Chapter 1

Production and Destruction of Platelets

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60678

1. Introduction

Platelet counts in blood are controlled by the rates of their production by megakaryocytes and the kinetics of their removal. Alterations in either process can lead to thrombocytopenia (TCP) or thrombocytosis. Under conditions of TCP, the spleen and liver are the sites for accelerated platelet destruction, and in thrombocytosis, the spleen can become a supplemental breeding ground for megakaryocytes, in addition to the bone marrow space. Humans produce and remove 10^{11} platelets per day. Senile or damaged platelets are detected as such and are removed from blood. Platelets must also be removed locally at diverse sites where they have discovered vascular damage, attached and become activated to prevent blood leakage. The mechanism of this local removal in the blood vascular system is not well described or understood. The removal of platelets from flowing blood mandates a system that detects changes that accumulate in the platelet surface with age and that responds by binding and removing the platelets when changes reach a critical level. Surface changes must either increase the availability of clearance ligands or remove anti-clearance signals such as CD47 [1]. Changes in the platelet surface that signal for removal include phosphatidylserine upregulation, deglycosylation of membrane glycoproteins, in particular Gp1bα of the vWf receptor, and Ig binding. Diseases that accelerate removal arbitrate their impact at the platelet surface.

This section highlights recent advancements in understanding how platelet lifetimes in blood are regulated and the discovery of a surprising feedback pathway that links platelet removal by hepatocytes in liver to platelet production in the bone marrow.

2. Sites and cells that remove senile platelets

Platelet senescence is driven by both an internal proteolytic clock and through external changes that occur on the cell surface as they circulate in blood. Internally, platelets have an apoptotic

mechanism that sets limits on the overall platelet lifetime at ~10 days [2]. At the time of birth, each mature platelet has a defined quota of the pro-survival protein Bcl-x that with time degrades, releasing its brake on the activities of Bak and Bax that subsequently induce mitochondrial lysis, cytochrome C release, and the activation of cytoplasmic capases. Capases disassemble the cytoskeleton and lead to the upregulation of phosphatidylserine to the platelet surface. Cells that have phosphatidylserine on their surfaces are avidly recognized and removed by the professional phagocytes, macrophages and immature dendritic cells. Multiple receptors have been identified for phosphatidylserine on phagocytes including CLM-1 or CD300f [3], Tim-4 [4], BaI1 [5], or Stabilin-1 receptor [6].

Platelets express CD47 on their surface. CD47, also called integrin associated protein [7], is a member of the immunoglobulin superfamily that associates with the integrins, αIIbβ3 and αvβ3, on platelets. A role for CD47 in maintaining platelets in circulation was first recognized in knockout mice, which have platelets counts reduced by ~20% compared to normal. This highly glycosylated surface protein is recognized by the SIRPα transmembrane protein on phagocytes that contains two immunoreceptor tyrosine-based inhibitory domains in its cytoplasmic domain. Hence, ligation by CD47 transmits anti-phagocytic signals to macrophages, independent of the phagocytic receptor engaged, helping to prevent macrophages for engaging phagocytic pathways involving Fc receptors.

One example where phosphatidylserine exposure can induce macrophages to remove platelets occurs with FlnA null platelets. Mice having megakaryocytes and platelets that lack FlnA are macrothrombocytopenic, with platelet counts reduced by 80-90%, compared to WT mice. FlnA null platelets are unstable because they lack FlnA's actin filament crosslinking activity and its membrane glycoprotein attachment sites, which link the actin cytoskeleton to the plasma membrane. FlnA constitutively binds to the GP1bα chains of the vWF complex, as well as certain β-integrin chains. The high density of the vWfR-FlnA interaction, ~12,000 per platelet, stabilizes the membrane by attaching it to the underlying actin cytoskeleton. Because the vWfR is linked to the sides of actin filaments it also regulates the topology of the vWf receptor, aligning the receptor in linear arrays on the platelet surface. When studied *in vitro*, FlnA null platelets spontaneously fragment into microparticles that are 100% positive for annexin V binding, which reports flipping of phosphatidylserine to the cell surface. Microparticles from the FlnA null platelets are, however, rarely found in freshly isolated blood, unless macrophages are first ablated in the mice using phagocytic liposomes filled with clodronate, a potent toxin when ingested. This finding demonstrates that macrophages normally clear both FlnA null platelets and microparticles derived from them *in situ*, most likely using phosphatidylserine recognizing receptors [8].

3. Sialic acid and platelet circulatory lifetime

Sialic acid terminates the N- and O-linked glycans of all cell surface glycoproteins. Desialylation of platelet glycans was the first recognized mechanism that can target platelets for clearance. In 1975, Greenberg and colleagues [9] showed that desialylation of platelets (and

erythrocytes) *in vitro* using neuraminidase resulted in rapid removal upon retransfusion. Subsequently, it was found that sialic acid is lost during platelet storage *in vitro* [10] and as platelets age *in vivo* [11]. Recently, the mechanism that removes sialic acid-depleted platelets has been identified. Studies from 2 independent labs have found that liver hepatocytes, using their asialoglycoprotein receptor (Asgr), remove desialylated platelets as well as certain desialylated plasma proteins.

The Asgr was one of the first receptors to be identified and characterized [12] over 40 years ago. Each surface Asgr is a heterotrimer composed of 2 HL-1 chains and 1 HL-2 chain. Knock out animals lacking in the expression of either chain, therefore, do not have functional Asgr receptors on their hepatocytes. The Asgr is a C-type lectin that recognizes exposed β-galactose, the underlying moiety to which sialic acid is linked in carbohydrate chains. Most glycoproteins have their glycans capped by α2,3-linked sialic acid. Galactose exposure is mediated by sialidases present in blood or released into blood by infectious bacteria. Interestingly and of high clinical impact, the Asgr can also bind to α2,6 linked sialic acid residues on glycoproteins.

Desialylated platelets are recognized and removed by the liver Asgr [13-15]. The specific function of the Asgr in platelet removal has been demonstrated by the following evidences: (1) HepG2 cells bind and ingest desialylated platelets *in vitro*; (2) Mice lacking functional Asgrs ($HL2^{-/-}$) have high platelet counts (increased by 50% over $HL2^{+/+}$) because their platelets circulate for longer lifespans. Platelet circulatory lifetime is lengthened by 35% in the $HL\text{-}2^{-/-}$ animals. Lectin profiling of platelets derived from the $HL2^{-/-}$ mice shows they have higher amounts of exposed β-galactose as expected. Hence, the lengthening of platelet lifetimes in these animals is caused by the loss of the removal system, not because the platelet surface escapes desialylation in the circulation. This finding is expected if the Asgr routinely removes desialylated platelets since the amount of desialylation will increase as the senile platelets continue to circulate; (3) Conversely, $St3gal4^{-/-}$ mice that lack the main sialyltransferase enzyme responsible for the linkage of sialic acid to galactose, have low platelet counts and short platelet circulation lifetimes. Once again, platelets harvested from these null mice have high levels of exposed galactose that bind the RCA I lectin avidly. Circulation times for these platelets can be extended to near normal by transfusion of them into $HL2^{-/-}$ mice. This again demonstrates the central role of the Asgr in recognizing and removing desialylated platelets. (4) Last, platelet desialylation *in vitro* using sialidases or *in situ* following injection of sialidase causes a rapid clearance of platelets from blood in WT mice, but not $HL2^{-/-}$mice, in a process that is not altered by macrophage ablation.

4. Feedback between liver and bone marrow

The importance of hepatocyte-platelet interaction extends beyond simple removal, as the recognition and ingestion of platelets by the Asgr generates cytoplasmic signals in hepatocytes that induce the formation and secretion of cytokines to promote marrow and megakaryocyte growth and maturation. In this case, the key cytokine produced in response to platelet ingestion is thrombopoietin [16]. Thus, the hepatocyte-platelet interaction directly feeds back to megakaryocytes in marrow, helping to stimulate platelet production.

5. Platelet reactive receptors on macrophages

In addition to its receptors that recognize phosphatidylserine, macrophage surfaces are festooned with receptors that can ingest damaged and/or diseased platelets. One group includes the lectin receptors that recognize carbohydrate alterations in platelet glycoproteins. The phagocytic integrin, $\alpha_M\beta_2$, recognizes and removes chilled and rewarmed platelets that release glycosylases causing β-GlcNAc moieties to expose on N-linked GP1bα glycans. In addition, a second domain on $\alpha_M\beta_2$ recognizes a different portion of GP1bα [17]. Mannose receptors are a second example of a receptor that detects glycan alterations, recognizing underlying mannose moieties exposed by glycosylases [18]. Fcγ receptors remove Ig-coated platelets from blood and when anti-platelet Igs are present their effectiveness leads to thrombocytopenia.

6. Diseases causing accelerated platelet clearance by macrophages

Accelerated clearance requires either the accumulation of opsonins on the platelet surface such as Igs and complement or the presence of agents in blood that remove protective molecules. Both types of mechanisms occur.

There are many examples of acquired or induced immune thrombocytopenia that cause platelet removal when anti-platelet Igs are generated, deposited on platelets, and are detected by Fcγ receptors on macrophages. These include congenital and drug or pathogen induced thrombocytopenia. In general, platelet clearance is primarily driven by splenic macrophages, a process that can result in splenomegaly. In many cases, patients having ITP, respond well to anti-Fc antibody treatment.

7. Platelet clearance mediated by hepatocytes

Bacterial-derived sialidases, released into blood during sepsis, cause platelet counts to drop precipitously. The target of the blood born bacterial sialidases is sialic acid that caps N-linked glycans on GP1bα, as demonstrated using mice lacking GP1bα [19], which are resistant to clearance induced by pneumococcal sepsis in WT mice. Cleavage of sialic acid on GP1bα exposes underlying galactose moieties that are recognized by the Asgr [13]. Animals lacking a functional Asgr do not accelerate their platelet clearance in response to sepsis.

A related process accounts for the circulation failure of platelets transfused after rewarming from refrigerated storage. Resting platelets contain sialidases that are stored in an internal compartment that can be released by activation [15, 20]. Rewarming from the cold releases a portion of the sialidase activity to the platelet surface and into the storage media, a process that mediates desialylation of the platelet surface glycoproteins. Since the accelerated clearance of chilled and rewarmed platelets is to a large extend ablated in mice lacking the Asgr

(HL-2$^{-/-}$ mice), it is the main receptor that recognizes and removes cold damaged platelets. Ablation of macrophage function in HL-2$^{-/-}$ mice further restores platelet circulation by 15-20%. Hence, macrophages also participate in clearance.

8. Accelerated platelet clearance in Wiskott-Aldrich syndrome

Mutation, truncation and/or deletion of WASp, a protein encoded on the X-chromosome and expressed by blood cells, results in a profound lymphocyte dysfunction that severely compromises the immune system. Severe thrombocytopenia (TCP) is also a signature component of the Wiskott-Aldrich syndrome; WAS platelets are small and have shortened circulatory lifetimes. WAS patients produce diverse autoantibodies and WAS platelets collect higher amounts of surface-associated immunoglobulins (Igs) than do normal platelets [21]. Many human WAS patients respond to splenectomy with increased platelet counts, despite the finding that all patients have been found to be refractory to anti-Fcγ antibodies. Unlike ITP, homologous platelets circulate normally in WAS patients strongly suggesting a more complex mechanism for removal that involves receptors other than Fc.

WASp KO mice have been shown to retain the key features of WAS disease, having T and B lymphocyte dysfunction, enlarged spleens, low platelet counts (70% of normal) and shortened platelet survival times in blood. It has been widely believed that platelet clearance is accelerated in these animals because the autoimmune aspect of the disease results in increased Igs bound to the platelets surface that led to recognition by splenic macrophages. However, as in the human conditions, normal platelets, when transfused into WASp KO mice, circulate normally indicating that a simple anti-platelet antibody mediated clearance is not the mechanism. In mice, splenectomy has been shown to be without effect on the clearance rate.

In efforts to identify the mechanism of removal, WASp Null platelets were transfused into mice lacking specific phagocytic receptors. A survey on macrophage receptors failed to reveal any in which the WASp null platelets had enhanced circulatory lifetimes. However, WASp KO platelets were found to circulate normally in Asgr null mice, a finding once again posits the Asgr as a central molecule involved in the recognition and removal of damaged platelets. The surface of WASp KO platelets is, however, not desialylated and lectin binding studies have instead revealed hypersialylation. This sialylation occurs specifically in the 2,6 linkage, not the normal 2,3 linkage. Critically, the Asgr also receptor recognizes this unique sialic linkage, leading to binding and platelet removal. The carrier of this sialic acid turns out to be surface bound Ig and sialylation of its Fc N-linked glycan shifts recognition of the Fc domain from macrophages to the hepatocytes.

Interestingly, the source of the 2,6 sialyltranferase (ST6Gal1) is liver hepatocytes, which make and secrete this enzyme into blood. This blood enzyme is an acute phase reactant protein, upregulated in liver in response to bacterial sepsis, cancer, or inflammation. In this case, platelet ingestion itself, feedbacks to upregulate ST6Gal1 mRNA transcription and translation and this increases by 3-4 fold the blood levels of this enzyme.

9. Cause of surface alterations that lead to the deposition of Igs on the WASp KO platelet

The modification(s) in WASp KO platelet surface that leads to Ig production by WASp KO B-lymphocytes and Ig-binding are not known. Since WT platelets do not collect Igs in WASp KO plasma or have accelerated clearance in WASp KO animals, surface alterations are specific for the WASp KO platelets. Because WASp is a protein that interacts with the actin cytoskeleton, it is likely that internal cytoskeletal changes in its absence result in an altered topology of platelet receptors or the expression of the neo-epitope. In general, platelet function in the absence of WASp is near normal although as the precision of assays increase, some differences have now been recognized. Active platelets lack small focal actin assembly sites in the absence of WASp, although spreading and filopodial formation are normal. In resting platelets, failure to express WASp alters the stability of microtubules, increasing their acetylation and slowing their turnover. How these internal changes alter the surface remains for future studies.

10. Platelet production

The basic processes involved in megakaryocyte commitment, maturation and platelet formation are well described although many precise details remain to be clarified. Megakaryocytes derive from a renewable population of hematopoietic stem cells that continuously enter the MK/platelet lineage and once committed to produce platelets, live for only a few more weeks before they are converted into hundreds of platelets. Proplatelet and platelet production requires a massive enlargement in MK size that is driven by high levels of mRNA transcription from their amplified polyploid nuclei followed by mRNA translation into platelet essential components. This includes the production of an abundant internal network of membranes called the demarcation membrane system (DMS) that dramatically increases the apparent membrane to surface ratio during proplatelet formation, platelet specific granules, and the synthesis of large amounts of the cytoskeletal machinery that is used to form and fill assembling platelets.

As MKs mature, they develop an extensive network of internal membranes called the DMS that are enriched phosphatidylinositol 4,5 bisphosphate and the vWf receptor [22] and are used as the primary membrane source for proplatelet elongation. Recent studies by Eckly et al [23] have begun to shed some light on DMS formation, describing how the DMS forms and matures. To form the DMS, the plasma membrane of megakaryocytes enfolds at specific sites and a perinuclear pre-DMS is generated. Next, the pre-DMS is expanded into its mature form by material added from golgi-derived vesicles and endoplasmic reticulum-mediated lipid transfer. This structural description is in accordance with the studies on platelet glycosyltransferases, which arrive early in the forming DMS and eventually make their way to the megakaryocyte and platelet surfaces [24]. Only a small number of proteins have been identified thus far to participate in the DMS formation process based on alterations in its structure in certain knockout animals. Gross disruptions of this network are found in megakaryocytes

isolated from either filamin A knockout or pacsin2 knockout mice, the latter of which connects GP1bα to the actin cytoskeleton and binds pacsin2, a molecule that deforms membrane. The relationship between the DMS and the platelet open canalicular system (OCS) is not clear. The OCS, like the DMS, is a unique anastomosing network of internal membrane tubes that is connected to the plasma membrane at multiple points. The internal canals of the OCS can be identified because they contain vWfR, and hence can be labeled with anti-GP1bα antibodies (Kahr et al).

To release platelets, megakaryocytes in the marrow space move to and nestle the marrow sinusoids where they project their proplatelet protrusions into the blood flow [25, 26]. Whether all proplatelets are directed to grow specifically into the sinusoids or if only some of the proplatelets elaborated by a MK find their way into the sinusoids is unknown, although living MKs in marrow have been observed to have many proplatelet projections, some of which project into the marrow space while others project into the sinusoids [27]. Studies have demonstrated that proplatelet fragments considerably larger than platelets are released by MKs into blood [26, 27] and that proplatelets can be both found, and can mature into platelets, in blood [28].

The state of our current knowledge of the mechanics of proplatelet production has come primarily from studies on MKs in culture. This work has clarified the essential role of microtubules, which were recognized early as the most prominent structure found within the MK projections [29] and that proplatelet and platelet production were adversely affected by MT poisons [30]. Subsequent gene expression studies established the importance of β1-tubulin, a tubulin isoform specific for MKs, to the maturation of MKs into proplatelet producing machines [31]. More recent studies using gene deleted MKs have begun to reveal the precise roles of specific proteins in proplatelet and platelet production and these are highlighted below.

Signals that initiate proplatelet formation, if present, remain undefined and it remains possible that the program to make platelets starts when the synthesis of cytoskeletal proteins for this process reaches a critical mass. From a mechanical view, centrosome dissolution precedes proplatelet extension, and the release of MTs from these multiple nucleating sites correlates best with the start of proplatelet elaboration. Released MTs first collect as bundles in the MK cortex where they are driven apart by their associated motor protein, dynein. These MT-dynein reactions deform the membrane outward and generate the structural motor of the proplatelet, which is a MT bundle that folds over in the proplatelet tip and runs back into the shafts. Each bundle is composed of many MTs that are continuously growing and shrinking from their ends. Six types of behaviors characterize the elaboration of proplatelets: elongation, branching, pausing, fusions, fragmentations, and retractions. While the average elongation rate for proplatelets over time is ~1 μm/min, extension normally occurs in bursts and pauses. Burst rates greatly exceed the average rates and under flow, and rates of >30 μm/min have been observed. These rates correlate well with the sliding rates of MTs within the bundles. Pauses in proplatelet extension are not caused by a stoppage in MT sliding, which continues at a 1-6 μm/min rate. Sliding reactions in paused proplatelets appear to build tension into the bundle which when released leads to branching and/or fragmentation. This implies that there are regions within the bundles where MTs are crosslinked to increase resistance or they are

pushing against resistive structures. Branching is a modified form of extension derived from tension asymmetry where a portion of the MT bundle detaches from the mother bundle and elongates rapidly forming a new tear shaped structure and proplatelet shaft. Retraction, where the sliding could either reverse or all crosslinking derived tension releases, could serve to subfragment the proplatelets.

In addition to playing a crucial role in proplatelet elongation, the microtubules lining the shafts of proplatelets serve a secondary function — tracks for the transport of membrane, organelles, and granules into proplatelets and assembling platelets at proplatelet ends [32]. Individual organelles are sent from the cell body into the proplatelets, where they move bidirectionally until they are captured at proplatelet ends. Immunofluorescence and electron microscopy studies indicate that organelles are in direct contact with microtubules, and actin poisons do not diminish organelle motion. Therefore, movement appears to involve microtubule-based forces. Bidirectional organelle movement is conveyed in part by the bipolar organization of microtubules within the proplatelet, as kinesin-coated beads move bidirectionally over the microtubule arrays of permeabilized proplatelets. Of the two major microtubule motors — kinesin and dynein — only the plus-end-directed kinesin is situated in a pattern similar to organelles and granules, and is likely responsible for transporting these elements along microtubules. It appears that a twofold mechanism of organelle and granule movement occurs in platelet assembly. First, organelles and granules travel along microtubules and, second, the microtubules themselves can slide bidirectionally in relation to other motile filaments to indirectly move organelles along proplatelets in a "piggyback" fashion

Although microtubules and associated motor and regulatory proteins power proplatelet motility, elimination of certain actin cytoskeletal-associated proteins have now been demonstrated to modulate this process. These include a number of actin-associated proteins, membrane contouring proteins, and signaling proteins.

11. Influence of actin-associated proteins on thrombopoiesis

Since proplatelets elongate, but do not branch, in the presence of the actin disrupting drug cytochalasin B, it is surprising that the deletion of certain actin associated proteins from the megakaryocyte lineage leads to macrothrombocytopenia. It seems likely that the removal of actin modulating proteins, alters and/or increases filamentous actin (F-actin) and cytoskeletal structure to have a dominant inhibitory effect proplatelet maturation and/or platelet release from proplatelets.

12. Actin crosslinking proteins

Three proteins, filamin A (FlnA), actinin 1 (Actn1) and spectrin, that crosslink F-actin using a related F-actin binding site, are critical components of the mature platelet cytoskeleton and regulate proplatelet formation and motility.

FlnA. FlnA, an elongated homodimer 160 nm in length, is an essential actin filament crosslinking and scaffolding protein that interacts with multiple platelet proteins including the vWfR, β-integrins, migfilin, Syk, and filGAP, in addition to crosslinking F-actin. Each FlnA subunit has an N-terminal actin-binding domain (ABD) that is followed by 24 Ig-like repeats, the last of which contains the self-association site for molecules. In platelets, FlnA crosslinks the actin filaments posited beneath the plasma membrane into a rigid network and using its 2 Gp1bα binding sites attaches actin filaments to the vWfR. Since there are 12,000 molecules of FlnA per platelet, and 25,000 Gp1bα chains, the amount of FlnA is sufficient to link 95% of the vWfR to actin since each FlnA binds two Gp1bα chains. Loss of this linkage, either by the failure to express stable $GP(1b\alpha\beta9)_2 5$ complexes on the platelet surface, or by the absence of cytoplasmic FlnA in conditional mice whose MK/platelet lineage lacks FlnA expression, results in a macrothrombocytopenia in which platelet counts are reduced by 80-90%. Not only does the loss of FlnA result in large platelets, the FlnA-null platelets are extremely fragile, and spontaneously subfragment within blood, resulting in short circulation lifetimes and accelerated removal of the fragments by macrophages. The enlarged platelet phenotype of the FlnA null platelets is very similar to that found in patients having Bernard-Soulier Syndrome, which because they do not express stable surface vWfR, also lack the important FlnA-mediated vWfR-cytoskeletal attachments. Human periventricular heterotopia patients with FlnA mutations and/or truncations have also been found to bleed and have low platelet counts [33-35]. Interestingly, failure to express FlnA in MKs disorganizes the DMS system. Alterations in the DMS in the absence of FlnA may involve its lipid-deforming partner, pacsin2 (see below). Proplatelet elaboration is accelerated in FlnA null MKs *in vitro*. Hence, FlnA imposes restraints on the timing of platelet production.

Actn1. Actinin dimers are short rods, 40 nm in length, built from an antiparallel, side-to-side alignment of its subunits. Each subunit chain has an NT ABD followed by 4 α-helical repeats, related in structure to those in spectrin, and last, an EF hand motif. In nonmuscle cells, binding of the actinin isoforms that are expressed (Actn1 and Actn4) to F-actin are regulated by calcium although the muscle isoforms have lost this sensitivity. Human patients with macrothrombocytopenia have been identified that express mutant actn1 molecules having sequence changes within their NT actin binding site [36]. Since these actn1 mutants reduce the proplatelet formation and cause MKs to elaborate thick proplatelets when expressed in mouse MKs, it is believed actinin function can alter the MK to proplatelet transition. Actn1 is expressed in platelets at levels equivalent to FlnA [37].

Spectrin. Platelets, like erythrocytes, have a planar membrane skeleton that laminates the plasma membrane and is linked to the underlying actin cytoskeleton. This skeleton is composed of twisted and elongated spectrin tetramers that interconnect by binding to the barbed ends of actin filaments, a interaction that is promoted by β-adducin, a molecule that binds to both spectrin, near its ABD, and the barbed filament end of an actin filament [38]. Tetramers are assembled from heterodimers; α,β dimers are 100 nm in length, are bipolar and side-to-side constructs, in which the β-chain donates the actin binding domain on one end (NT of the b-chain), with the self-association domain posited on the other end. Head-to-head binding of dimers forms tetramers, a process that is dynamic and can be disrupted using small fragments

of β-chain C-Terminal to compete for this binding site. When such negative dominant fragments are expressed into MK cultures, proplatelet elaboration is blocked. Similarly, the exposure of permeabilized proplatelets with this small C-Terminal protein construct results in blebbing and swelling, and ultimately complete loss of cytoskeletal integrity.

Myosin 2A and Rho/ROCK. MYH9 gene disorders (myosin 2a) cause May-Hegglin and the related Epstein, Fechtner, Sebastian, and Alport syndromes in humans, all of which have thrombocytopenia. However, the myosin 2A story is complex. Gene inactivation studies in mice have shown that loss of myosin 2a protein expression in MKs accelerates the production of large platelets, while MYH9 gene mutations in humans have dominant negative effects and reduce platelet production. The most severe mutations that effect platelet counts, posit within the myosin head motor domain, while mutations in the tail domain, allow somewhat higher, but still abnormal platelet counts. The general consensus on these myosin-mediated defects are that myosin activity is necessary to constrain proplatelet formation in MKs as they mature and that inactivation of myosin thus plays a role in initiating proplatelet elaboration. In addition, since myosin activity is regulated by MLC phosphorylation, modification of pathways that regulate either the kinases or phosphatase activities that are directed toward myosin can lead to phenotypes similar to those found for myosin mutant or loss of function.

Platelet specific RhoA deficiency causes macrothrombocytopenia with platelet counts reduced by ~50% from normal. The $RhoA^{-/-}$ platelets have many functional defects that cause animals to readily bleed, but they have normal lifetimes in blood [39]. One pathway by which RhoA deficiency may affect MK maturation and platelet formation is through ROCK modulation of myosin 2a activity in MKs. RhoA activates ROCK, inhibiting myosin LC phosphatase and hence leading to increased myosin LC phosphorylation. This activity is thought to restrain proplatelet elaboration and thus RhoA deficiency to release this brake, causing premature proplatelet formation and leading to enlarged and dysfunctional platelets.

Protein	Megakaryocyte phenotype	Proplatelet phenotype	Platelet phenotype	Clearance Phenotype	References
GP1ba KO			Large, fragile		[19]
Filamin A KO	Accelerated proplatelet elaboration, altered DMS	Accelerated	10-20% of normal: Large, fragile		[8]
Actinin 1 ABD mutants	Decreased proplatelet number, increased proplatelet thickness	Reduced, thickened	Large		[36]

Protein	Megakaryocyte phenotype	Proplatelet phenotype	Platelet phenotype	Clearance Phenotype	References
Nonmuscle myosin HC IIA KO		Increased, formation accelerated	Heterogenous, Reduced by 70%	Normal	[41]
Dynamin 2 KO		Reduced, thickened	20% of normal, size highly variable		
CIP4 KO		Reduced			[42]
Pacsin 2 KO			Normal		
WASp or WIP KO		Accelerated	Slightly small	Accelerated	[43]
β1-tubulin	Compensate by upregulating β2 and β5-tubulin	Proplatelet formation blocked	Amorphic, reduced by 70-75%		[44]
Rab27b					[45]
Cofilin-1			Large		[46]
ADF	normal		90% of normal, size normal		[46]
ADF/Cofilin1	Numbers increaseddecreased 3X; DMS and cytoplasmic structure altered		5% of normal count; sizes variable, amorphic		[46]
Actin interacting protein (Aip1/Wdr1)	Defective megakaryo-poiesis: small, DMS abnormalities		20% of normal: large	Normal	[40]
Profilin 1			Large, size variable		[47]
RhoA			50% of normal, large -125%	Slight increase in turnover rate	[39]
Spectrin		Proplatelets disrupted by dimer-dimer self-association inhibitor			[48]
Tmod3	Altered DMS	Enlarged tips	Large, decreased count		[49]

Table 1. Effect of Mouse KOs on Platelet Production and/or Platelet Clearance.

Cofilin-1/ADF and Aip1 (Wdr1]. Cofilin-1 and ADF are highly homologous protein isoforms in the ADF/cofilin family that accelerate actin turnover in cells. The third member of this family, m-cofilin or cofilin-2, is expressed only in muscle tissue. Cofilin/ADF bind along the sides of actin filaments, preferentially targeting ADP-subunits, and thereby, recognize the old portions of filaments. Binding induces a twist in the filament, causing its severing. Exposure of low affinity filament ends following the severing event, promotes dissociation from these ends, and this stimulates actin filament turnover in cells. Conditional mice lacking cofilin-1 have been produced. When cofilin-1 is specifically deleted form the MK/platelet lineage, large platelets result and platelet counts are mildly reduced (60-80% of normal). ADF$^{-/-}$ mice, on the other hand, have near normal platelet counts and normal platelet function. However, when ADF-/- mice are crossed with the mice carrying the floxed cofilin-1 allele, platelet production in the DKO offspring is severely diminished. The morphologies of the released platelets are highly variable, as the normal discoid space is distorted by their dense fillings of actin filaments. In agreement with these mouse studies, proplatelets are sparse on the double knockout MKs in vitro.

Cofilin is regulated by Aip1, a protein that binds and enhances filament severing /actin depolymerization activity. The importance of this protein to platelet production has been demonstrated [40]. N-ethyl-N-nitrosourea mutagenesis generated mice with profound thrombocytopenia, bleeding, and enlarged, amorphic platelets contain a gene mutation that was mapped to Wdr1 gene. The high degree of similarity to the phenotype of in the ADF/cofilin double knockout, strongly suggests both proteins are in the same thrombogenesis effector pathway.

Profilin1A and Wasp/Wip. Profilin is a small protein that promotes filament assembly. It binds ADP-monomers, converting them to ATP-monomers, which are permitted to only interact with the high affinity end of actin filaments (barbed end with respect to myosin S1 binding). Mice whose MKs/platelets specifically lack profilin 1 have macrothrombocytopenia with counts reduced by 40-50%. Platelet turnover is accelerated and the mature platelets have only a sparse internal actin cytoskeleton. Surprisingly, although their actin cytoskeleton is disrupted, profilin null platelets have a robust MT cytoskeleton with a thickened MT coil and more stable hyper-acetylated microtubules.

In certain ways, the profilin 1 KO phenotype is similar to the behavior of platelets in Wiskott-Aldrich syndrome, or in WASp or WIP KO mice. WASp is a signaling protein promotes actin assembly system, binding to Arp2/3 when activated by binding of GTP-cdc42. However, the bulk of studies performed on human WAS platelets or platelets derived from KO mice revealed normal and ever hyper-function for the WASp null platelets. Human patients have low platelet counts that are caused by accelerated platelet clearance, a process that lowers the platelet count in the Wasp/Wip KO mice. Recently studies have shown the MT ring in platelets from WAS patients to be hyper-acetylated, like the profilin 1 KO platelets. However, in the WAS syndrome, platelets in the circulation are small, not large, as is found in the Profilin null animals.

http://dx.doi.org/10.5772/60678

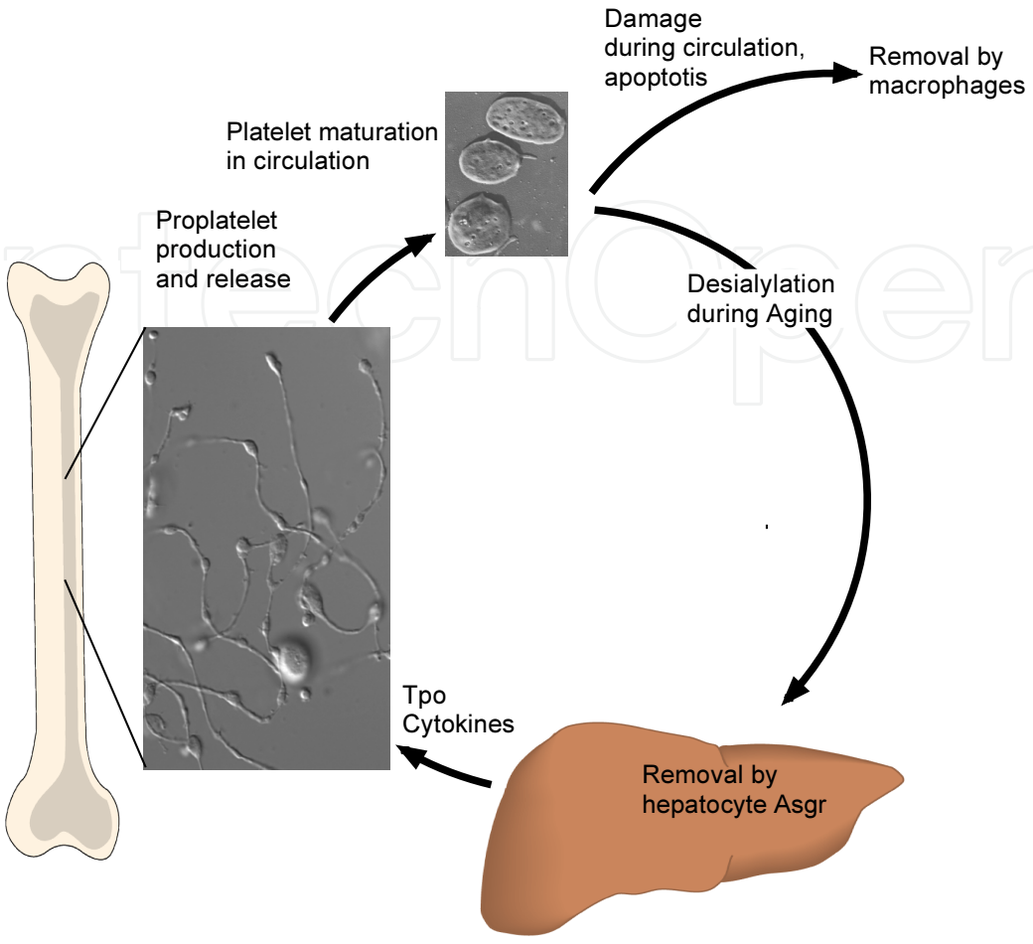

Figure 1. Life cycle of the platelet. Megakaryocytes in the bone marrow release proplatelets into the blood where they mature into platelets. If damaged, platelets are detected and removed by splenic macrophages and liver Kupffer cells. As platelets age in the circulation they become desialylated. The fate of desialylated platelets differs from that of acutely damaged platelets. Desialylated platelets are recognized and are removed from the blood in the liver sinusoids by liver hepatocytes using their Asgr. Platelet ingestion by hepatocytes stimulates them to produce TPO and release it into the blood.

CIP4/Pacsin2/Dynamin. Membrane deforming proteins that use F-BAR domains curve membranes, or GTP as an energy source to bud vesicles from membranes, are necessary for normal MK maturation and platelet release.

CIP4. CIP4 is a F-Bar containing, Cdc42 interacting and WASp binding protein. CIP4$^{-/-}$ mice have a mild thrombocytopenia with a 25% decrease in platelet counts. MK numbers and ploidy are normal in the KO mice but isolated MKs from these mice are less effective in producing proplatelets in culture.

Pacsin 2. Pacsin 2, the only family member expressed in mouse platelets, is a FlnA partner that has an F-BAR and SH3 domain that bindings dynamin and N-WASp. Pacsin2 plays an important role in organizing internal membranes in platelets and megakaryocytes. In platelets, it locates at the entrances of the OCS. In MKs it is dispersed throughout the internal anastomosing network of DMS membranes. FlnA binding is important for its functions as the

distribution of pascin2 and the arrangement of internal membranes is grossly altered in FlnA null MKS.

Dynamin. Dynamins are a family of three cytoplasmic mechanochemical proteins (DNM1-3) that regulate membrane dynamics with actin during such processes as cytokinesis, budding of transport vesicles, phagocytosis, and cell motility. Dynamin 2 and 3 are upregulated as MKs mature and iRNA knockdown of DNM3 has been reported to interfere with maturation and proplatelet production *in vitro*. Specific inactivation of the DNM2 gene in mice MKs/platelets profoundly diminishes platelet production and function.

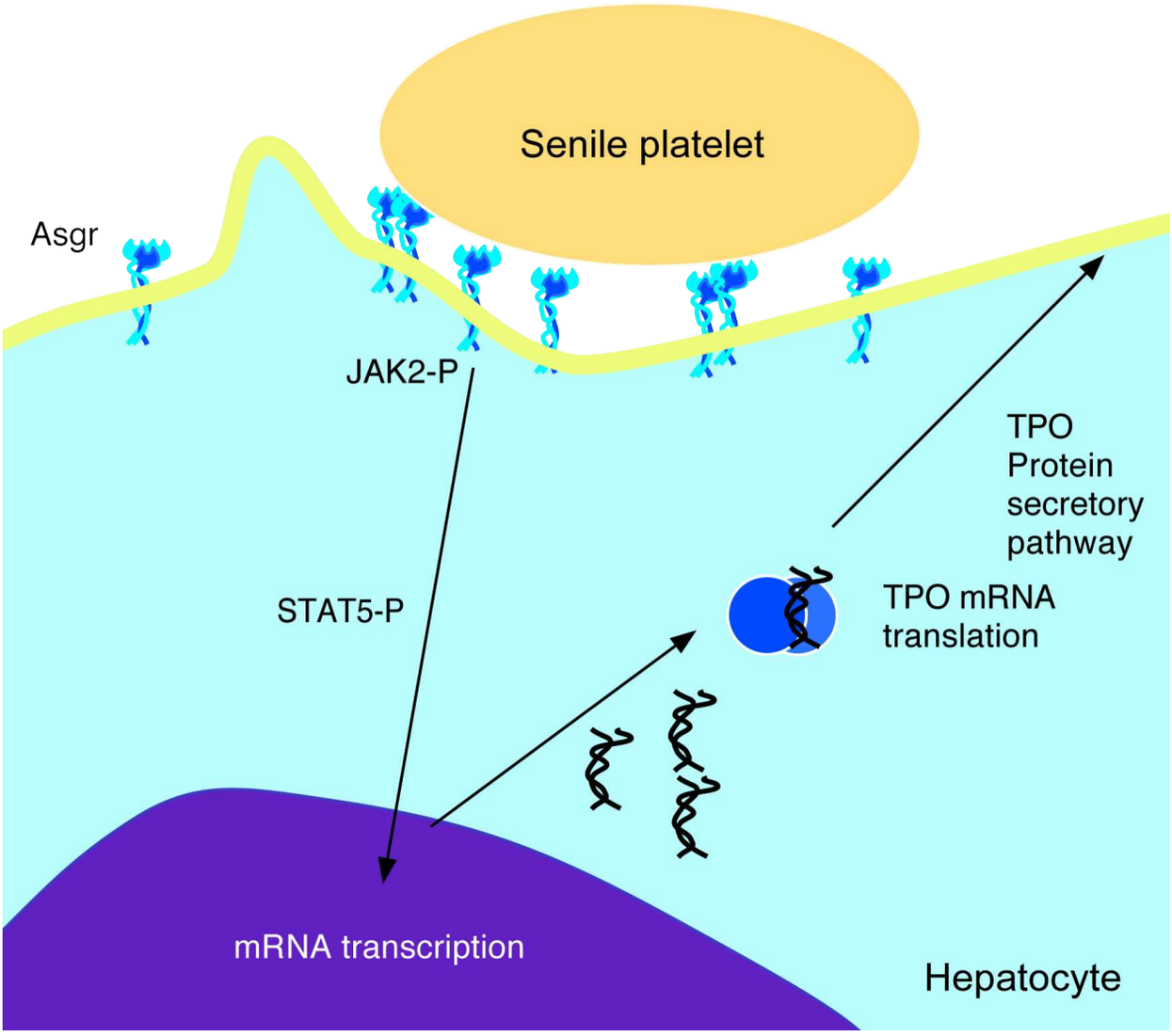

Figure 2. Platelet ingestion stimulates TPO signaling to the bone marrow. As platelets are detected and removed by the Asgr, the Jak-STAT pathway is activated. This leads to TPO mRNA transcription and translation, increasing blood TPO levels.

http://dx.doi.org/10.5772/60678

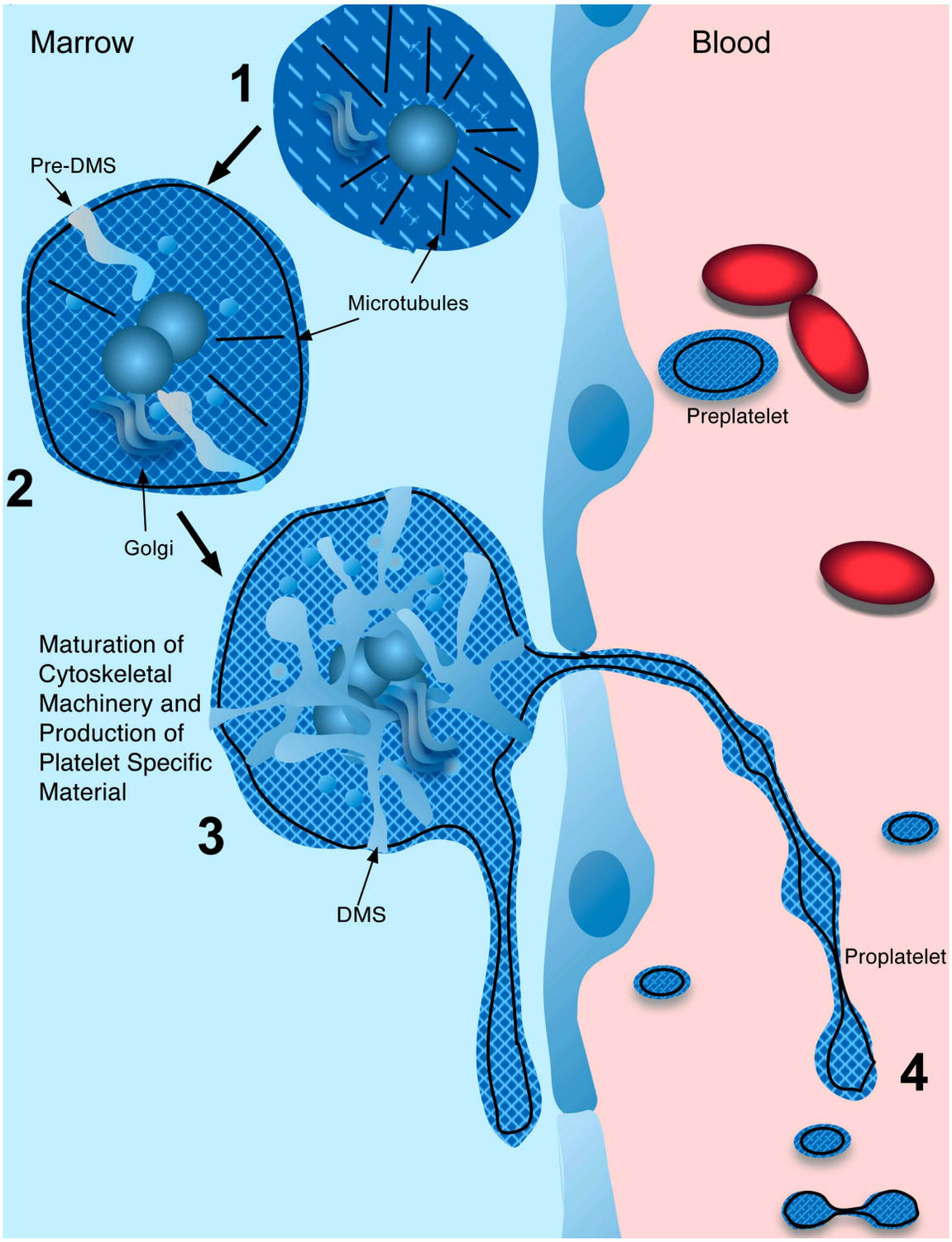

Figure 3. Steps in MK maturation and proplatelet release. (1) Immature MK. Microtubules radiate outward from centrosomes and DMS has not developed. (2) Ploidy increases, the pre-DMS begins to form, the centrosomes dissolve, and the released microtubules move out to the cell cortex and form bundles. (3) Ploidy increases to 8-16n, the DMS and platelet specific granules are produced, the cytoplasm has become enriched in cytoskeletal proteins, and the sliding of the MT within the bundles has begun. Sliding elongates proplatelets into the venous sinusoids and they are released into blood. (4) Platelets mature from proplatelets and preplatelets that release into blood.

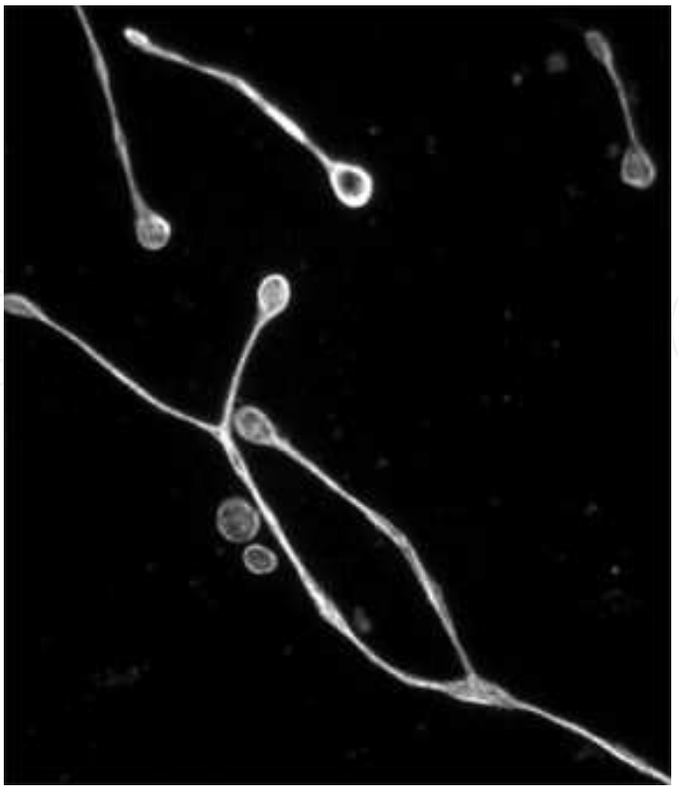

Figure 4. Localization of microtubules within proplatelets. Immunofluorescence studies on murine megakaryocytes grown in culture and labeled with β1-tubulin antibodies indicate that microtubules line the entire length of proplatelets. The hallmark features of proplatelets, including the tip, swellings, shafts and branch points are visible.

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