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Megakaryopoiesis

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Megakaryocytes (MKs) are differentiated through linage commitment from hematopoietic stem cells and terminally differentiated MKs release platelets. The processes of megakaryopoiesis and subsequent thrombopoiesis are unique and complex. The underlying mechanisms of MK development and platelet production, beginning with stem cells, are not fully understood because of the difficulty in obtaining sufficient numbers of MKs for analysis. However, recent advances in cell biology have furthered understanding of megakaryopoiesis and thrombopoiesis and allowed for great progress toward elucidating the mechanisms for MK differentiation and subsequent platelet production. In addition, a recently developed in vitro culture system for MKs and the technology for *ex vivo* production of platelets have resulted in a better understanding of MK and platelet biology and have highlighted their possible clinical applications. Platelet transfusions are widely used for patients with severe thrombocytopenia, and the supply of platelets is solely dependent on volunteer donors. Thus, the development of an efficient and stable system of *ex vivo* platelet generation from donor-independent sources is needed.

Keywords: Megakaryocytes, Platelets, Stem cells

Introduction

Platelets are essential for hemostatic plug formation and pathogenesis of arterial thrombosis. Platelets are released from terminally differentiated megakaryocytes (MKs) through lineage commitment beginning with hematopoietic stem cells (HSCs), and approximately 1×10^{11} platelets are produced each day in the human body.[1-3] HSCs have self-renewal capacities and differentiate into all types of blood cells according to an HSC hierarchy model (Fig. 1).[2] Other models of MK development that are not consistent with the traditional HSC hierarchy model have been suggested.[4] MK formation (megakaryopoiesis) and subsequent platelet production are complex processes, although the underlying mechanisms of MK lineage commitment are only partially understood. During MK differentiation, immature MKs further differentiate into mature MKs, which possess highly unique features such as large cell size, polyploidy, and an invaginated membrane system. Finally, mature MKs undergo terminal thrombopoiesis with proplatelet formation and platelet release.[1-3] In this chapter, we review the cellular aspects and molecular regulations of megakaryopoiesis. The current state of the ex vivo production of MKs and subsequent platelets is also described as a topic in MK research.

Stage-specific surface markers of MK differentiation

HSCs have dual abilities to self-renew and to differentiate into all types of blood cells. During hematopoietic development, HSCs produce cell progeny consisting of multipotent progenitors (MPP), common myeloid progenitors (CMP), and megakaryocyte-erythroid progenitors (MEP) (Fig. 1). MEP are bipotent progenitors for erythroids and MKs. Hematopoietic progenitors and mature cells during megakaryopoiesis are characterized by stage-specific surface markers. The phenotype of MEP is IL7R-/Lin-/c-Kit+/Sca1-/CD34-/FcγR- in mice and Lin-/CD34+/CD38+/IL3R-/CD45RA- in humans.[5] MK progenitor cells are divided into two stages: the primitive cells in the earlier stage, called burst-forming unit-megakaryocytes (BFU-MKs), and the more differentiated cells in the later stage, called colony-forming unit-megakaryocytes (CFU-MKs). The protein expression of HLA-DR is low in BFU-MK and high in CFU-MK. CD41 (integrin alpha IIb, also known as platelet glycoprotein IIb), a key platelet surface glycoprotein, is relatively specific for the MK lineage and is used as a specific surface marker throughout MK differentiation. CD42b (also known as platelet glycoprotein Ib alpha) is used as a surface marker for the late stage of MK differentiation and is associated with a marked increase in the expression of *MPL*, glycoprotein VI, glycoprotein IaIIa (α2β1 integrin), and CD36 (glycoprotein IV).

Cytokines and MK differentiation

Thrombopoietin (TPO) is a primary cytokine for the regulation of megakaryopoiesis via binding to its receptor, c-MPL. Since TPO was first identified,[6-9] we have learned much about platelet production and regulation.[10] Both TPO- and c-MPL-deficient mice show reduced MK numbers and thrombocytopenia. Recombinant human TPO and pegylated recombinant human megakaryocyte growth and development factor (PEG-rhMGDF) were developed for clinical use.[10] These recombinant agents have been widely used in a large number of studies and are effective in increasing platelet count. However, in 13 of 535 healthy volunteers, autoantibodies against PEG-rhMGDF and thrombocytopenia were observed. Eventually, production of both PEG-rhMGDF and recombinant human TPO for clinical use was stopped. Recently, a second generation of c-MPL agonists was developed and has been used for the treatment of immune thrombocytopenic purpura. Regarding other cytokines for regulation of MK differentiation, Avecilla et al. demonstrated that SDF-1 and fibroblast growth factor-4 enhanced megakaryopoiesis and platelet production in TPO/c-MPL-deficient mice.[11] Also, Salim et al.[12] re-

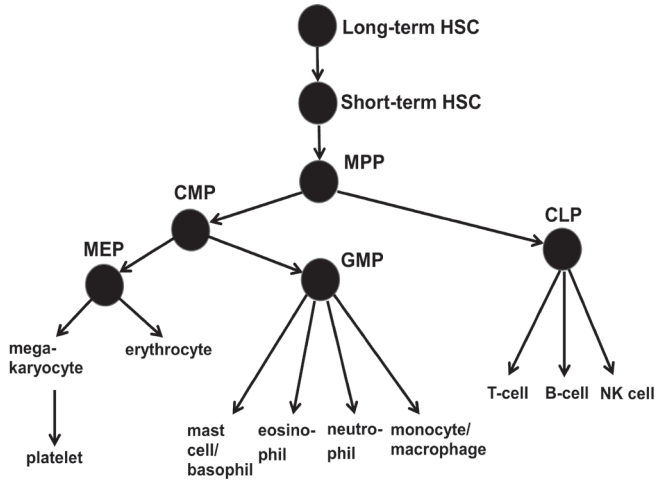

Fig. 1. Hierarchy in hematopoietic cells. HSC, hematopoietic stem cells; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythroid progenitor.

ported that dysregulation of SDF-1 and its receptor CXCR4 axis leads to thrombocytopenia.

MKs and endomitotic processes

Mature MKs initiate the synthesis of platelet proteins and undergo a proliferative 2 N stage as in other hematopoietic cells.[3] Additionally, MKs undergo a unique process called endomitosis, which allows them to support the large quantities of mRNA and protein that are packaged into granules. DNA polyploidy is a hallmark of MKs. Polyploid MKs achieve a nuclear content of 4 N to 128 N, with about half of the MKs in humans being 16 N. To form proplatelets, it is thought that MKs must be at least 4 N. The diameter of a 2 N MK is 21±4 μm, while that of a 64 N MK is 56±8 μm. Endomitotic MKs enter the mitotic cycle and proceed through to the initiation of the contractile ring, but they deviate from mitosis in late anaphase, after the initiation of the cleavage furrow. They develop mitotic spindles during the process, although the spindle of a polyploid MK is multipolar, with the number of poles corresponding to the ploidy level. Chromatids proceed toward each pole without spindle elongation. During the normal mitotic process, cells form a cleavage furrow and undergo fission into two daughter cells. In endomitosis, MKs exhibit regression of the cleavage furrow and re-enter G1 as polyploid cells, with each cell containing a single nucleus and a single nuclear membrane made up of multiple nuclear lobes.

The invaginated membrane system

As MKs mature, they develop an invaginated membrane system (IMS) serving as a reservoir for proplatelet formation.[13] This IMS was formerly called the demarcation membrane system (DMS) as it was thought to demarcate the MK cytoplasm into small platelet-producing parts. However, as the proplatelet model of platelet release was established and became more widely accepted, the IMS was considered to more accurately describe the unique characteristics of this structure because it is derived from the plasma membrane, maintains contact with the extracellular environment, and functions as a membrane reservoir for proplatelet formation.

Proplatelet transformation

Once MKs mature, they extend long tubular structures called pseudopods and transform into proplatelets.[13] The elongation of the pseudopod starts from one single region on the MK plasma membrane. Subsequently, the proplatelets develop platelet-sized small swellings in tandem arrays along the tubule. After that, the multi-lobed nucleus is extruded and degraded.

Transcription factors

Transcription factors play an essential role in cell fate decisions from stem cells to MK lineage cells. Several studies have indicated that various transcription factors regulate the expression of specific genes for MK lineages, including genes involved in the polyploidization process and proplatelet formation.[14,15] The promoter regions of MK-specific genes, such as glycoprotein IIb, platelet factor 4, and glycoprotein Ib alpha, and erythroid-specific genes contain a consensus binding sequence (WGATAR) for transcription factors of the GATA family. In addition, MK-specific genes have a consensus sequence (GGA/T) for transcription factors of the ETS family. Among the transcription factors of the GATA family, GATA-1 and GATA-2 have been shown to regulate differentiation of stem cells into blood cells.[16-20] These transcription factors share similar binding sequences. GATA-2 is expressed in erythroblasts and MKs. Previous studies suggest that GATA-2 is critical for driving MK lineages via downregulation of GATA-1. GATA-2 expression during erythrocyte differentiation is downregulated by GATA-1 activation. Enforced expression of GATA-2 during erythroid development impairs differentiation into mature erythrocytes through a possible mechanism that blocks GATA-1-mediated regulation. The occupancy pattern of GA TA during erythrocyte differentiation is seen in GATA target genes, including *GATA-2* and *KIT*. GATA-2 interacts with friend of GATA (FOG)-1, a hematopoietic cofactor, during MK development. GATA-2 and FOG-1 share the gene encoding PU.1, a critical transcription factor for development into CMPs and common lymphoid progenitors (CLPs), and they suppress its expression. Repression of PU.1 by GATA factors is necessary for MK development. Both GATA-1 and GATA-2 bind to FOG-1. GATA-1 is required for maturation of several blood lineages, including erythrocytes, MKs, eosinophils, and mast cells. Mutations in GATA-1 and ZFPM-1 are responsible for human X-linked congenital thrombocytopenia. GATA-1 is a critical factor for erythroid and MK development. GATA-1 might also have an overlapping function with GATA-2. Previous studies demonstrated that GATA-2 coordinates MK differentiation in GATA-1-deficient and mutant cells.

Runt-related transcription factor-1 (RUNX-1) is also known as acute myeloid leukemia 1 protein (AML1). Familial platelet disorder patients with RUNX-1 mutations have a high risk of developing myelodysplasia and leukemia. Several studies suggest that RUNX-1 regulates MK differentiation and platelet formation via constituents of the MK and platelet cytoskeleton. Serum response factor (SRF) is a MADS-box transcription factor regulating genes for growth factor-inducible factors and the cytoskeleton. Megakaryoblast leukemia 1 (MKL1) is an activator of SRF transcriptional activity and promotes MK differentiation. Recently, *MKL1/MKL2*-double knockout mice were shown to have less MK maturation and platelet formation.[21] Smith et al.[22] also reported that subcellular localization

and regulation of MKL1 are dependent on Rho-A activity and actin organization in MK development. Ecotropic virus integration site 1 (EVI-1) is expressed in hematopoietic progenitor cells, MKs, and platelets and has an inhibitory effect on the differentiation of HSCs into granulocytes and erythrocytes but enhances MK differentiation. Nuclear factor erythroid-derived 2 (NF-E2) p45 unit (*p45NF-E2*)[23-29] is a tissue-restricted subunit that forms a basic-leucine zipper heterodimeric complex with small Maf proteins that are widely expressed in many cells, a complex known as NF-E2. While NF-E2 was originally identified in erythroid cells, p45NF-E2-deficient mice show mild anemia but significant thrombocytopenia. Studies using p45NF-E2-deficient MKs suggest that the p45NF-E2 is important in terminal MK differentiation and platelet release, while *in vitro* and *in vivo* studies using p45NF-E2-overexpressing mouse bone marrow cells indicate that p45NF-E2 has additional roles in early megakaryopoiesis.[30] We recently demonstrated that fibroblasts transfected with *p45NF-E2*, its binding protein, v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian; *Maf*) *G,* and *Maf K* differentiate into MKs and platelets, whereas untransfected fibroblasts did not differentiate into MK lineage cells.[31] This finding indicates that *p45NF-E2, Maf G,* and *Maf K* are critical factors for megakaryopoiesis and thrombopoiesis.

MicroRNAs in megakaryopoiesis

MicroRNAs (miRNAs), non-coding RNAs of 20-22 nucleotides, bind to targeted mRNA sequences, leading to translational repression and/or degradation of mRNA. Aspects of miRNAs in megakaryopoiesis and their functionality have been assessed by experimental studies. MEP and MK development are reportedly regulated by miR-216, miR-10a, miR-10b, miR-17, miR-130a, miR-20, miR-146a, miR-34a, miR-181, miR-126, miR-109, miR-17, miR-20, and miR-155. Platelet production from MKs is regulated by miR-125b2 and miR-27a. Petriv et al. published the top 10 expressed miRNAs in murine CMPs, MEPs, and MKs [32]. For CMPs, miR-720 is first, followed by miR-16, miR-142-3b, miR-30b, miR-706, miR-136, miR-98, miR-18a, miR-15a, and miR-19b. For MEPs, the top 10 are miR-720, miR-142-3p, miR-16, let-7, miR-709, miR-19b, miR-106a, miR-19a, miR-15b, and miR-25. Similar to both CMPs and MEPs, miR-720 is most prevalent in MKs, followed by miR-142-3p, miR-16, miR-223, miR-98, miR-18a, miR-709, miR-15b, miR-706, and miR-19b.

During megakaryopoiesis and thrombopoiesis, miR-28 and miR-150 are involved in MEP development. miR-150 promotes differentiation from MEPs toward MKs,[33] and TPO induces miR-150 expression in UT7/TPO cells, which in turn targets expression of c-MYB.[34] Several studies show that miR-150 promotes late-stage MK differentiation and platelet production. Overexpression of miR-155 in K562 cells causes a block in megakaryocytic differentiation,[35] and enforced expression of miR-155 also impairs MK proliferation and development by targeting Ets-1 and Meis-1.[36] Transplantation of HSCs overexpressing miR-155 into irradiated mice leads to a reduction in the number of MKs. Several reports suggest that miR-155 has an inhibitory effect on megakaryopoiesis. MiR-34a inhibits cell proliferation and enhances MK differentiation by targeting c-MYB, a negative regulator of megakaryopoiesis, and CDK4 and CDK6, regulators of the cell cycle.[37,38] Several studies have reported that miR-146a level changes during the MK differentiation process, but its effect on gene regulation during megakaryopoiesis is controversial.[39] The effects of miR-146a on megakaryopoiesis depend on the experimental conditions. Girardot et al.[40] reported that miR-28 has an inhibitory effect on MK differentiation via targeting the c-MPL.

Ex vivo generation of platelets

Platelet transfusions are administered for a wide variety of conditions including thrombocytopenia after chemotherapy, and the clinical demand is greatly increasing. However, there are several important issues to solve to stabilize the platelet supply. First, platelet supply depends solely on volunteer donors who must undergo a lengthy process of apheresis. Second, the shelf-life of platelet concentrate is only 4 days in Japan (5 days in the USA) because it is stored at room temperature so as to maintain its functionality and shape; this storage method increases the risk of bacterial growth. Thus, there is great interest in the generation of platelets from stem cells for clinical use. New strategies for manufacturing MKs and subsequently platelets beginning with non-donor-dependent sources may obviate these and other platelet transfusion concerns.[41-44] Since TPO was first isolated and reported, it has been used to generate enriched populations of MKs using *in vitro* differentiation systems. Terminally differentiated cells of the MK lineage release platelets during thrombopoiesis. MKs and platelets have been differentiated from hematopoietic stem cells (HSCs),[44] fetal liver cells,[45] embryonic stem (ES) cells,[46-49] and induced pluripotent stem (iPS) cells.[50,51] Moreover, we have reported the generation of MKs and functional platelets beginning with subcutaneous adipose tissues and preadipocyte cell lines.[52-55] There has been initial success in producing platelets from umbilical cord blood stem cells and human embryonic stem cells (hESC). Experimentally, hESC-derived MKs reached a final ploidy of 32 N and released platelets with intact fibrinogen, although the number of MKs produced was small, and they yielded far fewer platelets than bone marrow-derived cells. In addition, hESCs do involve an ethical concern with regard to clinical use. Recently, induced pluripotent stem cells (iPSC) were also reported to be the source of *ex vivo* platelet production.[50,51] These iPSC are embryonic stage stem cells transformed from mature adult fibroblasts by retroviral transduction or plasmid transfection with four reprogramming genes, *POU5F1, SOX2, KLF4,* and *MYC*. IPSC-sacs are formed by co-culturing fibroblast-derived iPSC with C3H10T1/2 feeder cells, and they are replated with a cocktail of cytokines such as TPO in order to induce MK differentiation. After 22 to 26 days of culture, MKs develop and start to release platelets. Platelets released from iPSC are confirmed to form blood clots in laser-induced vessel injury in irradiated non-obese diabetic severe combined immunodeficient mice. The major drawbacks of both of hESC and iPSC are that they are both relatively complex and high-cost techniques that require several weeks of culture to derive MKs. One strategy to solve these issues is the establishment of stable immortalized self-renewing MK progenitors through the over-expression of BMI1 and BCL-XL to respectively suppress senescence and apoptosis and the constrained over-expression of c-*MYC* to promote proliferation. Immortalized MK cell lines can be expanded in culture over extended time periods and managed to produce CD42b+ platelets with functionality comparable to that of native platelets. However, there are some safety concerns in the clinical use of platelets derived from iPSC due to their potential oncogenicity because of the necessary gene manipulation. Recently, non-integrated and transgene-free induction methods were developed, which reduce the mutagenic potential of manufactured platelets. Also, fibroblasts transfected with *p45NF-E2, Maf G*, and *Maf K* differentiated into MKs

and platelets, whereas fibroblasts did not differentiate into MK lineage cells. Platelets created *ex vivo* from various kinds of cells successfully show aggregation functions, and their yield has been improving, although it is not yet sufficient for clinical application. To improve the efficiency of production, a better understanding of each rate-limiting step of *ex vivo* platelet generation is essential.

Conclusions

Megakaryopoiesis and subsequent thrombopoiesis are unique and complex processes involving various cytokines, transcription factors, and microRNAs for regulation. MKs are few in human bone marrow, and HSCs do not have a strong ability to proliferate *in vitro*. Thus, due to the difficulty in obtaining sufficient numbers of MKs for analysis, the underlying mechanisms of MK lineage commitment remain largely unclear. Recent advancements in cell biology have allowed for a broad spectrum of research into the mechanisms of megakaryopoiesis and thrombopoiesis. Hopefully, current successes and future breakthroughs will elucidate the mechanisms of megakaryopoiesis and thrombopoiesis and lead to a method of *ex vivo* platelet generation.

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