# The incredible journey: From megakaryocyte development to platelet formation

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Circulating blood platelets are specialized cells that prevent bleeding and minimize blood vessel injury. Large progenitor cells in the bone marrow called megakaryocytes (MKs) are the source of platelets. MKs release platelets through a series of fascinating cell biological events. During maturation, they become polyploid and accumulate massive amounts of protein and membrane. Then, in a cytoskeletal-driven process, they extend long branching processes, designated proplatelets, into sinusoidal blood vessels where they undergo fission to release platelets. Given the need for platelets in many pathological situations, understanding how this process occurs is an active area of research with important clinical applications.

## Introduction

Platelets are small anucleate cell fragments that have a characteristic discoid shape and range from 1 to 3 µm in diameter. Historically, platelets were referred to as cellular dust. We now know that they are indispensable for processes such as hemostasis, wound healing, angiogenesis, inflammation, and innate immunity. Platelets are formed from the cytoplasm of megakaryocytes (MKs), their precursor cells, which reside in the bone marrow (Pease, 1956). MKs are the largest (50–100 µm) and also one of the rarest cells in the bone marrow; MKs account for ∼0.01% of nucleated bone marrow cells (Nakeff and Maat, 1974). To assemble and release platelets, MKs become polyploid by endomitosis (DNA replication without cell division) and then undergo a maturation process in which the bulk of their cytoplasm is packaged into multiple long processes called proplatelets, and the nucleus is extruded. An MK may extend 10–20 proplatelets, each of which starts as a blunt protrusion that over time elongates, thins, and branches repeatedly. Platelets form selectively at the tips of proplatelets (Richardson et al., 2005). As platelets develop, they receive their granule and organelle content as streams of individual particles transported from the MK cell body (Italiano et al., 1999). Platelet formation can be arbitrarily divided into two phases: The first phase of MK maturation and development takes days to complete and requires MK-specific growth factors. During this time, massive nuclear proliferation and enlargement of the MK cytoplasm occur as the MK is filled with cytoskeletal proteins, platelet-specific granules, and sufficient membrane to complete the platelet assembly process. The second phase is relatively rapid and can be completed within hours. During this phase, MKs generate platelets by remodeling their cytoplasm first into proplatelets and then into preplatelets, which undergo subsequent fission events to generate discoid platelets. The time required for MKs to complete polyploidization, mature, and release platelets is ∼5 d in humans and 2–3 d in rodents (Ebbe and Stohlman, 1965; Odell and Jackson, 1968; Odell et al., 1970). Once released into the bloodstream, human platelets survive 7–10 d, whereas rodent platelets survive 4–5 d (Aster, 1967; Harker and Finch, 1969; Jackson and Edwards, 1977).

In this review, we outline the process of platelet production—starting with MK development and ending with terminal platelet formation (illustrated in Fig. 1). After a brief history and context of each step, we highlight some exciting recent findings and important unanswered questions pertinent to the cell biology of platelet formation.

## MK maturation and development

MKs develop from hematopoietic stem cells (HSCs) that reside mainly in the bone marrow but are also present in the yolk sac, fetal liver, and spleen during early development (Long et al., 1982; Gordon et al., 1990; Ogawa, 1993; Morita et al., 2011). During maturation, MKs increase in size, become full of platelet-specific granules, expand their cytoplasmic content of cytoskeletal proteins, and develop a highly tortuous invaginated membrane system (IMS; Behnke, 1968; Fig. 2).

**Thrombopoietin (TPO) directs MK development.** The discovery of TPO, and its MK-specific receptor c-Mpl, revolutionized the field of MK and platelet biology.

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Abbreviations used in this paper: HSC, hematopoietic stem cell; IMS, invaginated membrane system; ITP, idiopathic thrombocytopenic purpura; MK, megakaryocyte; TPO, thrombopoietin.



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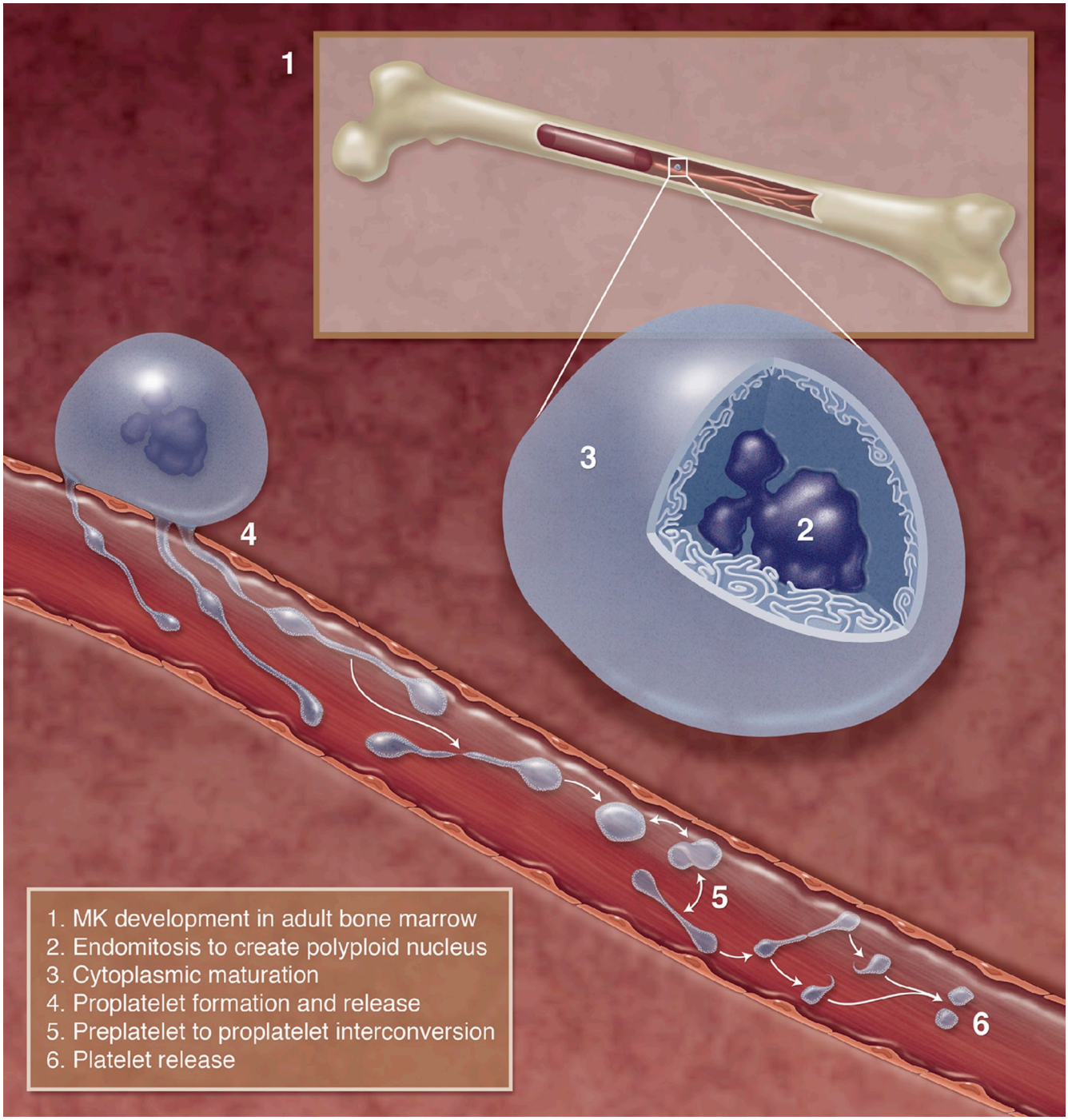


Figure 1. **Schematic of platelet production.** (1) HSCs in the bone marrow differentiate into MKs in a TPO-dependent manner. (2) MKs undergo endomitosis and develop nuclei ranging in DNA content from 2n to 128n. (3) As MKs mature, they develop a highly invaginated membrane throughout their cytoplasm, which is continuous with the external plasma membrane. This membrane serves as a reservoir for proplatelet formation. (4) MKs migrate to the vascular niche, where they extend proplatelets and release them into vascular sinusoids. The entire MK is converted into pre/proplatelets, and its nucleus is exuded and phagocytosed. (5) Once in the bloodstream, proplatelets interconvert into preplatelets. (6) A fission event creates two platelets from a barbell proplatelet.

TPO functions as the major regulator that promotes the growth and development of MKs from their HSC precursors (Bartley et al., 1994; de Sauvage et al., 1994; Kaushansky, 1994; Kaushansky et al., 1994; Kuter et al., 1994; Lok et al., 1994; Sohma et al., 1994; Wendling et al., 1994). Subsequently, this discovery facilitated development of in vitro cell culture systems that reconstitute MK differentiation, maturation, proplatelet extension, and platelet production and allowed study of the mechanisms that regulate these processes (Choi et al., 1995; Cramer et al., 1997; Lecine et al., 1998). Interestingly, although TPO is one key driver of MK differentiation, mice lacking either c-Mpl or TPO successfully produce platelets, indicating a role for other regulators in the process of proplatelet and platelet formation (Choi et al., 1995; Ito et al., 1996).

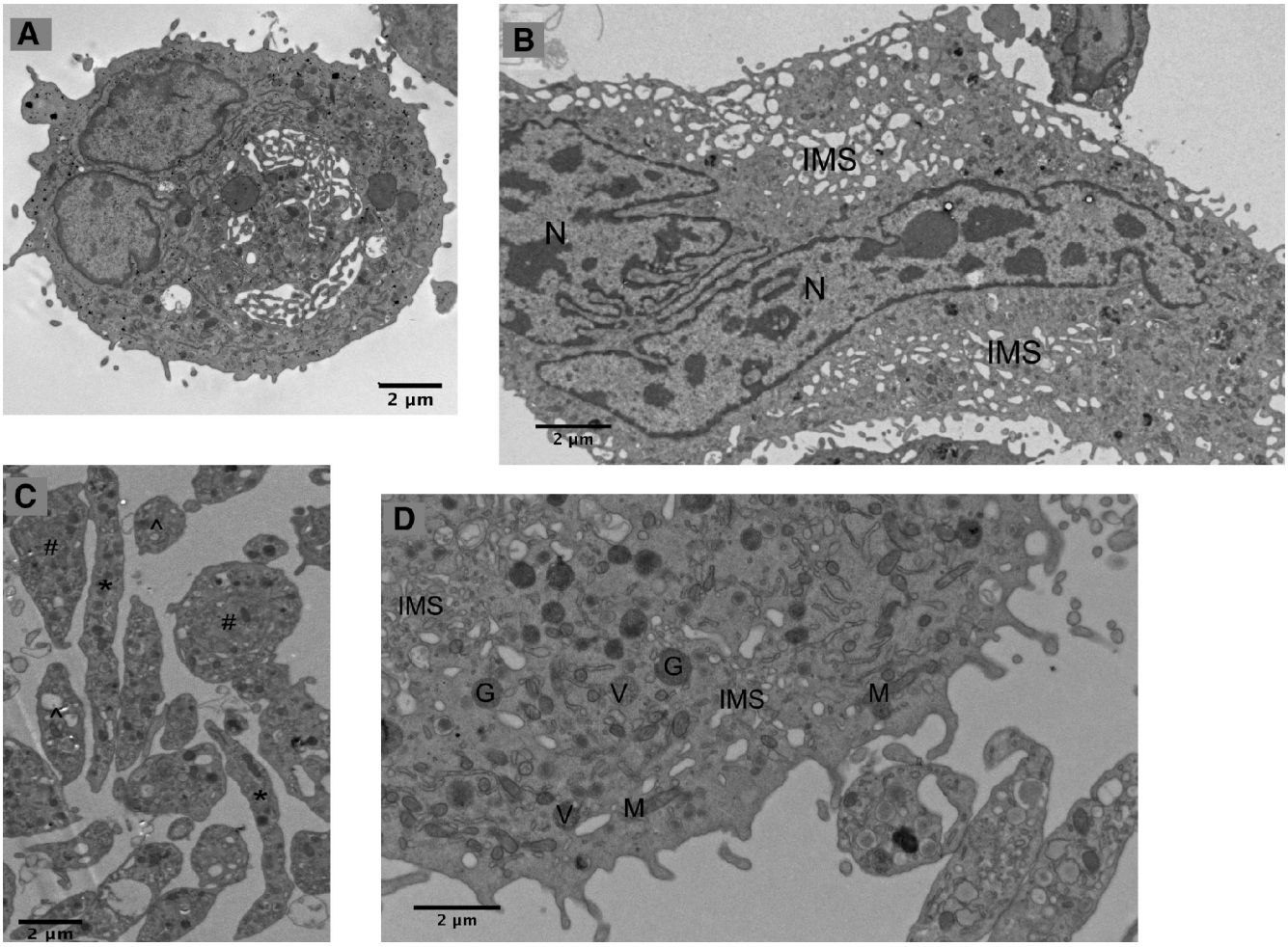


Figure 2. **Transmission electron micrographs of murine MKs, preplatelets, proplatelets, and platelets.** MK cultures generated from murine fetal liver cells were fixed with 1.25% paraformaldehyde, 0.03% picric acid, and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, postfixed with 1% osmium tetroxide, dehydrated through a series of alcohols, infiltrated with propylene oxide, and embedded in epoxy resin. Ultrathin sections were stained and examined with an electron microscope (Tecnai G2 Spirit BioTWIN; FEI Company) at an accelerating voltage of 80 kV. Images were recorded with a charge-coupled device camera (2K; Advanced Microscopy Techniques) using digital acquisition and analysis software. (A) Overview of one MK showing multilobulated nucleus and IMS. (B) MK with a highly developed IMS. (C) Released preplatelets (#), proplatelets (*), and platelets (^). (D) Detailed view of platelets (bottom right) and an MK, highlighting its contents. N, nucleus; IMS, invaginated membrane system; G, granule; M, mitochondria; V, multivesicular body.

**Endomitosis.** Endomitosis (Fig. 1, step 2) is a primarily TPO-driven process by which MKs become polyploid through cycles of DNA replication without cell division (cytokinesis; Ebbe, 1976; Gurney et al., 1994). The study of endomitosis was largely facilitated by the implementation of in vitro culture systems that used TPO. During their life cycle, MKs first undergo a proliferative 2n stage in which their progression through the cell cycle is identical to other hematopoietic cells. Subsequently, MKs begin 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 proplatelet formation (Zimmet and Ravid, 2000). Studies in megakaryocytic-transformed cell lines showed that the switch to polyploidization is related to the cell cycle and dependent on degradation of cyclin B and reduced activity of the cyclin B–dependent Cdc2 kinase (Datta et al., 1996; García and Calés, 1996; Zhang et al., 1996, 1998). Subsequent studies with primary MKs revealed that endomitosis occurs because of a defect in late cytokinesis that results in incomplete formation of the cleavage furrow, a contractile ring consisting of myosin II and F-actin that generates the mechanical forces necessary for cell separation (Geddis et al., 2007; Lordier et al., 2008). Specifically, down-regulation of nonmuscle MYH10 (myosin IIB heavy chain) in the contractile ring by RUNX1 (runt-related transcription factor 1) is required for the switch from mitosis (2n) to endomitosis (4n; Lordier et al., 2012). The importance of cleavage furrow inhibition in polyploidization was further underscored by examining the mechanism by which it forms. RhoA, a small GTPase that regulates the actin cytoskeleton, is required to generate the contraction force necessary to complete cytokinesis (Melendez et al., 2011). The microtubule-associated GEF-H1 activates RhoA at the cleavage furrow (Birkenfeld et al., 2007), whereas ECT2 (epithelial cell–transforming sequence 2) is involved in RhoA localization and activation (Petronczki et al., 2007). Gao et al. (2012) recently revealed that both GEF-H1 and ECT2 are down-regulated at the mRNA and protein levels during MK polyploidization. Interestingly, GEF-H1 down-regulation is required for 2n cells to become 4n,

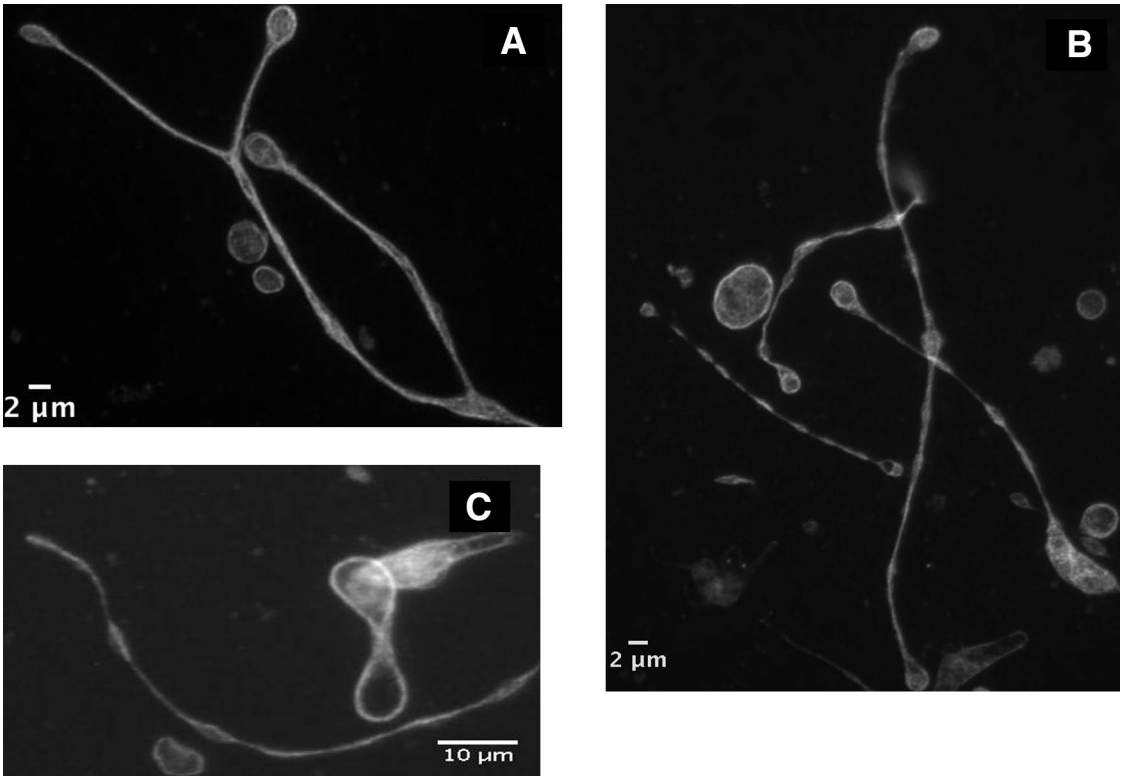


Figure 3. **Microtubules in proplatelets and platelets.** Microtubules composed of β1-tubulin line the shafts of proplatelet extensions and form a microtubule coil in nascent platelet tips and released preplatelets/platelets. To delineate the microtubule cytoskeleton, murine fetal liver MK-generated samples were incubated with a rabbit polyclonal primary antibody for β1-tubulin, washed, and probed with a secondary Alexa Fluor 488 nm–conjugated antibody. MKs were imaged on a microscope (Eclipse TE2000-E; Nikon) equipped with a 63× objective, NA 1.4, and 1.5× optivar. Images were acquired with a charge-coupled device camera (ORCA-II-ER; Hamamatsu Photonics). Image acquisition was under the control of MetaMorph software (Molecular Devices). (A–C) Images highlight the branching of proplatelets (A), heterogeneous mix of platelets, pre- and proplatelets released from MKs (B), and the figure 8 structure seen in preplatelet to proplatelet interconversion (C).

whereas ECT2 down-regulation is required for polyploidization beyond 4n. This suggests that different mechanisms may be regulating the initial 2n to 4n transition versus subsequent endomitotic events (Papadantonakis et al., 2008; Gao et al., 2012).

These studies do not, however, preclude a role for the cell cycle in endomitosis. Although inhibition of cleavage furrow formation physically prevents the MK from dividing, there still may be a separate process that regulates the repeated cycles of DNA replication. Multiple studies have identified roles for G1/S-phase regulators, such as the cyclins D and E, supporting the hypothesis that up-regulation of G1-phase components may be important in promoting cycles of endomitotic DNA synthesis to allow for the development of high ploidy MKs (Zimmet et al., 1997; Muntean et al., 2007; Eliades et al., 2010).

It is theorized that MKs are polyploid to produce the large quantities of mRNA and protein necessary to be packaged into platelets while still retaining their ability to perform multiple functions without the stress of mitosis and cytokinesis (Zimmet and Ravid, 2000). Although endomitosis and polyploidization are undoubtedly important for MK cytoplasmic maturation, the relationship between high nuclear DNA content and efficient platelet formation is still highly debated. For example, nicotinamide, a form of vitamin B3, is commonly used to increase the ploidy of human and mouse MKs in culture (Giammona et al., 2006; Panuganti et al., 2010; Avanzi et al., 2012; Leysi-Derilou et al., 2012). Although nicotinamide does increase ploidy, its effect on platelet formation is controversial. Some observed nicotinamide-induced increased proplatelet production in vitro (Giammona et al., 2006), whereas others saw reduced proplatelet formation (Giammona et al., 2006; Leysi-Derilou et al., 2012). Importantly, administration of nicotinamide to mice does not increase their platelet levels (Konieczna et al., 2013). In addition, overexpression of cell cycle regulators, such as cyclin D3, has been shown to enhance MK ploidy in vivo without any concomitant increase in platelet count (Zimmet et al., 1997). As we learn more about the process of endomitosis and how to manipulate it, we will be better able to reveal whether high nuclear content is causative or correlative to platelet formation.

**The IMS: A membrane reservoir.** One purpose of endomitosis is to generate the large quantity of protein and lipid synthesis necessary to create the IMS (previously referred to as the demarcation membrane system). The IMS (Fig. 1, step 3) is an extensive complex of cisternae and tubules distributed throughout the MK cytoplasm that is continuous with the plasma membrane and is thought to exist as a membrane reservoir for proplatelet formation (Yamada, 1957; Radley and Haller, 1982). Schulze et al. (2006) confirmed this hypothesis by demonstrating that the IMS is indeed the origin of the proplatelet and platelet surface. Furthermore, the force necessary for internal IMS migration relies on actin filament assembly via the WASP–WAVE

pathway at the IMS cytoplasmic face in response to phosphatidylinositol 4,5-bisphosphate signaling (Schulze et al., 2006).

Not surprisingly, the IMS also requires cytoskeletal support. Spectrin, a protein that forms the plasma membrane skeleton in many cell types, forms a 2D lattice in MKs that underlies and stabilizes the IMS; MKs expressing a dominant-negative spectrin peptide have an underdeveloped IMS with insufficient membrane to form proplatelets (Patel-Hett et al., 2011). Thus, a developed and mature IMS aided by the spectrin membrane skeleton helps establish and maintain proplatelets during platelet biogenesis (Patel-Hett et al., 2011). Although evidence suggests that the IMS functions as a membrane reservoir for proplatelet elongation, there are still several questions that need to be addressed. For example, IMS continuity with the plasma membrane in mature MKs may suggest that plasma membrane invagination is involved in IMS formation. In addition, other internal pools of membrane, such as the endoplasmic reticulum or Golgi, may contribute to IMS development and/or initiate this process.

## Terminal MK development and proplatelet formation

Mature MKs extend long branching processes called proplatelets into the sinusoidal blood vessels of the bone marrow. Proplatelets, which function as the assembly lines of platelet production, are comprised of platelet-sized swellings in tandem arrays that are connected by thin cytoplasmic bridges (Fig. 1, step 4; Italiano et al., 1999). Proplatelet production has been observed in vivo by imaging proplatelets extending into the sinusoidal blood vessels of bone marrow (Behnke, 1969; Becker and De Bruyn, 1976; Junt et al., 2007; Zhang et al., 2012a). Spontaneous proplatelet formation also occurs in vitro with MKs derived from murine fetal liver stem cells (Italiano et al., 1999; Patel et al., 2005a; Thon et al., 2010, 2012a) and human cell–derived MKs (Choi et al., 1995; Miyazaki et al., 2000; Chang et al., 2007; Dunois-Lardé et al., 2009). Currently, we have an insufficient understanding of the signals that trigger proplatelet formation in MKs, resulting in a significant gap in our knowledge of platelet production.

**The cytoskeleton powers proplatelet extension.** Accumulating evidence suggests that the cytoskeleton is the principal machinery for platelet production (Tablin et al., 1990; Hartwig and Italiano, 2006; Thon et al., 2010). This is not unexpected, considering the massive reorganization a mature, round MK goes through to extend proplatelets and release them into sinusoidal blood vessels. β1-Tubulin is the main tubulin isoform in MKs; its reorganization is essential for proplatelet formation and powers proplatelet elongation using cytoplasmic dynein, a microtubule minus end–associated motor protein (Fig. 3; Lecine et al., 2000; Patel et al., 2005b). Mice lacking β1-tubulin produce 60% fewer platelets, and the platelets that are produced show structural and functional defects, including reduced microtubule content and fewer microtubule coilings around the platelet periphery (Schwer et al., 2001). In humans, mutations in β1-tubulin result in an autosomal dominant macrothrombocytopenia (Kunishima et al., 2009).

Likewise, F-actin is present throughout proplatelets and forms the assembly points required for them to bend and bifurcate (Italiano et al., 1999; Patel et al., 2005b). Consistent with these data, mutations in the *MYH9* gene that reduce myosin II activity have been implicated in the May-Hegglin anomaly and related macrothrombocytopenias (Kelley et al., 2000; Seri et al., 2000). More recently, *MYH9* mutations have been associated with premature initiation of proplatelet formation within the bone marrow. This is mediated by disruption in the Rho–Rho kinase–myosin-IIA pathway and leads to decreased numbers of circulating platelets (Chang et al., 2007; Chen et al., 2007). These observations are supported by mouse models showing that mutations in the *MYH9* gene cause both cultured MKs and those in the bone marrow to produce fewer and shorter proplatelets with less branching (Zhang et al., 2012b). Recently, Bluteau et al. (2012) further highlighted the importance of myosin regulation by showing that RUNX1 can affect proplatelet formation by direct regulation of *MYL9*, *MYH10*, and *MYH9*. This study may explain one reason why patients with familial platelet disorder with predisposition to acute myelogenous leukemia, a familial platelet disorder characterized by germline heterozygous RUNX1 alterations, present with thrombocytopenia.

Although these studies suggest that regulation of the cytoskeleton is necessary for efficient proplatelet formation, their focus is largely on deficiencies or mutations that diminish proplatelet formation. In the future, it will be important to investigate whether direct manipulation of the cytoskeleton can augment or even initiate the process of proplatelet formation directly.

**The cytoskeleton highway connects the MK body to budding platelets.** In addition to playing an essential role in proplatelet elongation, microtubules lining the shafts of proplatelets serve a second function: transportation of organelles and granules into proplatelets and assembly of platelets at proplatelet ends. In MKs, granules are derived from budding of small vesicles containing granule cargo from the trans-Golgi network (Blair and Flaumenhaft, 2009). Vesicles budding from the trans-Golgi network may be delivered directly to multivesicular bodies, where proteins are sorted and eventually packaged into granules (Heijnen et al., 1998; Youssefian and Cramer, 2000). Organelles and granules are then sent individually from the MK cell body into the proplatelets, where they move bidirectionally until they are captured at proplatelet tips (Fig. 4; Richardson et al., 2005). Interestingly, this process bears striking resemblance to neuronal cells in which axons can reach millimeters in length and also need to transport organelles long distances. In MKs, immunofluorescence and electron microscopic experiments indicate that organelles are intimately associated with microtubules, and actin drugs do not affect organelle motion (Richardson et al., 2005). Thus, movement appears to be microtubule based (Richardson et al., 2005). The bipolar arrangement of microtubules within the proplatelet contributes to bidirectional organelle movement. The plus end–directed microtubule motor kinesin localizes in a pattern similar to organelles and granules, implicating this motor in transport of organelles along microtubules. There appears to be two aspects of this process: first, organelles and granules travel along microtubules, and second, the microtubules themselves slide bidirectionally in relation to other motile filaments, indirectly moving organelles along proplatelets (Richardson et al., 2005). Although microtubule highways appear to transport granules and organelles long distances along proplatelets, the mechanism by which

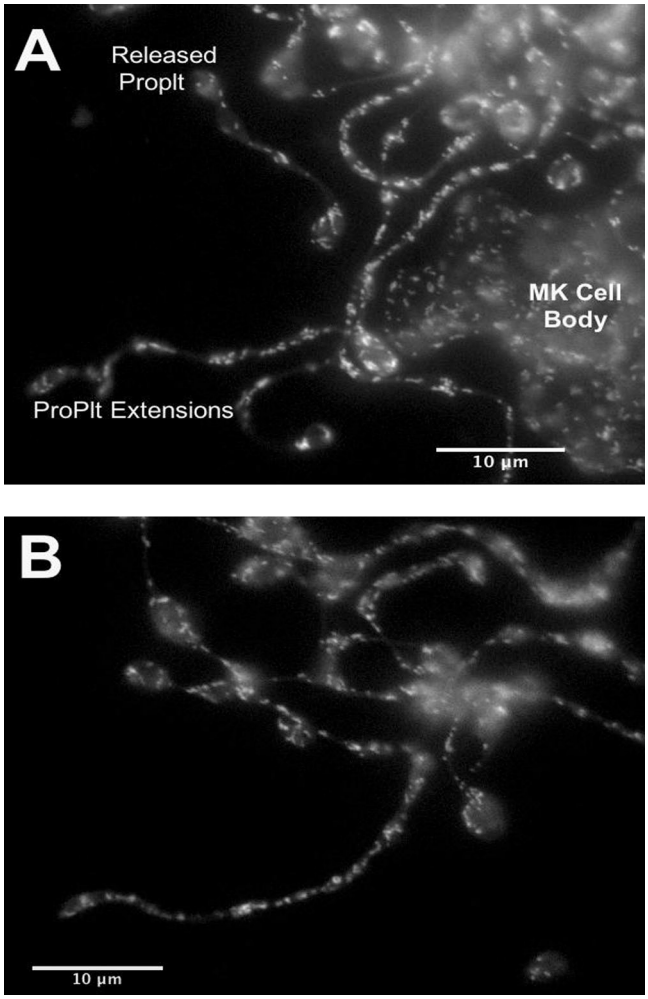


Figure 4. **Trafficking of α-granules.** Granules are packaged in MKs, trafficked along microtubules lining proplatelet (PropIt) shafts, and captured in nascent platelet tips. To visualize α-granules, murine fetal liver–derived MKs were incubated overnight with 150 µg/ml Oregon green 488 human fibrinogen conjugate, which they take up and package into their α-granules. MKs were then washed by albumin gradient sedimentation, and the resuspended pellet was placed in a video chamber. MKs were imaged on a microscope (Nikon) equipped with a 100× objective, NA 1.4, and were acquired with a charge-coupled device camera (ORCA-II-ER). Image acquisition was under the control of MetaMorph software. (A and B) Images visualize MKs actively releasing proplatelets (A) and released proplatelets (B).

the necessary organelles and granules are packaged into platelets remains unclear. In addition, the idea that granules may be sorted into heterogeneous populations in platelets remains controversial (Italiano et al., 2008; Kamykowski et al., 2011; Jonnalagadda et al., 2012). Furthermore, recent studies have identified heterogeneity in α-granule morphology and membrane protein composition as well as granule motility during platelet activation (van Nispen tot Pannerden et al., 2010; Peters et al., 2012). In the future, studies addressing this question may help us better understand the many unique roles platelets and their cargo play in health and disease.

## The role of the microenvironment in proplatelet formation

MKs cultured in vitro can form proplatelets in suspension, suggesting that direct interaction with the bone marrow environment is not required for platelet production. Nevertheless, the efficiency of platelet production in culture is decreased relative to that observed in vivo, suggesting that the bone marrow microenvironment plays an important role in stimulating and enhancing proplatelet formation and platelet release.

**The osteoblastic niche.** It has been hypothesized that the dynamic interaction of MKs with the different extracellular matrix proteins in the bone marrow compartmentalizes their maturation to specific sites. This is important to allow MKs to first develop from HSCs and then migrate to the vascular niche before beginning the process of proplatelet formation. Collagen I is the most abundant component of the osteoblastic niche (Reddi et al., 1977). Interestingly, binding of MKs to collagen through α2β1-integrin inhibits proplatelet formation (Sabri et al., 2004, 2006; Zou et al., 2009). This suggests that under normal physiological conditions, the osteoblastic niche inhibits proplatelet formation (Arai and Suda, 2007; Pallotta et al., 2009). Recently, an in vitro model to study MK function in the bone marrow environment was developed by differentiating human mesenchymal stem cells into osteoblasts and then co-culturing osteoblasts with HSCs and MKs (Pallotta et al., 2009). Using this model, Pallotta et al. (2009) found that HSCs form a niche that leads to collagen I deposition, creating an environment conducive for HSCs to differentiate through the megakaryocytic lineage but not to complete maturation and extend proplatelets. This supports the model that engagement of collagen I in the osteoblastic niche acts to suppress proplatelet formation while allowing for MK differentiation and maturation.

**The vascular niche.** Polyploid MKs localize to sinusoidal bone marrow endothelial cells in vivo, where they form proplatelets that migrate through bone marrow endothelial cells, and release platelets directly into the marrow intravascular sinusoidal space (Tavassoli and Aoki, 1989). Recent evidence suggests that up-regulation of the cytokine SDF-1 and its receptor CXCR4 may be important for the migration of MKs to the vascular niche (Avecilla et al., 2004; Pitchford et al., 2012). The vascular niche is comprised of extracellular matrix proteins, such as collagen IV, fibronectin, fibrinogen, and von Willebrand factor, which in conjunction with chemokine-mediated interaction of progenitors allow MKs to relocate to a microenvironment that is both permissive and instructive for the late stages of MK maturation and proplatelet formation (Avecilla et al., 2004).

Fibrinogen is a commonly studied extracellular matrix protein in bone marrow sinusoids that enhances proplatelet formation. MKs from bone marrow aspirates form significantly more proplatelets when plated on fibrinogen (Larson and Watson, 2006). The mechanism for this augmentation is thought to be through fibrinogen binding to the MK integrin $\alpha_{IIb}\beta_3$ (Eto et al., 2002; Larson and Watson, 2006). Although $\alpha_{IIb}$ is expressed in MK progenitor cells, and thus very early in MK development, its role throughout MK maturation remains controversial (Berridge et al., 1985; Prandini et al., 1996). A study by Eto et al. (2002) suggests that $\alpha_{IIb}\beta_3$ only exhibits agonist-induced fibrinogen binding in mature MKs. Aside from its role in binding fibrinogen, the integrin $\alpha_{IIb}\beta_3$ in itself may be important for proplatelet release; a study shows that antibodies directed against the integrin subunit $\alpha_{IIb}$ inhibit proplatelet formation when added to

cultured MKs (Takahashi et al., 1999). Interestingly, patients with Glanzmann's thrombasthenia, a disease characterized by either absent or nonfunctioning $\alpha_{IIb}\beta_3$, still have a normal level of circulating platelets (Caen et al., 1966). Therefore, $\alpha_{IIb}\beta_3$ may enhance but is not necessary for proplatelet formation.

In contrast, patients with Bernard-Soulier syndrome display thrombocytopenia with giant platelets in addition to functional defects like defective platelet adhesion to subendothelium and reduced platelet aggregation (Ware et al., 1993). Bernard-Soulier syndrome is linked to genetic lesions of the platelet membrane glycoprotein complex GPIb–IX–V, which contains the binding site for von Willebrand factor, a plasma glycoprotein important for platelet adhesion to the endothelium (Geddis and Kaushansky, 2004). Therefore, it appears that GPIb–IX–V may be necessary for efficient platelet formation. In fact, in vitro studies have shown that antibodies against GPIb–IX–V strongly inhibit proplatelet production and that MKs derived from patients with Bernard-Soulier syndrome do not extend proplatelets in vitro (Takahashi et al., 1999; Balduini et al., 2011). These studies suggest that one mechanism by which GPIb–IX–V mutations cause macrothrombocytopenia in patients is through defective proplatelet formation.

Fibronectin is another abundant protein in the hematopoietic microenvironment and is a proliferative stimulus for HSCs (Weinstein et al., 1989; Vuillet-Gaugler et al., 1990). Specifically, it plays an important role in megakaryocytopoiesis, proliferation, and differentiation through adhesion to fibronectin receptors VLA-4 (very late antigen 4) and VLA-5 (Han et al., 2004; Fox and Kaushansky, 2005). Recently, the role of these receptors in proplatelet formation was examined by Matsunaga et al. (2012), who found that fibronectin-activated VLA-4 and VLA-5 may contribute to proplatelet formation through enhanced activation of ERK1/2. Although preliminary, these data are the first to suggest a mechanism by which fibronectin augments proplatelet formation. In sum, these studies suggest a model in which the osteoblastic niche provides an environment that allows MKs to mature and develop, whereas the vascular niche enhances proplatelet formation.

## How proplatelets find their way into sinusoidal blood vessels

**Directed release of proplatelets by MKs.** In addition to functioning as the assembly lines for platelet production, the architecture and morphology of proplatelets provide a mechanism to deliver platelets to the bloodstream. Observations of MKs releasing proplatelets in vivo have led to the notion that there is directional release of proplatelets from MKs. It is only recently, however, that studies have begun to elucidate how MKs do this. Although MK receptor engagement of extracellular matrix proteins in the vascular niche is important, another less-studied interaction involves exposure of MKs and/or newly generated proplatelet extensions to blood components. Because of their unique position at the vascular interface, MKs are effectively exposed to a transendothelial gradient of blood components. Recently, an elegant study by Zhang et al. (2012a) exploited this phenomenon and identified S1P (sphingosine 1 phosphate) and its receptor on MKs, S1pr1, as important mediators of proplatelet extension and release. Once in the blood, proplatelets are exposed to a high S1P concentration, which initiates the subsequent shedding of platelets into the circulation. Using S1pr1 knockout mice and multiphoton intravital microscopy, they showed that the S1P gradient guides proplatelet extensions into the lumen of the bone marrow sinusoids and that mice lacking S1pr1 develop severe thrombocytopenia caused by both formation of extravascular proplatelets and defective proplatelet release inside the vascular space. Importantly and perhaps most exciting is that activation of S1pr1 signaling directly stimulated the release of new platelets; this is mediated through S1pr1, which triggers activation of the Gi/Rac GTPase signaling cascade. Therefore, this study identifies S1P and its receptor S1pr1 as important mediators of directional proplatelet elongation and terminal shedding of new platelets into the blood stream. The implications of this study are far reaching and open the door to many interesting questions. For example, as proplatelets extend into the lumen, could they also function to monitor circulating levels of proteins, such as TPO, or even platelet numbers? This would allow the MK to receive information and instruct the MK in processes such as protein translation, granule packaging, and platelet production. Understanding the impact of blood components on proplatelet formation is an exciting field for future work.

**Proplatelet extension into the vascular space.** Once the lumen is "sensed" by the MK, how are the proplatelets able to break through into the vascular space? In another recent study, it was revealed that podosomes, cylindrical actin-rich structures found on the outer surface of the plasma membrane, actively degrade the extracellular matrix and are therefore important for MKs to extend proplatelet protrusions across the basement membrane (Schachtner et al., 2013). This study showed that the mechanism by which MKs form podosomes is through actin polymerization via the Arp2/3 complex and WASP to create an F-actin core, which is then surrounded by rings of vinculin. Once assembled, podosomes degrade matrix proteins, such as fibrinogen, in a matrix metalloproteinase- and myosin-IIA–dependent manner. This study is the first to examine the role of podosomes in MKs and suggests that they may play a role in effective delivery of platelets into the bloodstream during proplatelet formation.

Similarly, the impact of blood shear forces on proplatelet formation is an emerging trend in the field of MK and platelet biology. In a pivotal study, Junt et al. (2007) observed proplatelet formation in real time in the bone marrow of mice by multiphoton intravital microscopy. Their observations in these live-cell experiments uphold the hypothesis that blood flow–induced shear stress helps separate proplatelet fragments from the MK cell body. This was supported by an in vitro model in which cultured MKs shed significantly more proplatelets when they were agitated compared with MKs in static cultures (Junt et al., 2007). In a complementary study, the role of shear on platelet release from human cord blood MKs adhered to a von Willebrand factor matrix was examined (Dunois-Lardé et al., 2009). During exposure to high shear rates (1,800 $s^{-1}$), cytoplasmic MKs produced extensions organized along the direction of flow in a manner dependent on both microtubules and the GPIb receptor (Dunois-Lardé et al., 2009). Together, these

results support the idea that intravascular release of fragments protruding from mature MKs is aided by fluid shear forces in bone marrow sinusoids. However, the majority of these studies have been performed using in vitro assays examining platelet rolling and adhesion in an open system. The development of microfluidic chips that recapitulate the bone marrow and vascular compartments will likely provide new insights into how shear influences proplatelet production and release.

### Terminal platelet formation and release

Once proplatelets are extended into the blood stream, what happens? Do small, platelet-sized objects or larger fragments get released into circulation? In light of recent work, it appears that MKs release a heterogeneous mix into the blood, indicating that terminal platelet formation may continue in the blood stream. The presence of proplatelet-like structures in blood has been long recognized, and it is therefore likely that proplatelets routinely fragment from the MK body, enter the blood, and mature into platelets while in circulation. Schwertz et al. (2010) revealed that platelets are capable of producing progeny; platelets produce "figure 8" barbell-shaped structures with two platelet-sized bulbs on each end that contain their own organelles and cytoskeletal system (illustrated in Fig. 3 C). This duplication occurs in vitro over a few hours, is dependent on an intact microtubular network, and is associated with increased protein synthesis. Subsequently, Thon et al. (2010) identified a new stage in platelet formation present in both cultured MKs and peripheral blood smears, the preplatelet. Preplatelets are anucleate discoid particles that are 2–10 µm in diameter that can reversibly convert into barbell-shaped proplatelets (Fig. 1, step 4). As in the study by Schwertz et al. (2010), Thon et al. (2012a) found that preplatelets are able to mature into platelets both in vitro and after transfusion into mice in vivo. Additionally, it was uncovered that this is a microtubule-mediated process; bidirectional polymerization of microtubules at each end of the barbell proplatelet forms two platelet-sized (2-µm diameter) microtubule coils at each end, which split into two individual platelets after an abscission event (Fig. 1, step 5; Thon et al., 2010).

**Regulation of terminal platelet size.** The conversion from pre- to proplatelet is driven by microtubule-based forces, which are governed by two major biophysical properties: microtubule coil diameter and microtubule coil thickness (Thon et al., 2012b). Interestingly, these forces both regulate and predict the size of circulating platelets generated by proplatelets, providing an explanation for the ∼2-µm diameter of platelets (Thon et al., 2012b). This supports a model in which circular preplatelets are released into the blood, rapidly and spontaneously convert into barbell proplatelets, and undergo fast rounds of abscission that result in mature platelets. Alternatively, preplatelets may become trapped in the microcapillaries of the bone marrow, lung, or spleen where intravascular shear forces drive proplatelet to platelet production. A study of higher platelet counts in postpulmonary vessels suggests that the lung may be a site of terminal platelet formation (Howell and Donahue, 1937). In addition, a study using rat models reveals that lung damage may reduce circulating platelets, suggesting that the lungs play an active role in the regulation of platelet formation (Xiao et al., 2006).

Platelet size correlates with platelet reactivity; larger platelets have greater prothrombotic potential. Elevated platelet size (mean platelet volume) is associated with increased platelet aggregation, increased expression of adhesion molecules, and elevated risk of cardiovascular and peripheral arterial diseases (Bath and Butterworth, 1996; Kamath et al., 2001; Berger et al., 2010; Chu et al., 2010; Slavka et al., 2011). Interestingly, a study of patients with acute coronary disease found a direct association between $\alpha_2$-integrin chain expression and mean platelet volume, suggesting that expression levels of integrin $\alpha_2\beta_1$ may be involved in the regulation of platelet size (Kunicki et al., 2012). Recently, this was supported by the creation of conditional MK-specific $\alpha_2$-integrin chain (*Itga2*$^{-/-}$)–deficient mice in which the resulting platelets have a significantly decreased mean platelet volume (Habart et al., 2013).

These observations begin to explain platelet size under normal, physiological conditions and also genetic variations that may result in macrothrombocytopenia. However, there is still much to be revealed about what regulates platelet size. A recent paper addressed this issue in a novel way; Gieger et al. (2011) conducted a high-powered meta-analysis of genome-wide association studies in >66,000 individuals. From this genome-wide association study, 68 genomic loci associated with platelet volume were identified, including both previously studied and novel regulators of platelet formation. Studies such as this pave the way for future research into genes that regulate platelet production, the results of which will be integral in understanding what molecular pathways regulate both platelet formation and size.

### Translating MK cell biology into medicine

Platelets are essential for hemostasis, and thrombocytopenia (platelet counts < $150 \times 10^9$/liter) is a major clinical problem encountered across several conditions, including idiopathic thrombocytopenic purpura (ITP), myelodysplastic syndromes, chemotherapy, aplastic anemia, human immunodeficiency virus infection, complications during pregnancy, and surgery. It is estimated that ∼1.5 million platelet transfusions to prevent severe bleeding are administered yearly, with each transfusion costing over $600 (Kaushansky, 2008). Therefore, because of the strong effect of TPO on platelet production, clinical trials evaluating the use of recombinant TPO to treat thrombocytopenia began in 1995 (Kuter and Begley, 2002). Unfortunately, some patients treated with recombinant TPO developed antibodies that created a paradoxical decrease in platelets. This led to the creation of TPO mimetics, such as romiplostim and eltrombopag, which are highly effective in raising the platelet count in ITP (Li et al., 2001). Although effective in treatment of ITP and other chronic conditions, TPO mimetics take 5 d to increase platelet counts and 12 d to reach maximum effect, making them less useful in acute situations (Kuter, 2010). In addition, one serious side effect of current TPO mimetics is development of bone marrow myelofibrosis (Kuter et al., 2009). Therefore, it is obvious that other alternatives (in addition to platelet transfusions) are necessary to instantaneously elevate platelet counts in situations such as surgery, sepsis, trauma, or disseminated intravascular coagulation.

The ability to make platelets from cultured MKs would be an extremely valuable clinical tool. Because of this, several

groups have begun to create in vitro MK cultures derived from either embryonic stem cells or induced pluripotent stem cells that have the potential to create a continuous supply of platelets for infusion. The idea being that creation of MKs from an immortalized progenitor cell will eliminate the need for platelet or embryonic/induced pluripotent stem cell donation. The first group to differentiate human embryonic stem cells into MKs created a co-culture system with human embryonic stem cells and stromal cells (Gaur et al., 2006). These MKs contain von Willebrand factor in their cytoplasm, express CD41a and CD42b on their surface, and have a DNA ploidy distribution consistent with that of human peripheral blood $CD34^+$ cells differentiated into MKs (Gaur et al., 2006). In the years that followed, the field began focusing on specific ways to stimulate cell lines to become induced pluripotent stem cells. In a breakthrough study, Takahashi et al. (2007) showed that human-induced pluripotent stem cells can be generated from human fibroblasts and other somatic cells by retroviral transduction of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc. Takayama et al. (2008, 2010) extended these findings to MKs by differentiating the induced pluripotent stem cells generated from human dermal fibroblasts directly into MKs, subsequently revealing that reduction of c-Myc expression is required for MK maturation and generation of functional platelets. Recently, this technology was also used in dogs: canine dermal fibroblasts were used to generate canine induced pluripotent stem cells, which were then differentiated into MKs that produced functional platelets in vivo (Nishimura et al., 2013).

In order for in vitro culture systems to be useful, however, they must create a large number of platelets because one effective platelet transfusion has $\sim 1–5 \times 10^{11}$ platelets (Cid and Lozano, 2010). The need for this large number of platelets is because platelets are made and consumed very rapidly in the body; $\sim 10^{11}$ platelets are made every day and have a life span of only 7–10 d (Harker, 1977). The process of thrombopoiesis in the bone marrow is very efficient to keep up with the constant need for platelets; data from MKs trapped in the lung led to the estimate that each MK releases $\sim 10^4$ platelets (Kaufman et al., 1965). Unfortunately, MKs differentiated from embryonic or induced pluripotent stem cells in culture have not approached this, as published studies show yields of ~20–400 platelets per MK (Takayama et al., 2008; Lu et al., 2011; Ono et al., 2012).

It is clear that progress is being made at differentiating various types of cells into MKs. However, the current model is to create MKs and then collect and analyze the platelets that are released in culture, hoping that they are plentiful and functional. Because so little is known about what initiates and regulates the process of proplatelet formation, there is no direct control over the process of proplatelet and platelet formation in these systems. As we learn more about platelet biogenesis, we may be able to create MKs that are ideal for increased proplatelet and platelet formation. Ultimately, we may be capable of generating platelets that are optimized for the job they are necessitated for, such as wound healing, immune response, or maintaining vascular integrity. In this way, platelet infusions could effectively correct pathological problems with minimal side effects.

### Future perspectives

We have made substantial progress on understanding the mechanisms that regulate thrombopoiesis and platelet formation. But, as is often the case in science, new discoveries lead to more questions. Interestingly, the field of platelet biology is beginning to move away from thinking of platelets as just mediators of hemostasis and starting to study their role in other processes such as inflammation, immunity, and cancer. Are there humoral regulators of proplatelet production and platelet release? Along with these "new" roles for platelets, we have yet to fully uncover what signaling pathways initiate and regulate various aspects of platelet production, in particular, proplatelet initiation, sliding, branching, and release. In addition to revealing fundamental cellular mechanisms, future studies of platelet production will enhance our understanding of how pathological processes in the body affect platelet production and may lead to improved treatments for thrombocytopenia.

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