CD34⁺ Cells

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Abstract

Platelet production occurs principally in the bone marrow in a process known as thrombopoiesis. During thrombopoiesis, hematopoietic progenitor cells differentiate to form platelet precursors called megakaryocytes, which terminally differentiate to release platelets from long cytoplasmic processes termed proplatelets. Megakaryocytes are rare cells confined to the bone marrow and are therefore difficult to harvest in sufficient numbers for laboratory use. Efficient production of human megakaryocytes can be achieved *in vitro* by culturing CD34⁺ cells under suitable conditions. The protocol detailed here describes isolation of CD34⁺ cells by magnetic cell sorting from umbilical cord blood samples. The necessary steps to produce highly pure, mature megakaryocytes under serum-free conditions are described. Details of phenotypic analysis of megakaryocyte differentiation and determination of proplatelet formation and platelet production are also provided. Effectors that influence megakaryocyte differentiation and/or proplatelet formation, such as anti-platelet antibodies or thrombopoietin mimetics, can be added to cultured cells to examine biological function.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56420/>

Introduction

Isolation of adequate numbers of primary human megakaryocytes (MK) for regular laboratory use is not feasible due to their low frequency in the bone marrow, where they account for ~0.01% of nucleated cells¹. A convenient alternative is the *ex vivo* expansion and differentiation of hematopoietic stem and progenitor cells in the presence of specific growth factors. A number of cytokines including stem cell factor (SCF; c-kit ligand) and interleukin (IL)-3 and IL-11 have been employed in culture systems to produce MKs. Thrombopoietin (TPO) is the most effective growth and differentiation factor for megakaryocytic cultures and is effective alone or with other cytokines, such as SCF and IL-3². TPO can act on stem cell populations to result in both the proliferation and maturation of MKs².

MK produce platelets from cytoplasmic protrusions called proplatelets and, *in vivo*, approximately 1 x 10¹¹ platelets are formed daily to sustain platelet counts of 150 - 400 x 10⁹/L. Platelet production *in vitro* is up to 1000-fold lower than the *in vivo* estimates³, and this has given rise to numerous culture conditions using CD34⁺ hematopoietic progenitor cells to improve MK and platelet production *in vitro*. The initial source of CD34⁺ cells used for MK differentiation was human peripheral blood⁴. Other cell sources include bone marrow^{5,6}, embryonic stem cells/induced pluripotent stem cells (ESC/iPSC)⁷, and umbilical cord blood (UCB)^{8,9,10}. Human bone marrow CD34⁺¹¹ and mouse lineage negative bone marrow cells⁵ produce MK and platelets *in vitro*; nevertheless, the lack of availability of human bone marrow limits its use as a source of CD34⁺ cells. In contrast, ESC and iPSC represent an unlimited source of cells for *in vitro* platelet production. Platelet production from these cells requires feeder cells such as murine OP9 cells and longer culture periods. Platelets derived in feeder-free conditions appear to be less functional¹². iPSC-derived platelets are likely to be of use in clinical settings since they can be expanded to a large scale. This process requires lentiviral-mediated transduction of transcription factors and long-term cell culture¹³.

UCB is an accessible source of CD34⁺ cells that can be readily used in research settings. TPO alone can promote differentiation of cord blood-derived CD34⁺ cells and this gives rise to highly pure, mature MKs without the need for serum supplementation or co-culture with feeder cells. Other cytokines such as SCF may decrease differentiation from UCB CD34⁺ cells, while Flt-3 ligand and IL-11 promote the production of immature megakaryocytes¹⁴. This protocol describes the production of highly pure MK cultures from cord blood CD34⁺ cells in serum-free conditions.

Protocol

This protocol was approved by the South Eastern Sydney Human Research Ethics Committee and ratified by the University of New South Wales' Human Research Ethics Committee. Umbilical cord blood obtained from healthy donors was provided by the Sydney Cord Blood Bank (Sydney, NSW, Australia). Volumes of approximately 100 mL were used for this procedure.

NOTE: Work in a Class II biosafety cabinet using aseptic technique. Decontaminate the exterior of the cord blood bag with 70% ethanol. Use sterile instruments (scissors, tweezers) for this procedure.

1. Cord Blood Cell Preparation and Isolation of CD34⁺ Cells

1. Prepare sterile separation buffer (SB) with phosphate buffered saline (PBS), at pH 7.2, and containing 0.5% bovine serum albumin and 2 mM EDTA.
2. Dispense 10 mL of SB into a 50 mL conical tube (one tube per 10 mL of blood is required). Using a G18 blunt needle mounted on a 10 mL syringe, draw 10 mL of blood from the bag and dispense into the 50 mL tubes containing 10 mL of SB.
3. Add 15 mL of lymphocyte separation media (see **Table of Materials**) to the bottom of the tube containing the diluted blood, creating two layers.
NOTE: Dispense media slowly at the bottom of the tube to avoid mixing the different layers.
4. Centrifuge tubes at $1,200 \times g$ for 30 min at room temperature (RT) with no break and no acceleration.
5. Transfer the layer near the center of the tube containing mononuclear cells (approximately 5 - 10 mL from each tube) into a new 50 mL tube using a Pasteur pipette. Add SB to each tube to a total volume of 50 mL.
6. Centrifuge at $400 \times g$ for 10 min at RT. Discard the supernatant.
7. Resuspend cell pellets carefully with a Pasteur pipette in 5 - 10 mL of SB. Combine the suspended cells and bring volume to 50 mL with SB.
8. Count cells using Trypan blue staining and a hemocytometer or automated cell counter (see **Table of Materials**). To determine the percentage of CD34⁺ cells in the sample, resuspend 2.5×10^5 cells in 100 μ L of SB and stain by adding 10 μ L of anti-CD34-PE antibody for 15 - 30 min at 4 °C. Analyze by flow cytometry (**Figure 1A**).
9. Centrifuge tubes at $400 \times g$ for 10 min at 4 °C. Discard supernatant.
NOTE: If not required immediately, mononuclear cells can be frozen at this stage.
10. Resuspend cells in 300 μ L of SB per 10^8 cells. Add 100 μ L of FcR human IgG (blocking reagent, see **Table of Materials**) and 100 μ L of CD34 magnetic beads per 10^8 cells. Mix gently and incubate at 4 °C for 30 - 40 min.
NOTE: A single cell suspension is required (if necessary, pass the cells through a 30 μ m filter before adding reagents). Use the reagent volumes described here for 10^8 cells or less. For more than 10^8 cells, scale up the reagents (SB, blocking solution, and CD34 magnetic beads) accordingly.
11. Prepare the LS separation column during the incubation period by placing the LS column in a magnetic holder and washing with 3 mL of SB. Discard the effluent.
NOTE: LS separation columns can be loaded with up to 2×10^8 total cells. Smaller and larger columns are available.
12. Add 5 - 10 mL of SB to the cell mixture and centrifuge at $400 \times g$ for 10 min. Discard the supernatant.
13. Resuspend cells in 1.5 mL of SB and load the cell suspension (1.5 mL) onto the LS column. Collect the flow through containing the unlabeled cells in a 15 mL collection tube (negative fraction #1). Let the liquid drain and wash the column with 1.5 mL of SB.
14. Load the collected flow through (3 mL) back into the column and collect the flow through in the same 15 mL collection tube (negative fraction #1). Wash the LS column 3 times with 3 mL of SB.
NOTE: If required, cells in the negative fraction (12 mL) can be stained with anti-CD34 antibody to ascertain capture of CD34⁺ cells by the LS column.
15. Remove column from the magnetic separator and flush cells slowly with a syringe plunger into a new 15 mL tube with 2 mL of SB.
16. Place column back on magnetic separator and load the 2 mL of cells back into the column. Collect the flow through and wash with 2 mL SB. This is the negative fraction #2 (4 mL).
17. Remove LS column from magnetic separator and add 2 mL of SB. Flush cells steadily and firmly with a syringe plunger to collect the CD34⁺ cell fraction. Count the cells using trypan blue staining and a hemocytometer or automated cell counter.
18. Centrifuge tube with the positive fraction at $400 \times g$ for 15 min at 4 °C. Discard the supernatant. Resuspend cells in serum-free media for CD34⁺ cells (SFM, see **Table of Materials**).
NOTE: If not required immediately, cells can be frozen in liquid nitrogen at this stage.

2. Purity Check of Isolated CD34⁺ Cells

1. Stain 2×10^4 cells from the positive fraction with 10 μ L anti-CD34-PE antibody and 20 μ L anti-CD45-PerCP antibody for 15 min at 4 °C (use a separate tube for isotype controls) (**Figure 1B**).
2. Add 1 mL of SB to wash. Centrifuge at $400 \times g$ for 10 min. Resuspend pellet in 200 μ L of SB.
3. Perform flow cytometric analysis and gate the live cell population to exclude debris (**Figure 1B**). Set the gate for PE and PerCP positive populations using the PE and PerCP isotype controls (**Figure 1B**) and determine the percentage of CD34⁺/CD45⁺ cells.

3. Megakaryocyte Differentiation

1. Seed 5×10^5 cells/mL CD34⁺ cells in 2 mL of SFM supplemented with 50 ng/mL recombinant human thrombopoietin (rhTPO) per well in a 12-well plate. Incubate cells at 37 °C, 5% CO₂ in a humidified atmosphere. If cells are confluent before they are required for analysis, harvest the cells and split into multiple wells with fresh media and rhTPO.
NOTE: Two or three wells should be prepared specifically to monitor differentiation at different time points (e.g., days 7, 9, and 10).

2. Harvest cells from the wells set aside to monitor differentiation without disturbing the cells in the other wells.
3. Stain cells with 20 μ L anti-GPIIb/CD41-FITC antibody and 10 μ L anti-GPIX/CD42a-Alexa Fluor 647 antibody in a final volume of 100 μ L. Set up a control tube using the respective isotype control antibodies. Incubate for 15 - 30 min at 4 °C.
4. Add 1 mL of SB to wash. Centrifuge at 400 $\times$ g for 10 min. Discard the supernatant and resuspend the pellet in 200 - 300 μ L of SB. Analyze by flow cytometry.
5. For each fluorophore, analyze the isotype controls to set the gating for FITC and Alexa Fluor 647 positive populations. Analyze the stained cell samples to determine the percent of CD41⁺/CD42a⁺ double positive cells, which represent mature MK (**Figure 1C**).
6. For microscopic visualization of cell surface markers stain cells as described in 3.3.
 1. Add 1 mL of SB to wash. Centrifuge at 400 $\times$ g for 10 min. Resuspend the pellet in 100 μ L of SB and spin onto a glass slide at 1,000 $\times$ g for 5 min. Fix cells on the slide by dipping in methanol for 30 s. Air dry, add 20 μ L of mounting media containing DAPI (see **Table of Materials**), cover with a coverslip, and visualize using a fluorescent microscope (**Figure 2A**).
7. For visualization of intracellular antigens, resuspend cells in PBS and fix with paraformaldehyde (1% final concentration) for 15 min at room temperature. To permeabilize the cells, add triton-X 100 (0.1%) and incubate for 15 min. Wash cells with 2 mL PBS/0.1% triton-X 100. Resuspend in 100 μ L of PBS/0.1% triton-X 100, add anti vWf and anti CD62p antibodies (1:200 dilution) and incubate for 30 min at room temperature.
 1. Wash with 2 mL PBS/0.1% triton-X 100 and centrifuge at 400 $\times$ g for 10 min, resuspend in 100 μ L of the same buffer, and add anti-mouse IgG-Alexa 594 and anti-rabbit IgG-Alexa 488 (1:100). Incubate for 30 min at room temperature, wash with 2 mL PBS/0.1% triton-X 100, resuspend in 100 μ L of the same buffer, and add 20 μ L of anti CD42b-APC. Then spin the cells onto glass slides as described in step 3.6.1 and prepare samples for microscopic visualization as described in step 3.6.1.
8. For ploidy determination, harvest cells at days 12 or 13 of differentiation. Add 1 mL of SB to wash. Centrifuge at 400 $\times$ g for 10 min and stain with 20 μ L anti-GPIIb/CD41-FITC antibody in a final volume of 100 μ L. Incubate at 4 °C for 30 min.
 1. Wash once with 1 mL of SB and resuspend pellet in 300 μ L of hypotonic citrate buffer (1.25 mM sodium citrate, 2.5 mM sodium chloride, 3.5 mM dextrose) containing 20 μ g/ml propidium iodide and 0.05% Triton-X 100. Incubate for 15 min at 4 °C protected from light.
 2. Add RNase to a final concentration of 20 μ g/mL and incubate for 30 min at 4 °C protected from light. Determine the intensity of propidium iodide by flow cytometry by collecting 30,000 to 50,000 events of the CD41-FITC⁺ population (**Figure 2C**).

4. Proplatelet Counting, Platelet Enumeration, and Platelet Activation

1. Harvest cells (from step 3.1) at days 8 or 9 of differentiation and seed at 1×10^4 cells/well in 48-well plates in 200 μ L of fresh SFM supplemented with 50 ng/mL rhTPO. Culture for 5 days at 37 °C, 5% CO₂.
NOTE: For quantitation purposes, seed wells in triplicate. This low density is required for visualization and counting of proplatelet-bearing MK. Proplatelets usually start appearing after 2 days of culture. The peak is between days 4 and 5.
2. Count the number of proplatelet-bearing MK in the whole well on an inverted light microscope using 10X or 20X objectives.
NOTE: A heated (37 °C) microscope stage is preferable, since keeping the cells at room temperature for extended periods causes shrinkage of the proplatelet extensions. Proplatelets are observed as long extensions from the MK body. Each MK may have several proplatelet protrusions. As proplatelets develop, the body of the MK decreases in size.
3. Harvest cells and centrifuge at 400 $\times$ g for 10 min at room temperature. Stain cells with 20 μ L anti-human CD41-FITC antibody, as described in steps 3.3 and 3.4. Calculate the percentage of proplatelet-bearing MK (pbMK): $\text{pbMK (\%)} = [(\text{Proplatelet-bearing MKs / well}) / (\text{Total CD41}^+ \text{ cells / well})] \times 100$
4. To count platelets released into the culture medium, gently mix the cells with a Pasteur pipette and collect 100 μ L at days 14 or 15 of culture.
 1. Stain with 20 μ L anti-human CD41-FITC antibody for 20 - 30 min at 4 °C. Set up a control tube using the respective isotype control antibody.
 2. Add 150 μ L of SB and 50 μ L of counting beads.
 3. For flow cytometric analysis, set the FSC and SSC to log scale. Use normal human blood platelets from platelet-rich plasma stained with CD41-FITC as described in section 4.4.1 to set the gating for platelets (**Figure 3A**).
 4. Analyze the stained platelets with counting beads by flow cytometry. Collect 1,000 events of counting beads using the FSC versus SSC scatter plot (**Figure 3A**). Calculate the number of platelets based on CD41-FITC positive events (**Figure 3B**) using the formula:
 $\text{Platelets per } \mu\text{L} = [(\text{number of CD41-FITC positive events}) / (1,000 \text{ beads})] \times [(\text{number of beads in } 50 \mu\text{L}) / (\text{sample volume})]$
5. To analyze platelet activation, gently mix the cells with a Pasteur pipette and collect 100 μ L at days 14 or 15 of culture. Add 1 mL of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM Na₂HPO₄, 12 mM NaHCO₃, 5.5 mM D-glucose, pH 6.5) and centrifuge at 200 $\times$ g for 5 min to pellet cells.
 1. Collect the supernatant and centrifuge at 800 $\times$ g for 10 min to pellet platelet-sized particles.
 2. Discard supernatant and resuspend in 100 μ L of Tyrode's buffer. Add 20 μ L of PAC1-FITC antibody and adenosine diphosphate (ADP) to a final concentration of 20 μ M. Incubate at room temperature for 20 min. Analyze by flow cytometry to determine the percentage of FITC positive events. Use fresh human platelets treated in the same manner as a positive control (**Figure 3C**).

Representative Results

This protocol allows the preparation of highly pure MK cultures from cord blood-derived CD34⁺ cells. The percentage of CD34⁺ cells in cord blood is approximately 1.3%¹⁵ (**Figure 1A**) and the total number of mononuclear cells (step 1.8) ranges from 90 - 300 x 10⁶ per UCB unit. The purity of CD34⁺/CD45⁺ cells after isolation ranges from 90 to 99% (**Figure 1B**). MK (defined as CD41⁺ cells) are observed early in serum-free CD34⁺ cell cultures in the presence of rhTPO. On day 7, the percentage of mature MK (CD41⁺ and CD42a⁺ cells) is usually 30 - 40% (**Figure 1C**). The highest levels of CD41⁺ and CD42a⁺ double positive cells (90 - 99%) are observed between days 10 and 12 of differentiation (**Figure 1C**). The variability observed depends mainly on the cord blood source and on the purity of the CD34⁺/CD45⁺ cells isolated in step 1.17. The yield of mature MK on day 10 ranges from 5 - 10 per input CD34⁺ cell. Mature MK (CD41⁺/CD42b⁺) observed under fluorescent microscopy are shown in **Figure 2A**. Cultured MK appear as large, usually multinucleated cells (**Figure 2A**, arrowheads). MK's granular content was determined by vWf (green) and CD62p (p-selectin, red) staining (**Figure 2B**). The ploidy distribution observed in cultured MKs is shown in **Figure 2C**.

Proplatelets are long, beaded fibers or filaments that extend from the MK body. Proplatelets can be several hundred micrometers long¹⁶ and contain branches and distended regions. A characteristic proplatelet-bearing MK is illustrated in **Figure 2D**. The percentage of proplatelet-bearing MK (pbMK) was 1.3 ± 0.17%.

Platelets can be analyzed and counted as described in the protocol. As shown in **Figure 3B**, most platelets in the cell culture supernatant fall within the analytical gate of peripheral blood platelets and are positive for the platelet marker CD41 (**Figure 3B**). The platelet yield from this method ranges from 19 to 42 platelets per MK. Platelets produced in culture can be activated by platelet agonists such as ADP as determined by increased binding of the activation-specific PAC1 monoclonal antibody (**Figure 3C**).

Figure 1: Flow cytometry plots of CD34⁺ cell isolation and MK differentiation in culture. (A) Mononuclear cells (gated as shown in the left panel) purified from human cord blood (step 1.8) were stained with anti-CD34-PE antibody to determine the percentage of the CD34⁺ cells in the sample (1.3%, right panel). (B) After separation, the positive fraction (step 1.17) was stained with anti-CD34-PE and anti-CD45 PerCP antibodies. The enriched CD34⁺ population is indicated in the figure (98.1%, right panel, upper right quadrant). (C) Phenotypic analysis of MK differentiated *in vitro* from CD34⁺ cells for 7 days or 11 days were stained with anti-CD41 and anti-CD42a antibodies. Mature MK are positive for both CD41⁺ and CD42a⁺ (upper right quadrant). [Please click here to view a larger version of this figure.](#)

Figure 2: MK staining, ploidy and proplatelet formation *in vitro*. (A) Fluorescent images of day 11 MK stained with anti-CD41-PE and CD42b-APC antibodies. Nuclei were stained with DAPI. Yellow arrowhead indicates multi-nuclear MKs. Scale bar, 30 μ m. (B) Fluorescent images of day 14 MK stained with anti vWf (green), anti CD62p (red) and anti-CD42b-APC (magenta) antibodies. Nuclei were stained with DAPI. Scale bar, 15 μ m. (C) Representative gating strategy showing the ploidy distribution of CD41⁺ events. The graph (lower panel) shows the observed distribution of ploidy classes (n = 4), error bars, SD. (D) The characteristic morphology of proplatelet-bearing MK is shown. The MK body is indicated by the arrow. The long cytoplasmic processes extending from the MK (proplatelets) are indicated by arrowheads. It may be unclear in some areas/fields of view whether one or two MK are producing these proplatelet extensions. This should be counted as one proplatelet-bearing MK. Images taken with an inverted microscope, 10X objective. Scale Bar, 50 μ m. [Please click here to view a larger version of this figure.](#)

Figure 3: Abundance of platelets produced *in vitro* and platelet activation. (A) Human platelets from platelet-rich plasma were used to set the analytical gate using log scale for forward and side scatter (left panel). The right panel shows cells from MK cultures. Platelets produced *in vitro* are observed in the analytical gate defined for human platelets. Cells and counting beads are indicated in the figure. Plt, platelet (B) Platelets produced *in vitro* were stained with anti-CD41 antibody. Human platelets from platelet-rich plasma were used to set the analytical gate using log scale for forward and side scatter and to compare the profile within the CD41-FITC gate. C-FITC, FITC isotype control (C) Platelet activation following treatment by ADP to a final concentration of 20 μ M. Human platelets from platelet-rich plasma were used as control (upper panels). Platelets produced in culture are shown in the lower panels. Binding of PAC1 antibody indicates platelet activation. C-FITC, FITC isotype control. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol described here is suitable for consistent production of MK and platelets in culture from umbilical cord blood. These cells can be used to study various processes such as the effect of drugs or biological activities on MK proliferation, differentiation, proplatelet formation, and platelet production.

A variety of culture media and cytokine combinations have been presented in the literature. Addition of cytokines such as stem cell factor, Flt-3 ligand, IL-3, and IL-6 supports CD34⁺ cell proliferation. However, this expansion results in reduced MK purity in the culture¹⁴. The method presented here, using serum-free media and rhTPO alone, does not allow significant expansion of the progenitor cells, but permits unilineage

megakaryocytic proliferation and differentiation and consistent production of MK (90 - 99% CD41⁺CD42a⁺ double positive cells) without contamination from other lineages. The culture period for MK formation is 10 to 12 days without the need for supporting stromal or feeder cells. This compares favorably with other methods that require larger culture periods (over 20 days)^{17,18}. The platelet yield from the present protocol is 19 - 42 platelets per MK or up to 420 platelets per input CD34⁺ cell. Most protocols result in lower platelet yields^{18,19}.

Although yield is high relative to other methods, large sources of CD34 cells are needed to produce sufficient numbers of platelets for therapeutic use. Cord blood MK mature at a lower rate, are generally smaller (of lower ploidy classes), and have reduced platelet production capacity²⁰. Nevertheless, fresh UCB is generally a more accessible resource and this represents a significant advantage for researchers. Other methods that can produce higher yields of MK and platelets with therapeutic aims have also been described^{13,17}.

There are some sources of variability to consider: A) Quality of the cord blood unit. Only cord blood collected within 24 h should be used as a source of CD34⁺ cells. Cord blood units containing clots should also be discarded. B) Percentage of CD34⁺ cells in the mononuclear cell fraction (step 1.8): using mononuclear cell fractions with less than 0.3% CD34⁺ cells may result in low yields of relatively low purity CD34⁺ cells. These cells are not recommended for MK differentiation. It is essential to let the liquid drain by gravity force only (e.g., steps 1.13, 1.14, 1.16). In case of column blockage, it is recommended to remove the column from the magnet, push the cells gently with the syringe plunger into a new tube, and reload on a new equilibrated column.

A number of conditions affect platelet number and function. Thrombocytopenia refers to a marked decline in platelet numbers that can lead to external and internal bleeding. Auto-immune conditions such as immune thrombocytopenia (ITP) and drug-induced thrombocytopenia (DITP) are well known causes of thrombocytopenia^{21,22}. Other immune diseases such as systemic lupus erythematosus and rheumatoid arthritis can also have detrimental effects on platelets. Non-immune causes of thrombocytopenia include cancer treatment, severe trauma, infections, bone marrow failure, and surgery. Due to the high utilization of platelets by patients undergoing chemotherapy or receiving stem cell transplants, platelet transfusion has steadily increased over the past decades. MK and platelet research will undoubtedly assist in the development of large scale platelet production for clinical applications. Availability of *in vitro* produced functional platelets would prevent platelet shortages and allow platelet transfusions into refractory patients. MK differentiation and platelet production *in vitro* are critical tools for the study and understanding of both pathological conditions and the physiological mechanisms that lead to platelet formation.

Disclosures

The authors have nothing to disclose.

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