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In vitro Culture of Murine Megakaryocytes from Fetal Liver-derived Hematopoietic Stem Cells

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Abstract

Megakaryocytes (MK) are specialized precursor cells committed to producing and proliferating platelets. In a cytoskeletal-driven process, mature MKs generate platelets by releasing thin cytoplasmic extensions, named proplatelets, into the sinusoids. Due to knowledge gaps in this process and mounting clinical demand for non-donor based platelet sources, investigators are successfully developing artificial culture systems to recreate the environment of platelet biogenesis. Nevertheless, drawbacks in current methods entail elaborate procedures for stem cell enrichment, extensive growth periods, low MK yield, and poor proplatelet production. We propose a simple, robust method of primary MK culture that utilizes fetal livers from pregnant mice. Our technique reduces expansion time to 4 days, and generates ~15,000–20,000 MKs per liver. Approximately 20–50% of these MKs produce structurally dense, high quality proplatelets. In this review, we outline our method of MK culture and isolation.

Method

Materials

15 mL Centrifuge Tube	- Corning	Catalog #: 430052
50 mL Centrifuge Tube	- Corning	Catalog #: 352098
10 cm Tissue Culture Dish	- BD	Catalog #: 353003
10 mL Syringe	- BD	Catalog #: 309604
19 Gauge Needle (1–1/2 in)	- BD	Catalog #: 305187
25 Gauge Needle (1–1/2 in)	- BD	Catalog #: 305122
40 µm cell strainer	- BD	Catalog #: 325340
0.45 µm vacuum filter	- Thermo	Catalog #: 168-0045
BSA (1.5% & 3% w/v in PBS)	- Roche	Catalog #: 03117057001
DMEM	- Corning	Catalog #: 10-013-CV
FBS (Performance Plus)	- Gibco	Catalog #: 16000-044
Penicillin (50 U/mL) / Streptomycin (10 µg/mL)	- Gibco	Catalog #: 15140-122

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PBS

- Corning Catalog #: 21-040-CV

Mouse Thrombopoietin (70 ng/mL, final)

A) Fetal Liver Cultures (Day 0)

1. Place DMEM (supplemented with 10% FBS & 1% Pen/Strep) in 37°C water bath.
2. Pipette complete DMEM into 3 × 10 cm tissue culture dishes (2 × 5 mL and 1 × 10 mL) and 1 × 50 mL centrifuge tube (5 mL DMEM) for each mouse. Leave plates in the 37°C incubator.
3. Take 50 mL centrifuge tube(s), diaper pad, forceps and dissecting scissors to the animal procedure room.
4. Euthanize pregnant mice following country & institutionally-accepted guidelines.
5. Clean dissection tools, prepare diaper pad and the carcass bag during asphyxiation.
6. Lay the mice on their back and disinfect (wipe or spray form) the stomach to sterilize; use scissors to mat down the fur.
7. Use forceps and scissors to cut the skin of the lower abdomen, taking care not to penetrate internal organs. Cut up/pull skin back to expose abdominal tissue.
8. Gently cut open the peritoneum to expose internal organs.
9. Locate the fetal sacs and remove them from the mouse by cutting away connective tissue. Place the fetal string in a 50 mL conical tube (w/ DMEM).
10. Place the dead mouse in the carcass bag & repeat for all mice.
11. Disinfect the tools, tie off and discard the carcass bag, and clean up the area.
12. Place the 50 mL conical tubes containing the fetal strings into the 37°C incubator.
13. Retrieve tissue culture dishes and a conical from the incubator, and transfer them to a sterile biosafety cabinet.
14. In a sterile biosafety cabinet, use ethanol-soaked forceps to transfer the fetal string from the conical into one plate of 5 mL DMEM (Figure 1a).
15. Use the forceps to dissect the fetuses from their embryonic sacs and place them into the second 5 mL culture dish (Figure 1b).
16. Be careful to only move fetuses into the second plate, as the red uterine tissue will be confusing for the next step.
17. Use the forceps to dissect the liver (large, red, multi-lobed organ) from the fetuses (Figure 1c). Remove all white connective tissue from the liver and

discard. Transfer the cleaned livers into a 10 cm dish with 10 mL complete media, and repeat for all fetuses (Figure 1d,e).

18. Homogenize liver tissue by drawing up media with livers into a 10 mL syringe using a 19 gauge needle. Draw up and purge back into the dish 3 times.
19. Switch to a 25 gauge needle and repeat the process, making sure to purge only once (Figure 1f).
20. Filter the homogenate through a 40 μ m cell strainer into a new 50 mL tube.
21. Repeat steps 15–21 for each mouse.
22. Centrifuge the 50 mL tubes of homogenate at 200 x *g* for 5 minutes.
23. During this time filter the DMEM. Pipette 20 mL of DMEM into a vacuum filter for each mouse + 5 mL to account for filtering/pipetting loss.
24. Add TPO at 70 ng/mL to the filtered media. TPO was derived from tissue culture supernatant from a fibroblast cell line engineered to secrete recombinant human TPO.(1)
25. Aspirate the supernatant from the centrifuged cells. Aspirate from the ‘elbow’ of the tube as the pellet is very loose.
26. Suspend each pellet in 2 mL DMEM w/ TPO, and pool together into one tube. If large fibroblasts (floating white tissue, strand-like) are found in suspension, re-filter the pooled homogenate through another 40 μ m cell strainer.
27. Combine all freshly suspended cells in filtered TPO media prepared in step 24, and swirl gently to mix.
28. Divide pooled resuspension into a new 10 cm culture dishes, 10 mL per dish.
29. Place culture dishes in 37°C incubator for 4 days. Figure 2 demonstrates the morphology of the cells in culture over days 0 (Figure 2a), 1 (Figure 2b), 2 (Figure 2c), 3 (Figure 2d), and 4 (Figure 2e). By day 4, approximately 3–5% of the entire cell population are MKs.

B) BSA Density Gradients (Days 4–5)

1. Make 1.5% and 3% BSA (weight/volume in PBS), and place with DMEM (no TPO) in 37°C water bath. You will need 1 mL of each (1.5%, 3% BSA, & DMEM) for each culture dish in the incubator.
2. Working in sterile biosafety cabinet, syringe filter each BSA tube into new tubes, making sure to label accordingly.
3. Pipette 1 mL of 3% BSA into a 15 mL conical tubes (1 for every culture, Figure 3a).
4. Create gradient by carefully tilting tube and pipetting the 1mL of 3% BSA (close to horizontal, Figure 3b). Next pipette 1.5% BSA very slowly, on top of it. Re-orient the tube very slowly, and repeat for all tubes (Figure 3c).

5. Gently place the gradient tubes aside and retrieve cultures from incubator.
6. Use a 10 mL pipette to gently resuspend cells on each dish and transfer the contents into individual 15 mL conical tubes. Take care to resuspend all the cells.
7. Centrifuge the 15 mL tubes at $200 \times g$ for 5 minutes.
8. Aspirate the supernatant and resuspend each culture in 1 mL DMEM.
9. Tilt gradient tubes as done in step 4, and carefully pipette 1 mL of resuspended cells over the 1.5% BSA (Figure 3d,e).
10. Gently place the gradient tubes into the incubator.
11. Ensure that each 15 mL conical tube sits for 30 minutes. After that time, the MKs will fall to the bottom in a loose pellet.
12. Aspirate the supernatant, taking care to not agitate the MK pellet.
13. Resuspend each MK pellet in 1 mL of DMEM (no TPO) and place the cultures in the 37°C incubator for later use (Figure 4a).
14. MKs (in DMEM w/o TPO) will produce proplatelets over the next 24 hours (Days 4–5, Figure 4b).

Discussion

Herein, we outline a method to culture murine fetal liver-derived MKs as a model to study MK maturation and platelet production *in vitro*. However, the study of these processes is of interest due to the prevalence of human platelet disorders, including thrombocytopenia. In humans, thrombocytopenia (platelet counts $< 150 \times 10^9/L$) can be life threatening due to bleeding complications and is a major clinical problem encountered in immune thrombocytopenic purpura (ITP), chemotherapy, surgery, aplastic anemia, human immunodeficiency virus infection, and complications during pregnancy and delivery. It is estimated that approximately 1.5 million platelet transfusions are administered yearly, with each transfusion costing over \$600.(2)

The discovery of the c-Mpl ligand, TPO, marked a positive shift in megakaryocyte (MK) and platelet research; for the first time MKs were able to be differentiated and cultured outside the body. (3, 4) Due to the scarcity of MKs within the bone marrow niche and the difficulties in accessing them, investigators have largely moved towards *in vitro* culture of MKs using TPO to study this microenvironment. The adoption of these differentiation and culture systems has permitted further study of the events leading to megakaryopoiesis and subsequent proplatelet formation.(5–7) In addition, *ex vivo* MK culture opened the door to the possibility of creating donor-independent platelets for transfusion.

The first human MK culture systems largely utilized CD34⁺-enriched progenitors originating from mobilized peripheral blood (mPB) or umbilical cord blood (UCB). Researchers tapped these sources to define methods for generating high-ploidy MKs with functional *in vivo* platelets.(8–11) Such groups exploiting CD34⁺ cells have concentrated on studying and sustaining MK differentiation and platelet production using various methods,

including bioreactors, co-culturing systems, and elegantly bioengineered scaffolding materials to recreate the complex bone marrow microenvironment.(7, 12–17) Stemming from these initial studies, promising techniques such as UCB-derived platelet perfusion systems were established shortly after, detailing production of up to $3.0 - 6.0 \times 10^{11}$ platelets (up to 20 platelets per starting HSC); a clinically acceptable amount for transfusion.(16) Both UCB & mPB MKs have been found to have similar levels of CD34⁺ expression, indicating comparable rates of MK production. However, the UCB MKs have lower levels of CD41 and ploidy, suggesting that the UCB-derived MKs are immature compared to mPBs. (18) This difference in MKs derived from fetal (i.e. UCB in humans and fetal livers in mice) versus adult progenitors (i.e. mPB in humans and bone marrow in mice) is worth noting and taking into account when planning model systems for experiments. Furthermore, additional studies characterizing differences in the transcriptome of MKs derived from different cell types are warranted.

Nonetheless, preliminary reports of artificial MK culture using these tissue sources have exposed a lack of consistent results owing to heterogenous hematopoietic stem cell (HSC) populations.(17) Ultimately, these elaborate procedures have been bottlenecked by dependencies on viable donor samples, variable effects of cytokines on MK differentiation, and the limited expansion of progenitors. Additionally, the low efficacy of post-transfusion platelets, fixed assembly limits, and tendencies for agonist-devoid activation have provided significant challenges.(19)

More recently, investigators have taken steps towards the directed differentiation and culture of functional MKs from induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs).(20) Prior techniques targeting large scale harvesting and differentiation of human-derived ESCs were capable of producing viable MKs, of which approximately 10% produced proplatelets.(21–23) However, the Lu *et al.* developed a method for ESC culture that propagated 6×10^8 CD41a⁺ cells (roughly 6.7 ± 0.4 platelets per starting cell).(20) This shows improvement in MK production, but is still a rate well below accepted *in vitro* production.

In addition, various groups have had success programming pluripotent stem cells to differentiate into MKs. This was first done by Nishimura *et al.* using canine iPSCs, with 17% of platelets from their culture supernatant positive for CD41/61, demonstrating the possibility of MK generation and the release of platelets from iPSCs.(24) In addition, Feng *et al.* generated platelets from human iPSCs in a simplified culture system free of serum and feeder cells; they generated an average of 16 MK progenitors per single iPSC.(25) Due to the promise of these advances, Nakamura sought to establish stable, immortalized MK progenitor cell lines for clinically applicable generation of platelets from iPSCs *in vitro*.(26) However, this technology currently relies on a relatively sophisticated and expensive reprogramming method, making scale-up to generation of clinically-relevant platelet numbers less feasible.(27)

Although these contemporary isolation methods highlight advancements in *in vitro* culturing of human cells, they involve time-intensive techniques such as immunomagnetic purification, cell sorting by flow cytometry, and continuous cycling between expensive

cytokine and medium combinations.(28–30) As such, murine models have proven to be a practical and ethical approach in researching MK differentiation and platelet production. The culturing of murine MKs eliminates dependencies on human donors and the benefits associated with controlling all facets of the murine growth cycle minimize many of the inconsistencies that accompany human-derived samples.

Lecine and colleagues were the first to successfully culture proplatelet-producing MKs from murine fetal livers in the presence of TPO.(31) Building from this initial protocol, we have established a robust method of culturing murine MKs, employing fetal livers recovered from embryonic day 13.5 fetuses.(32–35) Currently, we and others suggest that murine fetal livers are a suitable choice for high MK yield in comparison to other primary sources like spleen and bone marrow, which are limited in production and often incapable of generating proplatelets.(36)

As outlined above, culturing these cells with recombinant TPO and 10 % w/v Performance-Plus FBS results in high MK differentiation and proliferation by day 3 of culture. The subsequent 1.5% and 3% w/v albumin step gradient is a simple yet effective method of isolating MKs based on density. Although MKs isolated in this gradient are largely unsynchronized, approximately 15,000–20,000 cells are generated per fetal liver, of which 20–50% form proplatelets by day 5, consistent with previous publications.(37, 38) This technique has been used successfully for 20 years, with the advantages being pure, polyploid MKs, capable of generating robust amounts of proplatelets. While studies with murine MKs undoubtedly need to be validated in a human model, our *in vitro* method of murine culture grants researchers the ability to study the physiological mechanisms of proplatelet formation, with considerably fast turnover.

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Figure 1. Murine Fetal Liver Dissection

Images of mouse Embryonic Day 13.5 fetal livers derived from pregnant CD-1 mice showing (A) Mouse fetal liver string in first 10 cm dish containing 5 mL DMEM, (B) Intact, isolated fetal sacs in second 10 cm dish containing 5 mL DMEM, (C) Close-up of isolated fetal sacs, with arrowheads pointing to red fetal liver, (D) Isolated fetal livers, (E) Isolated fetal livers in third 10 cm dish containing 10 mL DMEM, and (F) 10 mL of homogenized fetal liver culture.

Figure 2. Megakaryocyte Differentiation Progression

Images were taken on an inverted Olympus CKX41 microscope using a 10x objective, showing megakaryocyte growth progression on (A) Day 0, (B) Day 1, (C) Day 2, (D) Day 3, and (E) Day 4. Scale bars represent 50 μm .

Figure 3. Albumin Step Gradient to Isolate Megakaryocytes

Images show the albumin step gradient setup in a 15 mL conical tube. (A) 1 mL 3% BSA at the bottom of 15 mL conical, (B) 15 mL conical with 3% BSA, tilted to gently layer 1.5% on top, (C) close-up of 1.5 and 3% BSA gradient (arrowheads point to faint lines indicating separation between layers), (D) 15 mL conical with 1.5 and 3% BSA, tilted to gently layer 1 mL of resuspended culture, and (E) completed gradient, pre-incubation.

Figure 4. Post-Gradient Megakaryocytes in Culture

Images taken on an inverted Olympus CKX41 microscope using a 10x objective, showing (A) gradient-isolated megakaryocytes on Day 4 and (B) proplatelet structure on Day 5. Scale bars represent 50 μm .