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Megakaryocyte biology and related disorders

Liyan Pang, Mitchell J. Weiss, and Mortimer Poncz

Division of Hematology, Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.

Platelets, derived from megakaryocytes, have an essential role in thrombosis and hemostasis. Over the past 10 years, a great deal of new information has been obtained concerning the various aspects of hematopoiesis necessary to maintain a steady-state platelet level to support physiologic hemostasis. Here we discuss the differentiation of HSCs into megakaryocytes, with emphasis on the key cytokine signaling pathways and hematopoietic transcription factors. Recent insight into these processes elucidates the molecular bases of numerous acquired and inherited hematologic disorders. It is anticipated that the growing knowledge in these areas may be exploited for new therapeutic strategies to modulate both platelet numbers and their thrombogenicity.

Development of the megakaryocyte lineage from HSCs

Like all terminally differentiated hematopoietic cells, megakaryocytes arise from common HSCs, which are responsible for lifelong production of all circulating blood cells (1). Hematopoietic cells are classified by 3 means: (a) by surface markers that are mainly detected by flow cytometry, (b) by their developmental potential assessed ex vivo in colony assays, and (c) by their ability to reconstitute host animals in vivo. Individual cells that reconstitute multilineage hematopoiesis for at least 6 months, termed long-term repopulating HSCs, are rare, constituting less than 0.1% of total nucleated marrow cells. In mice, these are highly enriched within a population of cells with surface markers Lin- Sca-1+ c-kit+ (2–5) (Figure 1). This population has been referred to as the LSK long-term HSC population, but for ease of reading we will refer to it below as the “HSC” population. The production of mature blood cells from HSCs involves a series of successive differentiation steps in which the developmental and proliferative capacities of progenitors become increasingly restricted.

The differentiation of HSCs has been tracked by the expression of cell surface markers including the tyrosine kinase cytokine receptor Flt3 (6), which is absent on the HSC (7). The classical model for hematopoiesis is that committed HSCs give rise to 2 lineages, a common lymphoid progenitor capable of producing lymphocytes, and a common myeloid progenitor with developmental potential restricted to myeloid, macrophage, eosinophil, erythroid, and megakaryocyte lineages (Figure 1) (8–10). Erythroid and megakaryocyte lineages arise from a common megakaryocyte-erythroid progenitor (MEP) derived from the common myeloid progenitor (11). However, recent studies reveal that progenitors that have surface markers similar to those of HSCs, but have become Flt3-positive, upon further differentiation into lymphoid and myeloid lineages do not produce megakaryocytes or erythrocytes in vitro or in vivo (Figure 1) (12). Thus FLT3-negative HSCs express markers of committed megakaryocytes and erythroid precursors and may directly give rise to MEPs (see below and Figure 1). When early stem cells become Flt3+, erythromegakaryocytic marker expression is lost, while lymphoid and myeloid marker expression and developmental potential are retained. These findings deviate from the classical model for hematopoiesis and indicate that loss of erythromegakaryocytic potential may represent a relatively early event in HSC differentiation in certain study models. This surprising finding highlights our incomplete understanding of hematopoiesis and the plasticity of the process.

The model presented in Figure 1 takes into account both the classical pathway, which predicts that HSCs split into common myeloid and lymphoid progenitors (8–10), and the newer findings suggesting a direct pathway from the HSC to the MEP (12). Improvement in fine mapping of cell lineages by flow cytometry analysis and the development of new approaches for studies of the earliest stages of hematopoiesis may further delineate the steps involved in lineage commitment under varied circumstances.

While erythroid and megakaryocyte lineages are believed to share a common MEP (8–10) (Figure 1), the signals that regulate the final separation of these lineages are not well understood. Erythroid and megakaryocytic precursors express both common and unique hematopoietic transcription factors. Among the latter, no single unique factors have been identified to determine lineage choice of the MEP. It is also possible that the final lineage of the MEP is determined by the combinatorial action of multiple nuclear proteins.

The first cells fully committed to the megakaryocyte lineage, termed CFU-Meg, are characterized by a unique cell surface phenotype (13) and form a small cluster of pure megakaryocytes in culture. CFU-Meg cells give rise to 2N megakaryocytes, which, in turn, undergo endomitosis and cytoplasmic differentiation, resulting in a pool of mature megakaryocytes recognized by their large size and characteristic morphology. In the normal human marrow, approximately 1 in 10,000 nucleated cells is a megakaryocyte, while in disorders associated with increased peripheral platelet destruction, such as immune thrombocytopenia purpura, the number increases about 10-fold (14).

Megakaryopoiesis is first noted in the embryonic yolk sac, although studies of animals with severe quantitative and qualitative platelet deficiencies, such as NF-E2–/– mice, showed that platelets are not critical for prenatal survival (15). In mice, fetal megakaryocytes appear to represent a distinct lineage with biologic features different from those of their adult counterparts (16). Several human disorders also support this point. One example is the thrombocytopenia and absent radius (TAR) syndrome, a disorder of unknown etiology characterized by moderate to severe thrombocytopenia in infancy that is typically outgrown in early

Nonstandard abbreviations used: CBFA2, core-binding factor α-2; EPO, erythropoietin; ET, essential thrombocythemia; MEP, megakaryocyte-erythroid progenitor; PP4, platelet factor 4; SDF-1, stromal cell-derived factor-1; TPO, thrombopoietin.

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Clearly cyclins are involved, though a combined knockout of cyclins D1, D2, and D3, while specifically affecting hematopoiesis not noted to affect megakaryopoiesis (24). On the other hand, the cyclin E–null mouse clearly had a defect in megakaryopoiesis and causing late midgestation fetal loss due in part to anemia, was megakaryocytes undergo an endomitotic cell cycle during which they replicate DNA but do not undergo anaphase or cytokinesis; cyte releases approximately 10⁴ platelets (21). Eventually, each megakaryocyte/platelet cytokine, termed Mpl ligand or thrombopoietin (TPO), was identified. This cytokine is discussed below, and dense granules, and organelle granular proteins that participate in platelet function, such as platelet-specific genes from “late-onset” megakaryocyte-specific genes from “late-onset” ones (29).

Despite their close relationship in hematopoietic phylogeny and numerous common hematopoietic transcription factors, erythroid and megakaryocyte lineages do not share many specific proteins or organelles. However, it is interesting to note that both lineages circulate in anucleate forms. The platelet equivalent in fish is called a thrombocyte and is a circulating nucleated diploid cell (30, 31). The purpose of platelet and erythrocyte enucleation in mammals is unclear. One possibility is that loss of nuclei increases flexibility and distensibility of circulating cells, optimizing delivery of specialized functions within small-caliber capillary beds.

Cytokines involved in megakaryopoiesis

In humans, homeostatic mechanisms regulate the normal platelet count within an approximately 3-fold range (150 × 10⁹ to 450 × 10⁹ per cubic micrometer). Disorders that consume platelets increase their production. Numerous hematopoietic factors regulate different aspects of megakaryocyte biology. Certain cytokines, including GM-CSF, IL-3, IL-6, IL-11, IL-12, and erythropoietin (EPO), stimulate proliferation of megakaryocytic progenitors (32). Other cytokines, including IL-1α and leukemia inhibitory factor (LIF), modulate megakaryocyte maturation and platelet release (32, 33). Many of these cytokines have broad effects on all hematopoietic lineages. Presently, the multilineage cytokine IL-11 (Neumega) is the only clinically approved drug for treating thrombocytopenia (34).

More than 10 years ago, a more potent and relatively specific megakaryocyte/platelet cytokine, termed Mpl ligand or thrombopoietin (TPO), was identified. This cytokine is discussed below, along with 2 chemokines, stromal cell–derived factor-1 (SDF-1; CXCL12) and PF4 (CXCL4), that have important effects on megakaryopoiesis and platelet production. A detailed Review by Kenneth Kaushansky that focuses on the role of TPO and its receptor in thrombopoiesis is part of this series on the biology of megakaryocytes and platelets (35).

TPO. Mpl is a GP130 family member previously identified as important for megakaryocyte formation in vitro (36). This pivotal observation led to identification of the Mpl ligand, termed TPO, which was determined to markedly stimulate megakaryocyte production (37–40). TPO is highly homologous to EPO in its N-termini half, reflecting a close evolutionary relationship between their respective receptor signaling pathways. Abrogation of either Mpl in development of trophoblasts, another cell line dependent on endomitosis (25). Other studies on chromosomal passenger proteins Aurora-B, survivin, and inner centromere protein showed normal levels overall in megakaryocytes (26), although one report suggests that survivin and Aurora-B may be mislocalized or absent during an important phase of endomitosis (27). The biologic importance of endoreduplication is unclear in terms of its necessity for cell size and for platelet release.

Cellular maturation of megakaryocytes is distinguished by accumulation of characteristic surface markers including Gpα/β3/GPIX/GPV receptors, a cytoplasmic demarcation system believed to participate in platelet formation, distinctive platelet organelles such as the α- and dense granules, and organelle granular proteins that participate in platelet function, such as platelet factor 4 (PF4) and vWF (28). Of note, the extent of polyploidization is not closely synchronized with cellular maturation, so that different degrees of ploidy are present at each stage. For this reason it has been hard to distinguish “early-onset” megakaryocyte-specific genes from “late-onset” ones (29).

Figure 1
Mega Karyopoiesis pathways. The figure extends from the HSC to platelets and offers a combination of a more “classical” pathway, leading to the common megakaryocyte-erythroid progenitor (MEP), and a proposed “direct” route from the HSC. Pathways leading to platelet production are indicated by blue arrows and other pathways by gray arrows. Surface markers of importance are noted in parentheses in red. LT-HSC, long-term HSC; ST-HSC, short-term HSC; Thy1, thymus 1 (“low” indicates low surface antigen and “–” indicates none detectable); Flt3, FMS-like tyrosine kinase 3; EPOR, erythropoietin receptor; CD41, glycoprotein IIb/IIIa or αIIb/β3 integrin receptor; G-CSFR, G-CSF receptor; CMP, common myeloid progenitor; ELP, early lymphoid progenitor; GMP, granulocyte/monocyte progenitor; CLP, common lymphoid progenitor.

Unique aspects of megakaryocyte maturation

The hallmark of megakaryocyte development is the formation of a large cell (~50–100 μm diameter) containing a single, large, multilobulated, polyploid nucleus (21). Eventually, each megakaryocyte releases approximately 10⁴ platelets (22). Unlike other cells, megakaryocytes undergo an endomitotic cell cycle during which they replicate DNA but do not undergo anaphase or cytokinesis; as a result, they acquire a DNA content of up to 256N per cell (23). The mechanisms regulating endomitosis are not fully understood. Clearly cyclins are involved, though a combined knockout of cyclins D1, D2, and D3, while specifically affecting hematopoiesis and causing late midgestation fetal loss due in part to anemia, was not noted to affect megakaryopoiesis (24). On the other hand, the cyclin E–null mouse clearly had a defect in megakaryopoiesis and childhood (17). Another example is transient myeloproliferative disorder and acute megakaryoblastic leukemia in Down syndrome, which develop nearly exclusively in the neonatal period and the first years of life, respectively (18). Interestingly, recent studies demonstrate that fetal megakaryocyte progenitors are uniquely sensitive to mutations in the transcription factor GATA-1, which accompany these disorders (19). Perhaps another example of the distinct nature of fetal/infant megakaryopoiesis is the well-known propensity of severely ill neonates to develop prolonged thrombocytopenia with slow marrow recovery of platelet production (20).
or TPO in mice decreases megakaryocyte numbers in the marrow and circulating platelets by approximately 85% (41–43). Clearly the TPO:Mpl axis is important but not essential for megakaryopoiesis. Furthermore, studies of Mpl knockout animals showed that the TPO:Mpl axis functions in early hematopoietic progenitors, including HSCs (44, 45) (Figure 1). Thus, the TPO:Mpl axis appears to be important for hematopoiesis in general and megakaryopoiesis specifically. The discovery of TPO has contributed greatly to platelet biology, because it permits relatively large and pure cultures of megakaryocytes to be generated in vitro. TPO remains under development as a potential clinical thrombopoietic and/or hematopoietic agent and as a drug to stimulate ex vivo expansion of HSCs (46).

In adults, humoral regulation of thrombopoiesis differs from that of erythropoiesis, where the kidneys produce EPO in response to tissue hypoxia. In contrast, TPO is produced constitutively, and its circulating levels are regulated by its end product, platelets. Circulating TPO remains under developmental control as a potential clinical thrombopoietic and/or hematopoietic agent and as a drug to stimulate ex vivo expansion of HSCs (46).

Defects in TPO:Mpl signaling occur in several human disorders. For example, Mpl mutations, mostly causing frameshifts and early termination, occur in congenital amegakaryocytic thrombocytopenia, a rare disorder of life-threatening thrombocytopenia and megakaryocyte deficiency in infancy (50–52) (Figure 2). Given the role of Mpl in HSC development, it is also possible that congenital amegakaryocytic thrombocytopenia patients are at risk for developing more diffuse hematopoietic defects, including aplastic anemia (53). Activating mutations in the TPO gene promoter (54) and the Mpl protein (55) occur in a subset of patients with familial essential thrombocythemia (ET) (Figure 2), a disorder characterized by increased numbers of hyperaggregable platelets. In contrast, the majority of patients with the more common acquired adult, myeloproliferative form of ET harbor somatic activating mutations in the JAK2 gene (56). These mechanistic differences could explain why familial ET carries an excellent long-term prognosis that differs from the high incidence of leukemic transformation in acquired ET (57).

SDF-1. SDF-1 enhances both megakaryopoiesis and homing of HSCs to the bone marrow during fetal development (58). SDF-1 stimulates megakaryopoiesis via TPO-independent CXCR4 receptor pathways by enhancing the chemotactic activity of their progenitor cells (49, 50). This activity of SDF-1 may be important for the movement of megakaryocyte progenitors from the proliferative “osteoblastic niche” to the “vascular niche” for platelet formation (61). Indeed, both in TPO−/− and in Mpl−/− mice, infusions of SDF-1 can rescue platelet production (61). There may be clinical utility for

Figure 2
Regulation of megakaryopoiesis by cytokines, chemokines, and transcription factors. In the middle panel, a scheme based on the classical pathway of megakaryopoiesis is shown. Cytokines and chemokines that influence that process are shown on the right side as green arrows to indicate the approximate level of development at which they have their influence. Open white areas in arrows indicate levels at which the cytokine is not known to act. Blue text refers to cytokine receptors of significance in megakaryopoiesis. Transcription factors that affect megakaryopoiesis are shown on the left side, and the lilac-colored arrows indicate the approximate point of their influence. Open white areas in arrows indicate levels at which the transcription factor is not known to act. Clinically relevant diseases linked to defects of these regulators are noted in red, italicized text. CFU-GEMM, CFU, granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-EM, CFU, erythrocyte, megakaryocyte; BFU-EM, burst-forming unit, erythrocyte, megakaryocyte; CFU-Meg, CFU, megakaryocyte.
Figure 3
Assembly of transcription factor complex at a megakaryocyte-specific gene. A schematic representation of the proximal promoter region of a hypothetical megakaryocyte-specific gene modeled after the $\alpha$Itgα2b gene. GATA-1 is shown in the middle binding to its known consensus sequence (69) and interacting with FOG-1, Fli-1, and CBFA2 of the RUNX complex (each interaction is indicated by a 2-headed arrow). Fli-1 and CBFA2 bind to their adjacent cognate on the DNA (89, 122), while FOG-1 is recruited through its interactions with the N-terminal zinc finger of GATA-1. In turn, FOG-1 recruits the NuRD complex and other nuclear factors.

SDF-1 infusions to improve platelet production. For example, HIV may cause thrombocytopenia by infecting megakaryocyte precursors through interactions with their CXCR4 receptors (62). Thus, SDF-1 mimetic drugs may improve HIV-related thrombocytopenia by competing with the virus for its megakaryocyte receptor (63).

$\alpha$PF4 and other chemokines. PF4 is an $\alpha$-granule protein that inhibits megakaryocyte development and maturation in vitro (64), as other CXC and CC subfamily chemokines have subsequently been shown to do (65). The in vitro findings for PF4 are corroborated by altered platelet counts in $\alpha$PF4–/– mice and transgenic $\alpha$PF4-overexpressing mice (66). Platelet $\alpha$-granules contain large stores not only of the platelet-specific chemokines PF4 and the closely related protein platelet basic protein (PB; CXCL7), but also of other chemokines, especially RANTES (CCL5) and ENA-78 (CXCL5) (67, 68). They negatively regulate megakaryopoiesis and are mild platelet agonists through cognate receptors on developing megakaryocytes. These are weak agonists of platelet activation and also may be important in linking thrombosis and inflammation. Release of $\alpha$-granular contents in the marrow could affect platelet numbers in pathologic states. For example, the discharge of chemokines during chemotherapy or radiation therapy may contribute to thrombocytopenia that occurs during these treatment modalities. In this case, strategies to inhibit this process could be used preemptively to prevent thrombocytopenia.

Transcription factors involved in megakaryopoiesis
Megakaryopoiesis is regulated by multiple cytokines influencing the survival and proliferation of increasingly committed progenitors as they transition from one hematopoietic niche to another in an organized fashion (61). During this process, a series of transcription factors coordinately regulate the chromatin organization of megakaryocyte-specific genes and prime them for expression en route to platelet formation. Some of these events are beginning to be understood, especially the hematopoietic-specific transcription factor complexes involved in terminal differentiation. Numerous nuclear proteins with important roles in megakaryocyte formation, growth regulation, and platelet release have been identified, mainly through loss-of-function studies in mice and analysis of human diseases. Important examples are discussed below.

GATA-1/FOG-1 complex. GATA-1 was first isolated as an essential 2–zinc finger, erythroid transcription factor that binds the DNA sequence WGATAR (69) (Figure 3). GATA-1 is also expressed and of functional consequence in megakaryocytes, mast cells, and eosinophils (70, 71). Transient expression reporter gene studies of megakaryocyte-specific proximal promoters defined several functionally important GATA-binding sites (72–74). A point mutation in a GATA-binding site of the $\alpha$GIP1bb proximal promoter region causes a form of Bernard-Soulier syndrome (74).

While targeted disruption of the GATA-1 gene in mice causes embryonic lethality due to anemia (75), a megakaryocyte-specific knock down of GATA-1 expression results in significant thrombocytopenia and increased numbers of immature and dysmorphic megakaryocytes (76). GATA-2 is a closely related transcription factor and is also hematopoietic-specific, but it is expressed earlier and participates in maintenance of HSCs and multipotential progenitors (77). GATA-1 and GATA-2 are believed to have both overlapping and unique functions (69, 78). Continued GATA-2 expression during early megakaryopoiesis may explain the partial ability for platelet formation in the GATA-1 knockdown mouse (Figure 2).

FOG-1 ($\text{Friend of GATA-1}$, or $\text{F}2$pm1) is a 9–zinc finger, hematopoietic-specific transcription factor isolated as a GATA-1 binding partner (79) (Figure 3). Targeted disruption of the FOG-1 gene markedly inhibits erythroid development, causing embryonic death from severe anemia. Detailed study of these animals and $\alpha$FOG-1–/– ES cells also demonstrated an early block to megakaryocytic development with no identifiable precursors. FOG-1 does not appear to bind DNA directly but, rather, associates with target genes indirectly through interactions with GATA proteins (Figure 3). FOG-1 is the only protein of its kind expressed in the erythromegakaryocytic lineages. The severity of the megakaryocyte defect in the $\alpha$FOG-1–/– mouse suggests that most or all critical GATA-1– and GATA-2–related activities require interactions with FOG-1. In support of this, GATA-1 and FOG-1 synergistically enhance the expression of the megakaryocyte-specific $\alpha$Dia2 gene (80, 81). These observations have been expanded to show that direct contact is needed between the N-terminal zinc finger of GATA-1 and FOG-1. This synergism applies to multiple megakaryocyte-specific genes and also involves a specific Ets family transcription factor (see below). The N-terminus of FOG-1 plays a unique, nonredundant role in megakaryocyte-specific expression (82), possibly through its ability to recruit the corepressor complex NuRD (83). The clinical importance of the GATA-1/FOG-1 interaction in megakaryopoiesis is demonstrated by the identification of patients with X-linked thrombocytopenia and variable anemia who have GATA-1 mutations that impair FOG-1 binding (84–86) (Figure 2).

An important role for GATA-1 in regulating the maturation and proliferation of megakaryocyte progenitors is further evidenced by the recent discovery of acquired somatic mutations associated with both megakaryoblastic leukemia and transient myeloproliferative syndrome in infants with Down syndrome (87, 88). These mutations occur in the first coding exon, causing early termination and production of a truncated protein, termed GATA-1short, via translation initiation from a downstream internal methionine. This mutant form of GATA-1 may act as a dominant oncogene by specifically stimulating the proliferation of fetal megakaryocyte progenitors (19).

Fli-1 and TEL. The proximal promoters of many megakaryocyte-specific genes contain tandem-binding sites for GATA and Ets proteins, suggesting functional interactions between these 2 classes of
transcription factors (71–73) (Figure 3). The Ets family is diverse with at least 30 members, all sharing an Ets-binding domain that recognizes a GGAA core sequence (89). Numerous Ets members are present in primary megakaryocytes and/or megakaryocytic cell lines (90–92). While a number of reports suggest a function for Ets-1 in megakaryopoiesis (93), the clearest story of a common transcriptional regulator appears to be that for Fli-1, an Ets transcription factor initially recognized to be important for T cell differentiation (94) and early hematopoiesis/vasculogenesis (95) (Figure 2). GATA-1/FOG-1 synergy for many megakaryocyte-specific genes appears to involve Fli-1 (81), and Fli-1 binds to the proximal promoter of these genes in vivo. The molecular basis of this synergy is still not fully known, but Fli-1 does bind GATA-1 (96) (Figure 2). "NF-E2 is a hematopoietic-specific transcription factor initially recognized to be important for T cell differentiation (94) and early hematopoiesis/vasculogenesis (95). The pointed domain of TEL is a subunit dimerized with a ubiquitous p18 subunit (104). In vitro (98), depending on the size of the transcription factor activity, initially identified in human T cell leukemias with multilineage features (91). Moreover, Fli1 gene–disrupted mice either have abnormal megakaryocytes with thrombocytopenia (97) or fail to develop recognizable megakaryocytes (98), depending on the size of the Fli-1 gene deletion. Fli-1 expression also inhibits erythroid differentiation (99). Thus, Fli-1 may not depend on steel factor for their generation. 1. Ogawa, M. 1993. Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844–2853. 2. Spangrude, G.J., Heimfeld, S., and Weissman, I.L. 1988. Purification and characterization of mouse hematopoietic stem cells. Science. 241:58–62. 3. Ikuta, K., and Weissman, I.L. 1992. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc. Natl. Acad. Sci. U. S. A. 89:1502–1506. 4. Li, C.L., and Johnson, G.R. 1995. Murine hematopoietic stem and progenitor cells. I. Enrichment and biologic characterization. Blood. 85:1472–1479. 5. Weissman, I.L., Anderson, D.J., and Gage, F. 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments and transdifferentiations. Annu. Rev. Cell Dev. Biol. 17:387–403. 6. Rosnet, O., Marchetto, S., deLapeyriere, O., and Bernbaum, D. 1991. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGF/R/CSF1R family. Oncores. 6:1641–1650. 7. Adolphson, J., et al. 2001. Upregulation of Flt3...