Induced Pluripotent Stem Cell–Derived Megakaryocytes and Platelets for Disease Modeling and Future Clinical Applications

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Abstract—Platelets, derived from megakaryocytes, are anucleate cytoplasmic discs that circulate in the blood stream and play major roles in hemostasis, inflammation, and vascular biology. Platelet transfusions are used in a variety of medical settings to prevent life-threatening thrombocytopenia because of cancer therapy, other causes of acquired or inherited thrombocytopenia, and trauma. Currently, platelets used for transfusion purposes are donor derived. However, there is a drive to generate nondonor sources of platelets to help supplement donor-derived platelets. Efforts have been made by many laboratories to generate in vitro platelets and optimize their production and quality. In vitro-derived platelets have the potential to be a safer, more uniform product, and genetic manipulation could allow for better treatment of patients who become refractory to donor-derived units. This review focuses on potential clinical applications of in vitro-derived megakaryocytes and platelets, current methods to generate and expand megakaryocytes from pluripotent stem cell sources, and the use of these cells for disease modeling. *Visual Overview*—An online visual overview is available for this article. (*Arterioscler Thromb Vasc Biol.* 2017;37: 2007-2013. DOI: 10.1161/ATVBAHA.117.309197.)

Key Words: blood platelets ■ hematopoiesis ■ humans ■ induced pluripotent stem cells ■ megakaryocytes

Why Do We Need iPSC-Derived Megakaryocytes?

The advent of human-induced pluripotent stem cell (iPSC) technology 10 years ago provided investigators with an important tool for the field of cellular therapeutics.[1] One goal in the hematopoietic field has been to generate donor-independent platelets to supplement current transfusion products. Platelets are cellular fragments derived from megakaryocytes that play a critical role in hemostasis and thrombus formation and mediate aspects of immunity, inflammation, and angiogenesis.[2–6] Although donor shortage has not been a frequent problem to date, the number of transfusions in first-world countries has steadily increased because of increased life span and a rise in hematologic malignancies.[7] Because of this, there is a drive to develop donor-independent sources of platelets for transfusions.

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One of the advantages of using iPSCs to generate platelets is that they are amenable to genetic manipulation. Two potential applications for generating donor-independent platelets are in patients refractory to donor-derived units because of alloimmunization and as a targeted delivery vehicle for compounds, such as clotting factors, to sites of vascular injury (Figure [A]). It is estimated that 28% to 44% of patients receiving long-term platelet transfusions become refractory to donor-derived units because of alloimmunization against human leukocyte antigens.[8–10] Generation of a human leukocyte antigen–universal iPSC line by silencing human leukocyte antigen class I epitopes would allow for a universal source of platelets with a low immunogenic profile.[11,12] Because natural killer cells are triggered in the absence of human leukocyte antigen class I expression, stable gene-editing techniques, such as CRISPR (clustered regularly interspaced short palindromic repeats) or zinc finger nucleases, may not be appropriate for this application.[13] Patients with hemophilia A receive frequent plasma transfusions to compensate for factor VIII deficiency, but it is not useful in patients with high titers of antifactor VIII antibodies.[14,15] Ectopic expression of factor VIII in platelets could be used to treat patients with hemophilia A with significant inhibitors.

Megakaryocyte Ontogeny

Primitive Versus Definitive Hematopoiesis

Two distinct hematopoietic programs, designated primitive and definitive, occur during embryonic/fetal development,

Nonstandard Abbreviations and Acronyms	
ESC	embryonic stem cell
HPC	hematopoietic progenitor cell
iPSC	induced pluripotent stem cell

and both give rise to megakaryocytes. These programs can be distinguished by the subtype of globin expressed in erythroid cells.[16] The first, primitive, wave of hematopoiesis develops from an extraembryonic mesoderm population in the yolk sac.[17,18] These yolk sac progenitors give rise to nucleated erythrocytes, macrophages, and primitive megakaryocytes.[19,20] Toward the end of the primitive wave, definitive erythromyeloid progenitors emerge from the yolk sac and later colonize the fetal liver, ultimately acting as the major source of hematopoiesis before the emergence of hematopoietic stem cells.[21] Another definitive wave of hematopoiesis occurs in the aorta-gonads-mesonephros region and is responsible for the generation of the long-term hematopoietic stem cells having multilineage hematopoietic potential, as well as enucleated erythrocytes, myeloid cells, lymphocytes,

and definitive megakaryocytes.[22] After specification, these hematopoietic stem cells colonize the fetal liver before transitioning to the bone marrow, where they reside for the remainder of adult life.[23]

Although megakaryocytes are generated in both the primitive and definitive waves of hematopoiesis, functional differences have been described.[19,24,25] Primitive megakaryocytes tend to be less proliferative and display a lower ploidy, with each megakaryocyte releasing a relatively small number of platelets.[26,27] Megakaryocytes generated through the definitive hematopoietic program undergo increased levels of endomitosis, where the cell does not divide after DNA duplication resulting in increased ploidy and efficient release of large numbers of higher functioning platelets, $\leq 10^{3-4}$ platelets per megakaryocyte in the adult.[27,28] Although neonatal and adult megakaryocytes are generated from the definitive program of hematopoiesis, the ploidy and functionality of megakaryocytes and their platelets do not peak until at least a year after birth.[29] Distinct gene expression signatures between megakaryocytes derived from human embryonic stem cells (ESCs), fetal liver, neonate, and adult
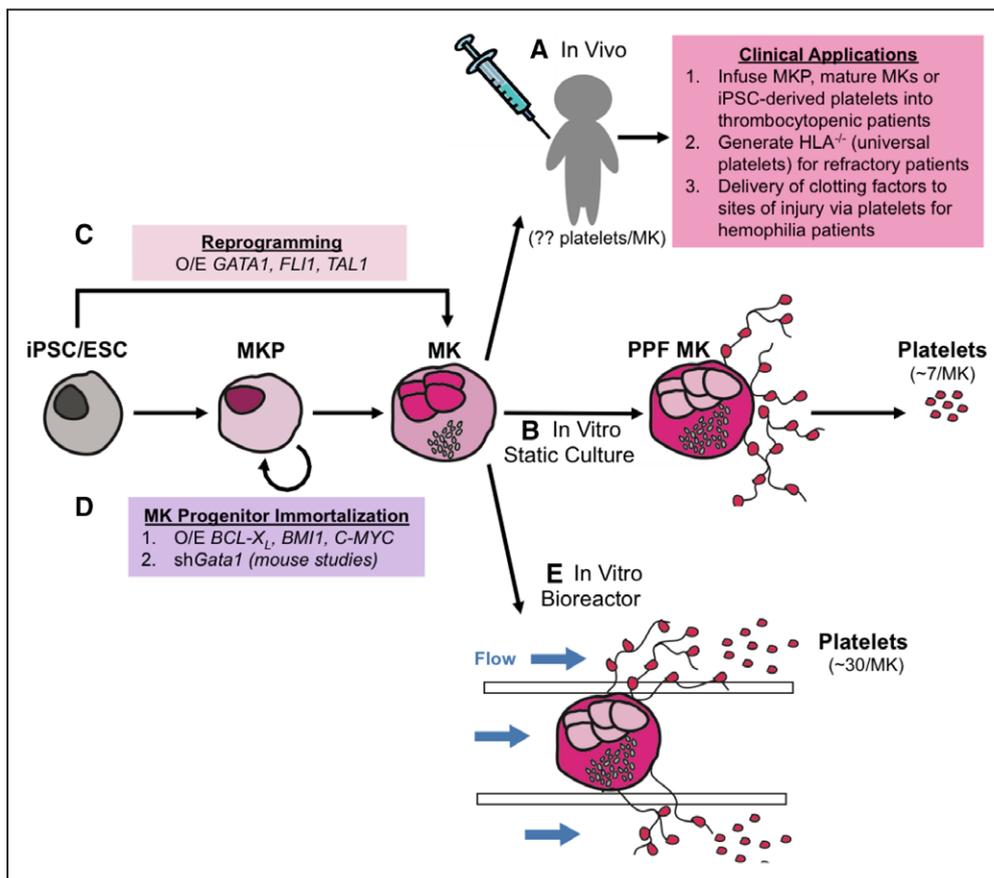
Figure. Current methods for generating stem cell-derived platelets. **A**, Megakaryocytes infused into human patients have the potential to act as an in vivo bioreactor, as seen in mouse studies. Megakaryocytes infused into mice release ≈30 platelets/megakaryocyte. **B**, In static culture, megakaryocytes differentiated from induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) form proplatelets. The shed platelets can be purified from the culture medium by centrifugation. This method yields ≈7 platelets/megakaryocyte. **C**, iPSCs and ESCs can be driven to the megakaryocyte lineage through overexpression of GATA1, FLI1, and TAL1. **D**, Megakaryocyte progenitors can be expanded by overexpressing BCL-XL, BMI1, and C-MYC. In mice, these progenitors are expanded by knockdown of Gata1. **E**, In vitro platelet bioreactors enhance proplatelet formation in megakaryocytes via endothelial cell contacts, extracellular matrix components, and shear stress. This method yields ≈30 platelets/megakaryocyte. HLA indicates human leucocyte antigen; MK, megakaryocyte; MKP, megakaryocyte progenitor; and PPF MK, proplatelet forming megakaryocyte.

progenitors supports the finding that megakaryocyte maturation progresses during ontogeny and results in increased ploidy and platelet release.[25] Recent evidence suggests IGF2BP3 as a master switch driving immature fetal/neonatal to adult megakaryopoiesis.[30] Overall, a better understanding of the regulatory and functional changes that occur during megakaryocyte ontogeny are critical for generating high-quality megakaryocytes and platelets for transfusion purposes.

Definitive Megakaryopoiesis and Thrombopoiesis

Megakaryopoiesis is the process by which a hematopoietic progenitor cell (HPC) differentiates into a large, polyploid megakaryocyte.[31] This process is dependent on the growth factor TPO (thrombopoietin) and transcription factors, such as FLI1, GATA1, and RUNX1.[32–34] In the adult, megakaryocytes are generated in the bone marrow and must undergo a series of maturation steps to release large number of functional platelets into the circulation. This complex maturation process involves generation of a large polyploid cell, the formation of an invaginated membrane system to serve as a cell membrane reservoir for platelet formation, and the synthesis of platelet specific granules, such as alpha and dense granules, which are distributed into platelets.[28,35–37]

After maturation, megakaryocytes are equipped to release platelets into the circulation—a process termed thrombopoiesis.[31] The exact mechanism of proplatelet formation and platelet release are not well understood, but there are 2 main models: proplatelet formation and megakaryocyte rupturing. The proplatelet formation model can occur in the bone marrow and in the lung capillary beds. In the bone marrow, mature megakaryocytes exit the osteoblastic niche and enter the vasculature where they extend proplatelet extensions into the bone marrow sinusoids.[38] Shear stress and high sphingosine 1-phosphate levels in the blood are proposed as mechanisms supporting the release of platelets into circulation.[39] Approximately half of all platelet release in the mouse occurs when megakaryocytes exit the bone marrow and enter lung capillary beds where they release platelets into the circulation via proplatelet extensions.[40–42] Although current studies examining platelet release in the lung have supported the lungs as a major site of thrombopoiesis, it is still unknown whether this holds true in the human. Other data suggest megakaryocytes can rupture through an interleukin-1a-dependent pathway, thus resulting in rapid release of a large number of platelets into the bone marrow where they enter the circulation rapidly after acute injury or inflammation.[43] With evidence for both models, it is possible to speculate that thrombopoiesis may occur differently depending on the physiological need of homeostasis versus acute injury.

Directed Differentiation of iPSCs to Megakaryocytes

The use of primary human megakaryocytes to study megakaryopoiesis and thrombopoiesis is difficult because these cells represent only ≈0.01% of the nucleated cells in the bone marrow.[44] Human stem cell sources, including ESCs and iPSCs, have been used successfully for the in vitro generation of megakaryocytes. However, an ongoing challenge is identifying conditions to optimize the quantity and quality of in vitro-generated megakaryocytes and released platelets.

Directed differentiation protocols have been developed for generating HPCs from ESCs/iPSCs with the overall design mimicking embryogenesis.[45] Primitive streak formation is induced through BMP4 and WNT pathway activation, followed by mesoderm specification.[45,46] Mesoderm is further specified to hematoendothelial mesoderm, through a still not fully understood mechanism, which ultimately gives rise to HPCs. HPCs expand on the addition of hematopoietic cytokines, such as SCF (stem cell factor), Flt3L (Fms-related tyrosine kinase 3 ligand), and TPO.[46–48] The primitive HPCs coexpress typical adult megakaryocyte and erythrocyte markers CD41 and CD235 and give rise to primitive erythrocytes, megakaryocytes, and myeloid cells.[46,49] Other widely used differentiation systems use coculture with stromal cell lines, such as OP9, to generate HPCs.[50,51] These static in vitro cultures generate ≈7 platelets per megakaryocyte (Figure [B]).

In recent years, differentiation protocols driving definitive hematopoiesis have been developed through timed modulation of the TGF-β (transforming growth factor-β) and WNT pathways.[52–54] Hemogenic endothelium can be purified from 3-dimensional embryoid body suspension cultures via cell sorting CD34+ cells that are CD73−CD184−.[52,53] This population possesses the ability to give rise to hematopoietic progenitors with lymphoid potential, confirming its definitive nature.[55]

With the identification of TPO, HPCs can be differentiated in vitro to the megakaryocyte lineage.[32,33] Studies comparing definitive or adult-like megakaryocytes derived from bone marrow CD34+ stem cells to primitive megakaryocytes derived from iPSCs show that the iPSC-derived primitive megakaryocytes are smaller, of lower ploidy, and release fewer platelets with a short half-life when infused into mice.[25,56] With the newer protocols driving definitive hematopoiesis, further studies will define the characteristics of megakaryocytes derived from these more mature progenitor populations.

Methods to Increase iPSC-Derived Progenitor and Megakaryocyte Numbers

With the goal of generating increased numbers of HPCs and megakaryocytes, systems using the overexpression of transcription factors to drive megakaryocyte progenitor generation and expansion have been developed. The Ghevaert laboratory found that overexpression of FLI1, GATA1, and TAL1 in ESCs led to a bias for the generation of megakaryocytes (Figure [C]).[57] These reprogrammed megakaryocytes had the ability to proliferate in culture for >90 days while still maintaining megakaryocyte purity, markers of maturity (ie, CD41/CD42a), and platelet production. Starting with 10^6 iPSCs, this methodology resulted in the production of $2×10^{11}$ megakaryocytes releasing a total of $1×10^{12}$ platelets. This is equivalent to ≈3 transfusion units, whereas current directed differentiation protocols would need ≈10^3 more iPSCs to generate a similar number of platelets. Although this system allows for large-scale production of platelets, these platelets are still inferior to donor-derived platelets because they are less mature and have a short half-life when infused into mice.

Two approaches have been developed to overcome the asynchronous nature of cell culture while generating self-renewing megakaryocyte progenitors. The Eto Laboratory modulated the expression of transcription factors known to regulate pathways related to apoptosis, senescence, and proliferation. Doxycycline-inducible expression of BCL-XL, BMI1, and C-MYC in human iPSC-derived HPCs promoted the expansion of immature megakaryocytes in culture (Figure [D]), termed immortalized megakaryocyte progenitor cell lines.[58] These megakaryocytes were capable of proliferating for ≤5 months, and removal of doxycycline allowed final megakaryocyte maturation to occur. However, these immortalized megakaryocytes shed fewer platelets with a less robust response to agonist stimulation when compared with unmanipulated megakaryocytes. The Weiss laboratory manipulated the expression of GATA1—a known master regulator of megakaryopoiesis and erythropoiesis—to generate expandable megakaryocyte progenitors (Figure [D]).[59,60] These studies were initiated due to the fact that patients with *GATA1* mutations have increased counts of proliferative, developmentally arrested megakaryocytes.[61–63] Their first system using *Gata1*-null mice[59] has been improved by enabling controlled temporal knockdown of GATA1.[60] Mouse ESCs were targeted to express a doxycycline-inducible shRNA targeting GATA1. Because with the original system, a biopotential progenitor expanded ≈10^3-fold in the presence of doxycycline. Withdrawal of doxycycline allowed for re-expression of endogenous GATA1 and further differentiation into megakaryocytes or erythrocytes. The generation of human systems that display the same phenomenon will be of interest.

Although all these systems are advantageous in that they generate large populations of megakaryocytes, there are risks involved in using factors with tumorigenic abilities, for example, C-MYC. Given that platelets are anucleate, the final product can be irradiated to eliminate residual live cells before infusing into patients, which may mitigate this concern. It is also unclear whether these systems are generating primitive or definitive megakaryocytes. To generate the best yield and functionality of platelets, it will be important to mimic later maturation events that occur after birth. For example, suppression of IGF2BP3 via small molecules or gene editing could allow for further maturation of megakaryocytes, or selective inhibition of the metalloproteinase ADAM17 could improve platelet functionality by preventing glycoprotein Ibα/CD42b shedding in vitro.[30,64]

iPSC-Derived Megakaryocytes for Disease Modeling

Although animal models are invaluable for studying the biology of human diseases, there are instances where they do not recapitulate the human disease phenotype. Therefore, human iPSCs are an important tool for modeling human disease in vitro. Detailed below are a set of megakaryocyte and platelet disorders that have been studied using human iPSCs.

Familial platelet disorder with a predisposition to acute myeloid leukemia is a rare genetic disorder resulting from heterozygous mutations in the transcription factor RUNX1.[65,66] As the name suggests, these patients present with thrombocytopenia and a >40% chance of developing hematologic malignancies by age 35.[65,66] Murine models have been used to study this disease but fail to display low platelet counts unless both alleles of *Runx1* are mutated.[67] With that, several laboratories have generated iPSCs from familial platelet disorder patients harboring RUNX1 mutations to further understand the mechanism of disease.[68–70] A consistent finding is that iPSCs derived from these patients display a severe decrease in megakaryocyte yield.[68–70] On lentiviral expression of wild-type RUNX1 or genetic correction of the mutation, the megakaryocyte phenotype was rescued.

Paris-Trousseau syndrome is caused by a large heterozygous deletion in chromosome 11q resulting in hemizygous expression of FLI1—a critical transcription factor for megakaryocyte differentiation and other hematopoietic lineages.[71] Patients with Paris-Trousseau syndrome have macrothrombocytopenia with large platelets circulating at reduced levels. FLI1 mutations have recently been ascribed to cause thrombocytopenia, but the link between FLI1 mutations and Paris-Trousseau syndrome had not been confirmed.[72,73] On differentiating iPSCs generated from these patients into megakaryocytes, the disease phenotype was confirmed with decreased megakaryocyte and platelet yields and poor platelet half-life and functionality when infused into mice.[74] When a wild-type iPSC line heterozygous for FLI1 knockout was differentiated into megakaryocytes, it displayed the same phenotype as the megakaryocytes and platelets obtained from the Paris-Trousseau syndrome patient iPSCs.[74]

Glanzmann thrombasthenia is a rare autosomal recessive disease resulting from a lack of functional αIIbβ3 (glycoprotein IIb/IIIa; CD41/CD61) because of mutations in ITGA2B and ITGB3.[75] This integrin complex found on platelets is a crucial receptor for fibrinogen resulting in platelet aggregation.[76] Although patients with Glanzmann thrombasthenia have normal platelet counts, the function of their platelets is altered resulting in increased bleeding. The iPSC lines generated from different Glanzmann thrombasthenia patients demonstrated a consistent physiological phenotype.[77–79] Megakaryocytes did not express αIIbβ3 receptors because of defective αIIb/CD41 but did express a mature megakaryocyte marker, CD42b.[77–79] By expressing wild-type ITGA2B under a glycoprotein Ibα promoter, αIIbβ3 receptors were restored to normal as shown by PAC-1 binding after agonist stimulation.[77,79]

Conclusion: Are We Ready for the Clinic?

Although significant progress has been made, much work still needs to be done to bring in vitro-derived megakaryocytes and platelets to the clinic. However, efforts are being made by numerous laboratories to increase the quality and yield of iPSC-derived megakaryocytes and platelets. In vitro bioreactors are being used to closely mimic the in vivo environment by incorporating extracellular matrix components, endothelial cells, and applying a flow to induce shear stress (Figure [E]).[80] These in vitro bioreactors yield ≈30 platelets per megakaryocyte, which is well below the in vivo yield of >2000.[81] One potential explanation for low platelet yield is that these bioreactors are mainly plastic-based; it is possible that silk bioreactors or lung mini-chip bioreactors would help to increase yields. Alternatively, iPSC-derived megakaryocytes could

be infused directly into patients to obtain maximal platelet release. Although infusing megakaryocytes directly increases safety risk concerns, unpublished studies in the Poncz laboratory show that megakaryocytes can undergo irradiation without a loss in efficacy (Figure [A]). Other laboratories are trying to further mature in vitro-derived megakaryocytes to increase ploidy and ultimately increase platelet release by genetically manipulating maturation factors.[30] Despite current progress, much more work remains to be done before iPSC-derived megakaryocytes and platelets make their way into the clinic.

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Highlights

- Human-induced pluripotent stem cell–derived megakaryocyte development using directed differentiation.
- Approaches to expand human-induced pluripotent stem cell–derived megakaryocytes.
- Use for studying human megakaryocyte and platelet disorders.
- Goals for supplementing current donor-derived platelet sources.

Arteriosclerosis, Thrombosis, and Vascular Biology

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