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Megakaryopoiesis

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Abstract

The process of megakaryopoiesis and platelet production is complex, with the potential for regulation at multiple stages. Megakaryocytes are derived from the hematopoietic stem cell through successive lineage commitment steps, and they undergo a unique maturation process that includes polyploidization, development of an extensive internal demarcation membrane system and finally formation of proplatelet processes. Platelets are shed from these processes into vascular sinusoids within the bone marrow. Megakaryocyte differentiation is regulated both positively and negatively by transcription factors and cytokine signaling. Thrombopoietin is the most important hematopoietic cytokine for platelet production. Clinically, acquired and inherited mutations affecting megakaryocytic transcription factors and thrombopoietin signaling have been identified in disorders of thrombocytopenia and thrombocytosis.

Transcriptional regulation of megakaryopoiesis

Megakaryocytes, like all blood cells, derive from the hematopoietic stem cell (HSC) (reviewed ¹). During hematopoietic differentiation, the HSC gives rise to progressively committed progenitors, including the common myeloid progenitor (CMP) and the megakaryocyte-erythroid progenitor (MEP) (Figure 1, reviewed ²). MEPs are bipotential precursors that give rise to cells of both megakaryocytic and erythroid lineages. Multiple transcription factors including Runx1, Gata1, Fli1 and c-Myb form complex networks that regulate the differentiation of megakaryocytes both positively and negatively. For example, in addition to its requirement during embryogenesis for the formation of HSCs, Runx1 has an important role in megakaryocyte development, and inducible deletion of Runx1 in the adult mouse leads to thrombocytopenia and impaired megakaryopoiesis ³. Runx1 interacts with additional megakaryocytic factors including Gata1 and Fli1 ^{4,5}. Gata1 and its co-factor Friend of Gata1 (Fog1) are critical in promoting megakaryocyte-erythroid differentiation, while at the same time inhibiting expression of Pu.1 and myeloid differentiation ^{6,7}. Binding sites for Gata1 and Fli1 can be found in the enhancers of many megakaryocyte-specific genes ⁸, and Fli1 enhances the activity of Gata1 at megakaryocytic promoters and represses the activity of erythroid factors at erythroid promoters ⁹. Thus Fli1 expression may act to restrict the MEP to the megakaryocytic lineage. Accordingly, deletion of Fli1 is associated with loss of mature megakaryocytes in mouse models ¹⁰. In contrast, expression of the proto-oncogene c-Myb in the MEP favors erythropoiesis, and c-Myb expression is down-regulated during megakaryopoiesis ¹¹. The miRNA miR-150, expressed during megakaryocyte maturation, acts

post-transcriptionally to decrease c-Myb expression, and may influence whether the bipotential MEP adopts either the megakaryocyte or erythroid fate ¹².

Endomitosis and proplatelet formation

As megakaryocytic cells become progressively differentiated they lose their proliferative ability and become polyploid through a variation of the cell cycle called endomitosis (reviewed ¹³). During differentiation, diploid promegakaryoblasts give rise to tetraploid megakaryoblasts and then successively larger and more polyploid promegakaryocytes and megakaryocytes. Mature megakaryocytes can be 150 μ M or more in diameter and are the largest hematopoietic cells in the bone marrow. Although polyploidy is a defining feature of mature megakaryocytes, the purpose for acquiring multiple chromosome complements is unknown. It is theorized that polyploidization facilitates the massive protein and membrane synthesis required for later platelet production through functional gene amplification ¹⁴. In support of this notion, there is some correlation between the degree of megakaryocyte ploidy and platelet production; for example, neonatal megakaryocytes are typically of low ploidy and may have a reduced capacity to produce platelets relative to their adult counterparts ¹⁵. However, when mouse strains in which the modal megakaryocyte ploidy varies have been compared, there does not appear to be a direct relationship between ploidy and steady state platelet counts ¹⁶.

The mechanism of endomitosis is an enigma. Following G1 and S phases, endomitotic megakaryocytes enter mitosis and anaphase, separate their chromosomes, and begin the process of cleavage furrow formation ^{17,18}. Subsequently, nuclei fail to completely separate and cleavage furrows regress before cytokinesis can be completed, resulting in the formation of a single cell with a multilobulated, polyploid nucleus ¹⁸ (Figure 2). Furrow regression may be due the failure to properly localize or activate RhoA at the contractile ring ¹⁹, but what regulates the switch from mitosis to endomitosis and how the polyploid cell avoids apoptotic triggers and continues to cycle remain important questions.

Following nuclear polyploidization, megakaryocytes undergo cytoplasmic maturation involving the formation an extensive demarcation membrane system (DMS) as well as numerous alpha and dense granules (reviewed ¹⁶). The DMS is an elaborate membrane network that ultimately fills the megakaryocyte cytoplasm except for a narrow band at the periphery of the cell. The DMS is continuous with the plasma membrane of the cell and is in contact with the external environment ²⁰, and it has been proposed to serve as a reservoir of membrane to support proplatelet formation ²¹. Alpha granules are formed in the Golgi complex and contain both endogenously produced proteins and proteins derived from the extracellular environment through receptor-mediated endocytosis and pinocytosis. These granules as well as other megakaryocyte organelles such as mitochondria and mRNAs will be transferred into the nascent platelets.

Two models have been proposed to explain platelet formation from mature megakaryocytes. In the fragmentation model, megakaryocytes travel from the bone marrow to the lung where they are broken up into platelets in the microvasculature ²². Alternatively, in the proplatelet model, megakaryocytes in the bone marrow develop multiple branching processes that extend into the marrow sinusoids to release platelets into the circulation ²³. In support of the latter model, Italiano and colleagues used live cell microscopy to document the elaboration of a network of branching proplatelets by cultured megakaryocytes ²⁴. Junt and colleagues later extended these results by imaging the bone marrow compartment *in vivo*, demonstrating that fluorescently-labeled megakaryocytes release platelet-like particles into marrow vascular sinusoids ²⁵. These studies confirmed that the marrow is a site of platelet production, although a contribution from the lung cannot be excluded. The process and regulation of proplatelet formation is discussed in more detail elsewhere in this issue (see Thon and Italiano chapter).

TPO signaling in megakaryopoiesis

Multiple growth factors support megakaryopoiesis, the most important of which is thrombopoietin (TPO). TPO belongs to the four-helix bundle family of cytokines, which includes erythropoietin, G-CSF, growth hormone and leukemia inhibitory factor among others. The TPO receptor *c-Mpl* was identified based on its homology to the oncogene *v-Mpl*, already known at the time as the transforming factor of the murine myeloproliferative leukemia virus ²⁶. TPO and *c-Mpl* are critical for megakaryocyte growth and development, and in mouse models where one or the other is absent, platelets and megakaryocytes are reduced to approximately 10% of normal values ^{27,28}. In addition to megakaryocytic cells, HSCs also express *c-Mpl* and depend on TPO signaling for their maintenance and expansion ²⁹. Consequently, *c-Mpl* and *Tpo*-null mice are not only thrombocytopenic but have reduced HSCs and progenitors of all lineages ³¹.

The *c-Mpl* gene encodes a 635 amino acid protein consisting of a 25 amino acid signal peptide (1-25), a 465 amino acid extracellular domain (26-491), a 22 residue transmembrane domain (492-513) and an intracellular domain containing two conserved motifs termed box 1 (528-536) and box 2 (565-574). The extracellular domain is composed of two repeating modules; intriguingly, the membrane distal module appears to have an inhibitory effect on signaling, as its deletion results in constitutive activation of the receptor ³². *c-Mpl* does not have intrinsic kinase activity, but instead associates with the cytoplasmic tyrosine kinase Janus kinase 2 (Jak2) through its box 1 domain ³³. Additional elements regulate receptor internalization and subsequent degradation following TPO binding. These include dileucine repeats located within box 2, Tyr₅₉₁ and Tyr₆₂₅ ^{34,35}.

TPO signaling depends on the activation of Jak2 (Figure 3). Jak2 associates with box 1 of *c-Mpl* through its FERM (band 4.1/ezrin/radixin/moesin) domain. Based on X-ray crystal studies of the erythropoietin receptor ³⁶, it is believed that in the unliganded state *c-Mpl* exists as a homodimer, and that TPO binding results in a conformational change that brings the cytoplasmic tails of the receptor into closer proximity. Consequently, the Jak2 molecules associated with the receptor are brought close enough to each other to become activated through trans-autophosphorylation ³⁷. Active Jak2 then phosphorylates itself on multiple residues and phosphorylates *c-Mpl* on at least Tyr₆₂₅ and Tyr₆₃₀ ³⁸. These phosphotyrosine residues provide docking sites for src homology 2 (SH2)-domain-containing signaling proteins that modulate receptor signaling.

Following the activation of Jak2, multiple signaling molecules are activated and mediate the cellular response to TPO. These include members of the signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways (reviewed ³⁹). Jak2 directly phosphorylates STAT family members including STAT1, 3, 5a and 5b ⁴⁰. Once phosphorylated, these STAT proteins dimerize and translocate to the nucleus of the cell where they can bind to STAT-responsive transcriptional elements within genes such as p21 ⁴¹, Bcl-xL ⁴² and cyclin D1 ⁴³. Constitutive activation of the Jak2/STAT pathway can lead to cytokine-independent growth and contribute to transformation, as demonstrated by the finding of mutant Jak2 in myeloproliferative disorders, translocations involving Jak2 in lymphoid leukemias, and constitutively active STAT5 in leukemic cell lines ^{44,45}.

Jak2 also activates the small GTPase Ras and the MAPK cascade, culminating in the activation of extracellular signal-related kinase (ERK)1/2. Multiple studies have demonstrated the importance of TPO-induced MAPK signaling in megakaryocytic differentiation ⁴⁶⁻⁴⁸. The classical pathway by which TPO signaling is thought to activate Ras depends on the binding of the adaptor protein Shc to phosphorylated *c-Mpl* Tyr₆₂₅ ^{38,49} and the assembly of a complex

containing the adaptor protein Grb2 and the guanine nucleotide exchange factor SOS^{50,51}. Ras then activates Raf-1, mitogen-induced extracellular kinase (MEK) and finally Erk 1/2 (reviewed⁵²). It is intriguing that although activation of MAPK is significantly reduced in the absence of c-Mpl Tyr₆₂₅ and Tyr₆₃₀, it is not eliminated⁵³, suggesting that activation of Erk1/2 can be mediated either through a Shc-independent mechanism, possibly through Grb2/Sos complexes recruited to Jak2⁵⁴. Alternatively, the small GTPase Rap1 can activate Erk1/2 via B-Raf independent of Ras⁵⁵.

The PI3K pathway is also essential for megakaryopoiesis⁵⁶. PI3K is composed of a kinase (p110) and a regulatory subunit (p85). TPO induces the formation of a complex between phosphorylated p85 and the adaptor Gab, although this complex has not been found to bind directly to c-Mpl⁵⁷; alternatively, PI3K may become activated indirectly through Ras⁵⁸. TPO-induced PI3K phosphorylates and activates the serine/threonine kinase Akt whose substrates include Forkhead, glycogen synthase kinase 3 beta (GSK-3 β) and Bad^{56,59,60}, collectively promoting survival and proliferation of megakaryocytic cells. PI3K also activates mammalian target of rapamycin (mTOR), whose targets SK6 and 4E-BP1 increase proliferation and maturation of megakaryocytic progenitors^{61,62}. PI3K is itself negatively regulated by phosphatase and tensin homolog (PTEN), a tumor suppressor that promotes quiescence in hematopoietic stem cells (HSC)⁶³. Although PTEN regulates the activity of Akt and mTOR, its role in TPO signaling and megakaryopoiesis has not yet been defined.

Negative regulation of TPO signaling and megakaryopoiesis

As with all proliferative stimuli, checks on TPO signaling and megakaryopoiesis are required to maintain homeostatic balance and prevent the development of thrombocythemia or leukemia. To ensure that signals are appropriately terminated, many positive regulators also induce their own inhibitors. For example, activation of Jak/STAT pathway induces the transcription of members of the suppressor of cytokine signaling (SOCS) family^{64,65}. This family includes at least 8 members that can inhibit Jak signaling in a variety of ways, including binding to the activation loop of Jak and targeting it for degradation, acting as a pseudosubstrate for Jak, or binding to phosphorylated tyrosines within the cytokine receptor itself (reviewed⁶⁶). Importantly, induction of a SOCS response from one receptor can negatively regulate another, thereby providing a mechanism for cytokine cross-talk; this is illustrated by the finding that treatment of megakaryocytes with interferon- α induces SOCS1, which then down-regulates TPO signaling through inhibition of Jak2⁶⁷. Some cancer cells may be able to avoid downregulation of Jak2 and this may be a strategy enabling them to grow; for example, the activity of mutant Jak2 V₆₁₇F appears to be resistant to inhibition by SOCS3⁶⁸.

Jak2 has other binding partners that regulate its activity. For example, Lnk is an adaptor protein that inhibits growth in HSCs, erythroid and megakaryocytic cells^{69,70}. Lnk binds to phosphorylated tyrosines within Jak2 through its SH2-domain⁷¹; however, the exact mechanism by which it inhibits TPO signaling is not yet understood. In addition to binding negative regulators, Jak2 may be phosphorylated within the FERM domain, inducing its dissociation from c-Mpl and thus providing another mechanism to 'turn off' signaling⁷².

Clinical correlates

Congenital and familial thrombocytopenia syndromes are models for understanding the function of specific genes in megakaryopoiesis. For example, consistent with the importance of the transcription factors Runx1, Gata1 and Fli1 in megakaryocyte differentiation, mutations involving each of these factors have been found in association with an inherited thrombocytopenia syndrome. Runx1 mutations are associated with Familial Thrombocytopenia Disorder with Predisposition to AML, an autosomal dominant

thrombocytopenia in which affected family members are prone to the development of myeloid leukemias^{73,74}. Reflecting the requirement for Gata1 in both megakaryopoiesis and erythropoiesis, mutations of Gata1 are associated with X-linked thrombocytopenia accompanied by anemia and red cell abnormalities such as thalassemia, dyserythropoiesis or porphyria (reviewed⁷⁵). Mutations typically are located within the N-terminal zinc finger of Gata1 and disrupt binding to its cofactor Fog1. Although specific Fli1 mutations have not been reported in the clinical setting, the expression of Fli1 is affected in patients with Jacobsen syndrome. This congenital disorder is characterized by thrombocytopenia, heart defects, mental retardation and hemizygous deletion of the terminal portion of chromosome 11q that includes Fli1 located at 11q23-24⁷⁶. Although other deleted genes might contribute to the platelet phenotype, over-expression of exogenous Fli1 in bone marrow cells from a patient with Jacobsen syndrome rescued defective megakaryopoiesis *in vitro*⁷⁷, supporting the conclusion that the thrombocytopenia in Jacobsen syndrome is due to the hemizygous loss of Fli1.

Altered TPO signaling contributes to both disorders of thrombocytopenia and thrombocytosis. The classic syndrome of deficient TPO signaling is seen in congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is an autosomal recessive disorder in which c-Mpl is either absent or minimally functional^{78,79}. Circulating TPO levels are highly elevated due to the lack of receptor-mediated uptake, and without a functional receptor megakaryopoiesis is severely impaired. Children present at birth with severe thrombocytopenia and megakaryocytopenia, and because TPO is vital for maintenance of the HSC their disease nearly always progresses to aplastic anemia^{80,81}. Definitive treatment requires allogeneic HSC transplantation. Curiously, although in murine models the deletion of TPO phenocopies that of c-Mpl³¹, no humans have been identified with an inherited deficiency of TPO.

Conversely, inherited mutations that result in increased TPO signaling have also been described in families with thrombocythemia and myeloproliferative disease. In familial thrombocythemia there is autosomal dominant transmission of high platelet counts. In some kindreds this is due to a mutation causing aberrant splicing of TPO mRNA resulting in a form that is translated more efficiently than the wild type transcript, leading to excessive TPO production^{82,83}. The clue to this diagnosis is that circulating TPO levels are elevated despite thrombocytosis. Additional families have been described in which mutations in c-Mpl result in constitutive activation of the receptor⁸⁴. Although these individuals also have thrombocytosis, circulating TPO levels are low because TPO is not being overproduced and is being taken up and degraded by the increased platelet mass.

Acquired mutations altering TPO signaling are characteristic of Philadelphia chromosome-negative myeloproliferative diseases, including polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). Activating mutations of Jak2, most frequently the substitution Val₆₁₇Phe⁸⁵⁻⁸⁷ but rarely mutations within exon 12⁸⁸, are found in nearly all patients with PV and about half of those with ET and IMF. A minority of patients with ET and IMF carry activating mutations not of Jak2 but of c-Mpl⁸⁹. Complications of myeloproliferative disease include thrombosis, myelofibrosis and transformation to leukemia. Activated Jak2 seems to be an independent risk factor for thrombosis, as thrombosis in patients with Jak2 Val₆₁₇Phe mutations can precede the development of overt overproduction of blood cells^{90,91}. In contrast, the development of myelofibrosis may be secondary to factors secreted by the increased numbers of megakaryocytes rather than a direct effect of c-Mpl and Jak2 signaling^{92,93}. Surprisingly, recent studies of patients with MPD who develop leukemia suggest that at least in some cases, the leukemic clone is derived from a cell that does not carry the Jak2 mutation⁹⁴. This, as well as studies of families with heritable MPD, provides evidence that Jak2 Val₆₁₇Phe may not be the mutation that initiates this disease but may occur as a secondary mutation in a genetically predisposed cell^{95,96}.

Conclusions

Much has been learned about megakaryopoiesis since the discovery of TPO and its receptor. Furthermore, advances in genotyping have enabled researchers to identify the causative genes in several acquired and inherited disorders of platelet production. New tools, such as platforms that enable large scale studies of protein-protein and protein-DNA interactions, tissue-specific promoters that permit the deletion of megakaryocytic genes in mouse models, and developmental models using embryonic stem cells, will continue to expand the boundaries of our knowledge.

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Figure 1. Overview of megakaryopoiesis. Megakaryocytes are derived from the hematopoietic stem cell and proliferate and differentiate under the influence of TPO.

Figure 2.

Endomitosis. Diploid megakaryocyte progenitors progress through S phase and enter mitosis. Although anaphase is initiated, with separation of paired sister chromosomes and formation of the cleavage furrow, prior to completion of cytokinesis the cleavage furrow regresses, resulting in a tetraploid cell that re-enters G1.

Figure 3.

TPO signaling. c-Mpl is a homodimeric cytokine receptor that associates with the cytoplasmic tyrosine kinase Jak2. Following the binding of TPO, a conformation change occurs that leads to the activation of Jak2 and downstream signaling pathways including STAT, MAPK and PI3K.