

# Megakaryopoiesis and platelet production: insight into hematopoietic stem cell proliferation and differentiation

Tianyu Guo<sup>1\*</sup>, Xuejun Wang<sup>1\*</sup>, Yigong Qu<sup>1</sup>, Yu Yin<sup>1</sup>, Tao Jing<sup>1</sup>, Qing Zhang<sup>1,2</sup>

<sup>1</sup>State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China; <sup>2</sup>Research Institute of Sun Yat-Sen University in Shenzhen, Shenzhen 518057, China

\*These authors contributed equally to this work.

Correspondence to: Qing Zhang. State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China. Email: lsszq@mail.sysu.edu.cn.

**Abstract:** Hematopoietic stem cells (HSCs) undergo successive lineage commitment steps to generate megakaryocytes (MKs) in a process referred to as megakaryopoiesis. MKs undergo a unique differentiation process involving endomitosis to eventually produce platelets. Many transcription factors participate in the regulation of this complex progress. Chemokines and other factors in the microenvironment where megakaryopoiesis and platelet production occur play vital roles in the regulation of HSC lineage commitment and MK maturation; among these factors, thrombopoietin (TPO) is the most important. Endomitosis is a vital process of MK maturation, and granules that are formed in MKs are important for platelet function. Proplatelets are firstly generated from mature MKs and then become platelets. The proplatelet production process was verified by novel studies that revealed that the mechanism is partially regulated by the invaginated membrane system (IMS), microtubules and Rho GTPases. The extracellular matrices (ECMs) and shear stress also affect and regulate the process while the mature MKs migrate from the marrow to the sub-endothelium region near the venous sinusoids leading to the release of platelets into the circulation. This review describes the entire process of megakaryopoiesis in detail, illustrates both the transcriptional and microenvironmental regulation of MKs and provides insight into platelet biogenesis.

**Keywords:** Hematopoietic stem cells (HSCs); megakaryopoiesis; platelet production; endomitosis; microenvironment

Received: 03 January 2015; Accepted: 06 February 2015; Published: 14 February 2015.

doi: 10.3978/j.issn.2306-9759.2015.02.01

View this article at: <http://dx.doi.org/10.3978/j.issn.2306-9759.2015.02.01>

## Introduction

Megakaryopoiesis is a complex process that involves the commitment of hematopoietic stem cells (HSCs) to the megakaryocyte (MK) lineage, proliferation of the progenitors, MK maturation and terminal differentiation that produces platelets. HSCs are multipotent cells that can either self-renew or differentiate into various blood lineages including the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The CMP further differentiates into the granulocyte/macrophage progenitor and the megakaryocyte-erythrocyte progenitor (MEP), which are responsible for the production of MKs

and erythroid lineages in the bone marrow (BM). MKs then undergo a unique maturation process to generate platelets. This complex process occurs in specialized niches in the BM where MKs align adjacent to vascular endothelial cells, form proplatelet projections and release platelets into the circulation (1). Because platelets are the second most abundant cell type in the blood and play pivotal roles in hemostasis and thrombosis, understanding megakaryopoiesis and platelet production has significant implications for human health. Many experiments have focused on megakaryopoiesis and platelet production, and some important results have been obtained. This review provides an overview of the development of MKs including

endomitosis, granule formation and the genesis of platelets.

### Transcriptional regulation of megakaryopoiesis

Megakaryopoiesis involves multiple extrinsic and intrinsic signals and is ultimately controlled by transcription factors. Some of the reported transcription factors that control of megakaryopoiesis include GATA-1, Friend of GATA-1 (FOG-1), GATA-2, Fli-1, PU.1, NF-E2, and RUNX1 (AML1, CBF $\alpha$ 2, or PEBP2 $\alpha$ B).

A series of experiments have shown that GATA-1 is a central regulator of MK differentiation and maturation. GATA-1 knockout MKs are delayed in their cellular maturation, exhibit marked hyperproliferation and generate fewer than normal enlarged platelets *in vivo* (2). An analysis of heterozygous female mice (which bear either an active wild-type or mutant GATA-1 allele) showed that GATA-1 was required for the terminal differentiation of definitive erythroid and megakaryocytic cells (3). Wechsler *et al.* merged the investigation of normal and malignant megakaryopoiesis by providing exciting evidence that links AMKL of Down syndrome with the loss of normal GATA-1 function (4). GATA-1 participates in the regulation of megakaryopoiesis by recruiting diverse coregulators to chromatin, which mediate transcriptional activation and repression (5). FOG-1, a multitype zinc finger protein, interacts with GATA-1 physically and stimulates or inhibits GATA-1 activity depending on the cell and promoter context, and this interaction is critical for megakaryocytic differentiation (6,7). FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1 during megakaryopoiesis (8,9), and the molecular basis of this interaction has been revealed (10).

Fli-1 is a member of the ETS family of winged helix-turn-helix transcription factors that bind a purine-rich consensus sequence, GGA (A/T). It has been shown to be an essential regulator of megakaryopoiesis (11). Fli-1 null mice embryos died at midgestation, and the MKs, both of fetal liver origin and AGM region origin, were shown to be blocked in early differentiation stage based on a colony forming assay (12,13). More in-depth studies have shown that Fli-1 is a major regulator of the late stages of megakaryocytic differentiation (14) and can work together with GATA-1 to activate the expression of genes associated with the terminal differentiation of MKs (15). Another member of the Ets family of transcription factors, Pu.1, has been shown to interact with GATA-1 physically, and Pu.1 and GATA-1 inhibit each other's functions (16). A

number of studies have suggested that changes in PU.1 concentration play a role in directing cell fate decisions during hematopoiesis. In particular, a reduction in PU.1 concentration is required for the normal development of megakaryocyte-erythroid progenitors (17).

The transcription factor NF-E2, a heterodimeric protein complex composed of p45 and small Maf family proteins, originally identified as an erythroid transcription factor, is also crucial for the proper differentiation of MKs. The enforced expression of p45-NF-E2 selectively enhances many aspects of MK differentiation, including MK maturation, proplatelet formation, and platelet release. In addition, p45 overexpression increases MK commitment during early megakaryopoiesis, while inhibiting white blood cell differentiation (18). Mice lacking p45 NF-E2 (NF-E2) exhibited profound thrombocytopenia resulting from a maturational arrest of mature MK with <5% of the number of platelets that are detectable in wild-type peripheral blood (19).

*In vitro* studies and findings in human diseases, including leukemias, myelodysplastic syndromes and familial platelet disorders with predisposition to acute myeloid leukemia (AML), suggest that Runx1 plays a pivotal role in adult hematopoiesis (20). In RUNX1-deficient BM megakaryocytic maturation was inhibited (21). The depletion of RUNX1 in UT-7/GM cells resulted in up-regulated expression of megakaryocytic markers and polyploidization; however, cell proliferation was down regulated, and the over-expression of RUNX1 decreased the activity of the megakaryocytic gene promoters. These results suggest that RUNX1 down-regulates the terminal differentiation of MKs and promotes the proliferation of megakaryocytic progenitors (22). RUNX1 interacts with other transcription factors such as GATA-1 (23,24) and Fli-1 (25). A genome-wide occupancy profile of RUNX1 was based on cell line models. The results suggested that in differentiating megakaryocytic cell lines, RUNX1 cooperates with GATA1, AP-1, and ETS to orchestrate cell-specific transcription programs through dynamic transcription factor partnerships (26).

With the advances in sequencing techniques and genomics, an increasing number of new genes are identified to participate in the regulation of megakaryopoiesis. A high-powered meta-analysis of genome-wide association studies (GWAS) in up to 66,867 individuals of European ancestry was performed, followed by extensive biological and functional assessment. By doing this, researchers identified 68 genomic loci that are reliably associated with platelet counts and volume mapping to established and putative novel

regulators of megakaryopoiesis and platelet formation (27).

### Microenvironment regulation of megakaryopoiesis

The processes of megakaryocytopoiesis and platelet production occur within a complex BM microenvironment in highly specialized osteoblastic and vascular niches, where gradients of chemokines, growth factors, calcium, oxygen and adhesive interactions regulate megakaryocytopoiesis and MK migration (11). The factors have been used to stimulate MK maturation *in vitro* and *in vivo* include human interleukin-3 (IL-3), interleukin-6 (IL-6), human stem cell factor (SCF), and thrombopoietin (TPO). The cytokine TPO is the major regulator of megakaryopoiesis. *In vitro* studies showed that TPO was important for MK maturation, after the elimination of TPO prevented the full maturation of IL-3-induced MK (28). The results showed that at a limiting dilution, TPO had direct proliferative biologic activity, but none of the other cytokines tested (SCF, IL-6, and erythropoietin) were effective, whereas IL-3 showed a mild effect (29). TPO acts by binding to a specific cell surface receptor, the cellular homologue of the myeloproliferative leukemia (Mpl) virus oncogene, leading to receptor dimerization, activation of intracellular signal transduction pathways, and target cell responses. There are numerous MPL mutations that can lead to various diseases. A total of 59 different MPL mutations were recently summarized and divided into four different groups according to the associated diseases and mutation rates (30). A recent study reported a novel activating variant of TPO receptor in human megakaryoblastic leukemia Dami cells, which may play a role in the pathogenesis of megakaryoblastic leukemia (31).

Direct evidence of the importance of the microenvironment to megakaryopoiesis was produced by Slayton *et al.* (32). They found that neonatal stem cells that produced small MKs with low DNA content in the newborn liver were capable of producing MKs that were adult-sized and had adult ploidy levels when transplanted into an adult environment. It is likely that the balance between the factors that inhibit megakaryopoiesis, such as transforming growth factor- $\beta$  and platelet factor-4, and factors that stimulate megakaryopoiesis, such as granulocyte-macrophage colony stimulating factor, TPO, IL-6, or IL-11, is tightly regulated and is different in each organ and at each stage of development; this leads to diverse microenvironments and contributes to the observed changes in MK size and ploidy during development (30).

Intriguingly, von Willebrand factor (VWF) may modulate megakaryopoiesis through a uniquely determined mechanism. VWF is an essential mediator of platelet adhesion to the vessel wall. It has long been regarded as being expressed in MKs but stored in  $\alpha$ -granules. Nurden *et al.* reported that enhanced VWF-GPIb interactions in patients with von Willebrand disease type 2B (VWD2B) modify platelet production (33). Previously, they found that cultured MKs produced self-associated and interwoven proplatelets, and immunolocalization showed that VWF was not only associated with platelets but also located on the MK surface and within internal channels (34). In addition, a recent publication now identifies VWF expression as a discriminating marker of a HSC state that is primed for platelet production in response to TPO but also subject to developmental and other, as yet undefined, cues (35). These findings suggest that VWF may have unique function in megakaryopoiesis and platelet production.

### Endomitosis and granule formation

During differentiation, MKs undergo repeated incomplete cell cycles in which mitosis is aborted in late anaphase with failure of both karyokinesis and cytokinesis, termed endomitosis (36). MKs first undergo a proliferative 2N stage in which their progression through the cell cycle is identical to other hematopoietic cells. Then, MKs undergo endomitosis and accumulate a DNA content of 4N, 8N, 16N, 32N, 64N, and even 128N in a single polylobulated nucleus before proceeding with their final maturation and subsequent proplatelet formation (37). Lordier *et al.* demonstrated that the switch from mitosis to endomitosis corresponds to a late failure of cytokinesis accompanied by a backward movement of the two daughter cells from one MK (38). In another experiment, a defect in karyokinesis was found during MK endomitosis. By analyzing the nuclear kinetics during endomitosis, Lordier *et al.* observed the presence of nucleoplasmic bridges (NPM) in most multipolar endomitosis cells from telophase until cytokinesis failure, indicating that MK endomitosis presents a karyokinesis defect; this may explain why polyploid MKs display a single polylobulated nucleus along with an increase in ploidy (39).

Small GTPase RhoA plays a multifaceted role in MK maturation. The RhoA pathway is a central player in the assembly of the contractile ring during cytokinesis. Activated RhoA regulates actin polymerization and myosin activation at the midzone through interactions

with different effectors (32). The inhibition of Rho kinase (ROCK) signaling leads to increased polyploidization in umbilical cord blood-derived MKs (40). Deleting RhoA in MKs *in vivo* resulted in significant macrothrombocytopenia. RhoA-null MKs were larger, had higher mean ploidy, and exhibited stiff membranes with micropipette aspiration (41). Consistently, guanine exchange factors GEF-H1 and ECT2, which are essential for RhoA activation during cytokinesis, must be down-regulated for Mk polyploidization (42).

Moreover, as varieties of cyclins participate in the regulation of MK endomitosis, such as D-type cyclins (43), cyclin E and cyclin A (44), some cyclin-dependent kinase inhibitors have also been found to play a role in endomitosis arrest including p19INK4D (45) and p21Cip1/Waf1 (46).

Mature platelets contain abundant stores of secretory vesicles that include dense granules, lysosomes, and  $\alpha$ -granules (50-80 per platelet), which carry endogenously synthesized or endocytosed cargo and arise from budding vesicles in the MK trans-Golgi network that mature into multivesicular bodies and nascent  $\alpha$ -granules that are transported into proplatelets (47). Platelet function is largely dependent on the cargo carried in their granules. Most studies of granule biogenesis were based on other endocrine cells rather than MKs. Coat proteins and biogenesis of lysosome-related organelle complexes are commonly accepted as vital players in granule biogenesis. Here we highlight the specific findings related to MK granule formation.

The  $\alpha$ -granules are the most abundant vesicles in platelets. Gray platelet syndrome (GPS) is an inherited bleeding disorder characterized  $\alpha$ -granule-deficient platelets (48), which makes it a good model to study  $\alpha$ -granule biogenesis in MKs specifically. Mutant Nbeal-2 is identified as the cause of GPS according to gene sequencing and knock-out mouse experiments (49-51). VPS33B, a Sec1/Munc18 protein discovered because of arthrogyryposis, renal dysfunction, and cholestasis (ARC) syndrome, has been shown to be involved in intracellular vesicle trafficking (52). Its binding protein, VPS16B, has a similar function to VPS33B, and is essential for the development of platelet  $\alpha$ -granules (53). Further research showed that the levels of VPS33B and VPS16B in Nbeal2(-/-) platelets were normal, suggesting that NBEAL2 acts independently of VPS33B/VPS16B at a later stage of  $\alpha$ -granule biogenesis (47).

Dense granules, which are members of a family of tissue-specific, lysosome-related organelles, originate from early endosomes in MKs (54). Hermansky-Pudlak syndrome (HPS), a genetically heterogeneous disorder of lysosome-

related organelle biogenesis, has been widely used to study dense granule formation in MKs. There are more than 16 spontaneous mouse models of HPS. Five of the HPS genes encode known vesicle trafficking proteins, whereas nine genes are novel and are found only in higher eukaryotes and encode members of three protein complexes termed biogenesis of lysosome-related organelles complexes (BLOCs) (55). Other researchers focused on GTPase proteins. Clear evidence shows that some GTPase proteins play critical roles in dense granule biogenesis of MKs, including Rab38 (56), Rab27a and Rab27b (57).

### Platelets production

The underlying mechanisms of platelet production have been a matter of debate for several years. However, due to recent studies both *in vitro* and *in vivo*, currently, scientists widely accept that platelets are released from MKs via the formation of proplatelets from mature MKs as an intermediate before terminal platelet production (58-60). This event occurs when mature MKs in the marrow start to form multiple long cytoplasmic extensions which originate from a single site on the MK plasma membrane; these extensions are initially thick but then elongate to produce thin and beaded forms of a proplatelet shaft that lead to complex branching structures (58). In addition, the multi-lobed nucleus remains in the mature Mk until the entire Mk cell body is transformed into proplatelets (37). An *in vitro* model of MKs derived from murine fetal liver stem cells supported the existence of the proplatelet formation process (58,61-63). Physiological evidence of proplatelet production *in vivo* has also been based on images and videos of proplatelets extending into the sinusoidal blood vessels of the BM (60,64,65). There is evidence shows that MK nucleus undergoes apoptosis and remains surrounded by a rim after platelet shedding. The nucleus is engulfed and phagocytosed by macrophages. A mutation in cytochrome C that intensifies its apoptotic activity has been reported in a form of hereditary thrombocytopenia (66). However, a recent report published in *Nature Communications* proposed raises new questions about platelet biogenesis. Using mice lacking both BAK and BAX and mice with a MK-specific deletion of Caspase-8, the authors find that MKs contain a FasL-responsive Caspase-8-mediated extrinsic apoptosis pathway that is dispensable for platelet production (67). Thus, the role of apoptosis in platelets shedding remains unclear. After the development of proplatelets, platelets can be shed from proplatelet extension directly into marrow

sinusoids, or large proplatelets can also be released into the blood stream where they form platelets by fragmentation at their bulbous ends as shown by *in vivo* imaging (60). The drivers of the platelet formation process described above have been a research hotspot for decades. Experiments have been performed to identify the factors in this complex process and have led to some insight into the factors.

There are three principal actors that are partly involved in the platelet formation; these include the invaginated membrane system (IMS), microtubules and Rho GTPases (68). As an elaborated membrane system formed during the maturation of MKs, IMS still maintains contact with the extracellular environment and serves primarily as a membrane reservoir for proplatelet formation (58,69). In addition, proplatelet formation also relies on the function of microtubules. According to Tablin's study, treatment of MKs with drugs that disassemble microtubules, such as colchicine, vincristine or nocadazole, blocks proplatelet production (70). Microtubules play a distinctive role in various stages of proplatelet production. Polymerization of tubulin molecules is initially vital; in contrast, the sliding of overlapping microtubules acts as a primary motor for proplatelet elongation (71). Some Rho GTPases were shown to play certain roles in platelet production. The Rho GTPase RhoA reported to be involved in MK maturation, which we discussed earlier, has also been suggested to regulate platelet production after MKs mature by controlling actin cytoskeletal forces (72). This argument was supported by experiments performed on a separate transgenic mouse line with MK and platelet-specific RhoA knockout, in which an obvious macrothrombocytopenia could be detected (73). Furthermore, the author also indicated that the macrothrombocytopenia observed in the mice may not be solely the cause of defective proplatelet formation but may also result from increased platelet clearance (41). On the other hand, another new study implied a completely different role of the other Rho GTPases RACGAP1 (Rac) and CDC42 whose effects, unlike the Rho GTPase RhoA, seem to include regulating microtubule stabilization and the dynamics of proplatelet formation (74).

In addition, it is accepted that MKs are located in the marrow. The microenvironment not only influences MKs maturation but also regulates platelet production. After MKs mature in the osteoblastic niche, they migrate to the sub-endothelium region near venous sinusoids where proplatelets release nascent platelets directly into the bloodstream (65). The interactions of MKs with the various adhesive proteins of the BM during this process play an

important role in regulating platelet formation (75,76). A growing body of evidence indicates that adhesion and migration on ECMs are vital aspects of the regulation of platelet formation by the BM environment. Specifically, the vascular niche is comprised of ECM proteins such as collagen type IV, fibronectin, laminin, fibrinogen, and VWF, which appears to upregulate proplatelet reorganization and platelet formation (75,76). Whereas at the level of the osteoblastic niche, collagen type I completely inhibits proplatelet formation (60). These findings suggest a fine control of the ECM-Mk receptors-cytoskeleton is required for functional platelet production. However, we still have limited knowledge of the molecular mechanisms involved in platelet production from MKs; therefore, insight into MK functions would be instrumental for developing novel methods of understanding and controlling cell maturation and migration related to platelet functions both in normal and disease scenarios (77).

Furthermore, apoptosis seems to be related to proplatelet formation. Previous studies claimed that caspase activation led to increased proplatelet formation (78). Caspase activation with nitric oxide was shown to increase the number of released proplatelets (79). Both these findings suggest the positive effects of the intrinsic apoptosis pathway on MKs to adequately form proplatelets and release platelets (80,81). However, recent studies involving knockout mice have indicated that a lack of both pro- and anti-apoptotic proteins did not affect the production of platelets and that caspases are not necessary for the generation of platelets (82). From this angle, the exact correlation between apoptosis and MK proplatelet formation still remains unclear. Finally, in the final generation of platelets from MKs, shear stress plays a decisive role in regulation. According to Dunois-Larde, compared with cultured MKs under static conditions, the counterpart exposed to shear stress exhibits a more productive proplatelet formation and better function of released platelets (83). Although the precise mechanisms of MK interaction with shear forces are not completely understood, it is possible that these forces regulate the interaction between MK glycoprotein receptors, such as GPIb/IX, and matrix proteins, such as VWF, which along with high shear rates can increase the amount of proplatelets produced from MKs compared to static culture (84).

## Conclusions

The importance of megakaryopoiesis and platelet production is clear. We summarized some typical results

offering an overview of the complicated regulation system that underlies megakaryopoiesis and platelet production and several novel studies providing new insights into this area. Our understanding of the regulation of MK maturation and platelet formation has greatly progressed but is still limited. *Ex vivo* production of platelets from stem cells is still a challenge. In addition, a better understanding of HSC lineage commitment, which is an earlier developing stage of megakaryopoiesis, is important for discoveries related to medical therapy.

Fortunately, with the advancements in genomics, we are likely to unveil the molecular basis of thrombopoiesis. Moreover, a novel technique for *in vivo* investigations, termed CLARIFY, has been established and has provided great breakthroughs (85). Together with updated imaging systems such as light-sheet fluorescence microscopy, it will be a powerful tool for observing cells' development processes and complex structure-function relationships. A combination of genome-wide sequencing and functional screening will help expand our knowledge of these processes.

## Acknowledgements

**Funding:** This work was supported by National Natural Science Foundation of China Grant 30770923 and 31371163.

## Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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doi: 10.3978/j.issn.2306-9759.2015.02.01

**Cite this article as:** Guo T, Wang X, Qu Y, Yin Y, Jing T, Zhang Q. Megakaryopoiesis and platelet production: insight into hematopoietic stem cell proliferation and differentiation. *Stem Cell Investig* 2015;2:3.