Role of Rho-GTPases in megakaryopoiesis

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ABSTRACT
Megakaryocytes (MKs) are the bone marrow (BM) cells that generate blood platelets by a process that requires: i) polyploidization responsible for the increased MK size and ii) cytoplasmic organization leading to extension of long pseudopods, called proplatelets, through the endothelial barrier to allow platelet release into blood. Low level of localized RHOA activation prevents actomyosin accumulation at the cleavage furrow and participates in MK polyploidization. In the platelet production, RHOA and CDC42 play opposite, but complementary roles. RHOA inhibits both proplatelet formation and MK exit from BM, whereas CDC42 drives the development of the demarcation membranes and MK migration in BM. Moreover, the RhoA or Cdc42 MK specific knock-out in mice and the genetic alterations in their downstream effectors in human induce a thrombocytopenia demonstrating their key roles in platelet production. A better knowledge of Rho-GTPase signalling is thus necessary to develop therapies for diseases associated with platelet production defects.

Abbreviations: AKT: Protein Kinase B; Rho/Rac Guanine Nucleotide Exchange Factor 2; ARP2/3: Actin related protein 2/3; BM: Bone marrow; CDC42: Cell division control protein 42 homolog; CML-MK: Colony-forming-unit megakaryocyte-CPI4; Cdc42-interacting protein 4mDia: Diaphanosus DiaphA1; Diaphanosus diapholog 1Ect2: Epithelial Cell Transforming Sequence 2FLNA: Filamin AGAP: GTPase-activating proteins or GTPase-accelerating proteins; GD1: GDP Dissociation Inhibitor; GF/E: Guanine nucleotide exchange factor; HDAC: Histone deacetylase; LIMK: Lim kinase; MAL: Megakaryoblastic leukemiamarker; Myristoylated alanine-rich C-kinase substrate; MKL: Megakaryoblastic leukemia marker; Myosin light chain; MRTF: Myocardin Related Transcription Factor OTT: One-Twenty Two Protein; PACSIN2: Protein Kinase C And Casein Kinase Substrate; PAK: P21-Activated Kinase; PI3K: Phosphoinositide 3-kinase; PKC: Protein kinase C; POD: Phosphotyrosine phosphatase receptor type; Rac: Ras-related C3 botulinum toxin substrate; RBC: Red Blood Cell; RHO: Ras homolog; ROCK: Rho-associated protein kinase; SCAR: Suppressor of cAMP receptor signaling; SRC: Serum response factor; SRF: SarcTAZ: Transcriptional coactivator with PDZ motif TUBB1: Tubulin B VEGF: Vascular endothelial growth factor; WASP: Wiskott Aldrich syndrome; WAVE: WASP-family verprolin-homologous protein; YAP: Yes-associated protein

Megakaryopoiesis: a unique model of cell differentiation

Megakaryopoiesis is the cell process that leads to platelet production and is one branch of the hematopoietic hierarchy initiating from a multipotent hematopoietic stem cell (HSC) in adult [1]. This is a highly dynamic cellular differentiation system that produces $10^{11}$ platelets daily in human adult. Platelets are anucleate cells, which arise from the cytoplasm fragmentation of the megakaryocytes (MKs), their bone marrow (BM) precursors. They have a short life of around 8 days in human with normal counts ranging from 150,000 to 450,000/μL of blood, explaining the need for this important daily production.

The early stages of megakaryopoiesis consist in the commitment of a HSC towards the MK lineage [2]. There is increasing evidence of the existence of a murine MK/platelet-biased HSC capable to directly generate a MK-committed progenitor, eventually in the absence of any mitosis. This direct commitment enables rapid platelet production in stress conditions or during aging [3-5]. Recent evidence suggests that 50% of MKs and...
platelets are derived from MK/platelet-biased HSCs in mouse [6]. The other half of the platelets are produced from HSC progressive MK commitment through several stages including the presence of a bipotent erythroid/MK progenitor (MEP) preceding a MK progenitor and underscoring the proximity between erythroid and MK differentiation [7-10]. These early differentiation steps are all characterized by an important proliferation without morphological criteria of cellular differentiation. However, some platelet proteins such as the von Willebrand factor (vWF) and the GPIIb/IIIa complex (αIib/β3 integrin)/CD41 may be expressed by some HSCs, more particularly by MK/platelet-biased HSCs [3,4]. MK progenitors are called CFU-MKs, when defined by clonal semi-solid culture assays [1], or MkPs, when defined by surface markers [11], but the two classifications may not exactly match as for example in humans only a fraction of the CFU-MKs expresses the CD41.

The late stages of MK differentiation are unique and implicate major cytoskeleton reorganization. In mammals, when MK precursors enter late stages of differentiation, they stop to divide and enter a process of polyploidization called endomitosis [12]. Endomitosis is close to a normal cell cycle, but with a defect in the cleavage furrow leading to a failure of cytokinesis and of karyokinesis [13-16]. There are several rounds of endomitotic cycle with a duplication of the DNA at each cycle [17,18]. This leads to a cell with a 2^n N ploidy (x being the number of cycles of endomitosis ranging from 1 to 6: 2N, 4N, 8N, 16N, 32N, 64N) and a modal ploidy of 16N. In parallel, the cell size increases with polyploidization, especially the cytoplasm expands at the end of endomitosis. Indeed, all alleles remain functional during polyploidization [19]. Thus, polyploidization leads to a large highly metabolic cell with a unique polylobulated and polyploid nucleus.

During polyploidization, the development of an important membrane system, called demarcation membrane system (DMS) arises from a tubular invagination of the plasma membrane, but also from a direct integration of Golgi-derived vesicles in the invaginated membranes [20,21]. DMS fold in the middle zone of the cytoplasm while maintaining continuity with the extracellular space. An interaction between the DMS and the F-actin cytoskeleton is indispensable to obtain a correct positioning of the DMS and a fully-developed network [22,23]. A link exists between pre-DMS formation and the endomitotic process [21]. However polyploidization is dispensable for DMS development as observed in microMKs (2N mature MKs) derived from cord blood or in pathologies [24-26]. At the end of maturation, the large MK contains three main regions: a perinuclear zone with the Golgi, the ER and mitochondria, a middle zone containing the DMS and the granules and a peripheral zone devoid of organelles. The peripheral zone is separated from the inner cytoplasm by an actin and microtubule network, which impedes the DMS to move to the periphery of the cell. At this stage the MK appears as a spherical cell.

At the terminal stage, MK produces platelets by a cytoplasmic fragmentation. Mainly based on in vitro experiments, it has been assumed that platelet production occurs via proplatelet formation [27-29]. Proplatelets are long pseudopods that contain all the MK organelles [30,31]. This process requires profound changes in the cytoskeleton. Indeed, proplatelets arise from the unfolding of its DMS. In vitro, the microtubules play a central role in the proplatelet extension serving as the motor of the elongation process. In contrast, the main role of the F actin cytoskeleton including actomyosin concerns the stages preceding the proplatelet formation (loss of the peripheral cytoplasmic zone, DMS development, MK polarization), but also the branching of the proplatelets to increase the efficiency of platelet production [22,23,32,33]. In vivo, proplatelet formation has been also observed, but differs from in vitro proplatelet formation due to the complex microenvironment and the need to create transendothelial pore in order to extend the initial protrusion. Microtubule organization is also different with heterogeneously distributed microtubules in vivo whereas they form thick arrays all along the proplatelet in vitro [27,28]. The role of microtubules as a power for elongation seems less important in vivo than in vitro with actin rich shoulders being also involved in the extension of the proplatelet [34,35]. However a recent study suggests that only a minority of adult bone marrow MKs underwent proplatelet formation. Thus there is a growing body of evidence that other mechanisms are responsible for platelet biogenesis in vivo. In stress conditions, it has been shown that platelets are produced by MK rupture associated with membrane blebbing and the activation of caspase 3 [36]. In basal condition, membrane budding seems to be the predominant mechanism of platelet production [37]. It leads to the release of buds with the same size as platelets [37]. In addition, in vivo MKs may extend very large protrusions in the sinusoids with a different microtubule organization than proplatelets [38,39].

Megakaryopoiesis takes place in BM. It is usually considered that MKs migrate from an “endosteal” to a “non-endosteal” niche, near the sinusoid [40]. In fact, there is evidence that the entire megakaryopoiesis takes place away from the endosteal niche except for the early stages, but at the end of maturation MKs migrate to be in close contact with the endothelium of the sinusoids [41]. Thus, there is a limited migration of MKs, whose role is not completely understood, inside the bone
marrow environment. The elongation of proplatelets through the endothelium barrier requires the degradation of the extracellular matrix and of the native basement membrane. The formation of podosomes plays a central role in this process [42-44]. The proplatelets are released in the blood circulation and are fragmented by the blood shear. They are first fragmented in preplatelets that are subsequently fragmented into two platelets [45]. The actin and tubulin cytoskeleton plays an important role in this fragmentation process. Alternatively but rarely, the entire MK migrates through the endothelial barrier and fragments in the blood circulation, more particularly in the lung circulation [46-48]. Very surprisingly, the lung was recently shown to be a main site of megakaryopoiesis and thrombopoiesis responsible for half of the daily platelet production in mice [49]. The presence of MKs in the extravascular space of the lung has been confirmed recently [50,51]. These lung MKs are hypoploid cells specialized in immune response, different from bone marrow MKs, but close to Antigen Presenting Cells such as monocytes or dendritic cells. [50] Whether these extravascular lung MKs really contribute to platelet production remain to be determined. Therefore in mice, it can be hypothesized that two different types of MKs may be present in the lung: one extravascular involved in immune response, another intravascular, probably of bone marrow origin, producing platelets through proplatelet formation [52]. In non-human primates, MKs are also detected in the lung parenchyma [50]. In human, MKs are enriched in the lung circulation, but it has not been yet demonstrated whether MKs are also present in the lung parenchyma [48]. Recently megakaryocyte nuclei have been detected inside the pulmonary venous circulation in severe COVID patients [53].

**Rho-GTPases as main regulators of the cytoskeleton and megakaryopoiesis**

Rho-GTPases are main regulators of the F-actin cytoskeleton dynamics, but also of microtubules. They play a central role in many cellular processes including cytokinesis, cell migration, cell polarity, and cell adhesion. In addition, they are involved in complex processes such as axonal elongation, which exhibits some similarities with proplatelet formation [54,55]. This crucial role of Rho-GTPases on cytoskeleton dynamics explains that they are important regulators of the MK

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**Figure 1.** Rho-GTPases, their activators and effectors involved in cytoskeleton remodelling during megakaryopoiesis.
terminal differentiation as well as of platelet function (see reviews [56-58]) (Figure 1).

Rho-GTPases belong to the RAS superfamily of small GTPases and include 20 members that are classified in classical and atypical Rho-GTPases [54,59]. The role of three main classical Rho-GTPases (RHOA, RAC1/RAC2 and CDC42) and their effectors on MK biology has been studied in details. Most Rho-GTPases cycle from an inactive GDP- to an active GTP-bound form under the regulation of specific guanine exchange factors (Rho-GEFs). In contrast, they are inactivated by GTPase-activating proteins (Rho-GAPs) that catalyze GTP hydrolysis. Under a GTP-bound form, the different Rho-GTPases bind and activate their downstream effectors. In addition, the RHO guanine nucleotide dissociation inhibitors (Rho-GDI) control their localization and degradation [54]. Their level of expression is also regulated at the transcriptional and post-transcriptional levels and their activity by different post-translational modifications.

Classically in fibroblasts, RHOA induces stress fiber, RAC1/RAC2 lamellipodia and CDC42 filopodia formation. In addition, CDC42 plays a central role in polarized migration and transmigration. However, their role on cytoskeleton remodeling is highly cell-dependent. The two main RHOA effectors are ROCK, which regulates actomyosin contractility through myosin II A activity and cell adhesion, and the mDia1/DIAPH1 form, which is both an actin nucleation (formation of stable actin multimers) and an elongation factor. These two RHO effectors cooperate more particularly in stress fiber formation and in the regulation of microtubule functions. CDC42 and RAC1/RAC2 activate the ARP2/3 complex, which is a nucleation factor that branches a new actin filament on a preexisting actin filament, in contrast to the RHOA-activated formins. CDC42 activates the ARP2/3 complex through the hematopoietic-specific WASP and the ubiquitously-expressed N-WASP. RAC1/RAC2 also activate the ARP2/3 complex via other members of the WASP family, the WAVE/SCAR proteins. Both CDC42 and RAC1/2 share PAKs as common effectors. Activation of PAKs induces the phosphorylation of LIMK that inactivates the coflin protein family involved in the disassembling of actin filament. Interestingly, LIMK are also activated by the RHOA/ROCK pathway.

RHOA, RAC1 and CDC42 regulate numerous genes involved in the cytoskeleton by controlling SRF transcriptional activity through the nuclear translocation of its partner MKL1 (also called MAL or MRTFA) [60]. These three Rho-GTPases also regulate the YAP/TAZ transcription pathway [61]. Additionally, RHOA and RAC1 interact with the NF Kappa B and Wnt pathways [62,63].

The role of Rho-GTPases on megakaryopoiesis has been studied by several approaches:

- The use of knock-out (KO) mice with a 'specific' deletion of the targeted gene in the MK lineage (see for review [56]). Mice with a floxed candidate gene were crossed with the Ptf4-Cre transgenic mice [64]. This approach is considered to be the gold standard to have a clear in vivo phenotype on platelet production and MK number and morphology [56]. However, this approach has some limitations: 1) The in vivo system alone is limited to studying the molecular mechanism, except if coupled to in vitro techniques or other approaches, 2) The Ptf4 promoter is not strictly MK specific [65,66] and in some cases the phenotype can be related to a non-cell autonomous mechanism [66,67], and 3) There is increasing evidence that several cytoskeleton components and regulators such as WASP, HDAC6/Cortactin and eventually DIAPH1 have different functions in mouse and human megakaryopoiesis [68-72]. Thus, results obtained in mouse models cannot be entirely extrapolated to normal and pathological human megakaryopoiesis. As an alternative model to mouse, the zebrafish has been used to screen the role of the genes identified in genomic approaches of inherited thrombocytopenia [73].
- In vitro studies of MK differentiation from human CD34+ cells or mouse BM Lin− cells. This approach includes several advantages such as the study of human megakaryopoiesis with an easy access to all stages of MK differentiation including the role of shear forces and migration. However, the cell culture techniques may modify the terminal differentiation and thus may not be totally indicative of what occurs in vivo [34]. In addition, in primary cells, the use of genetic manipulations such as genome editing remains limited despite recent progress. The MK differentiation of iPSC now allows access to genome editing approaches [74-76] and in some conditions to large numbers of MKs, facilitating molecular studies [77,78]. However, the haematopoiesis derived from iPSC is mainly embryonic, thus HSC independent, and does not totally reflect adult megakaryopoiesis. It is presently not possible to efficiently transplant haematopoietic cells derived from iPSC into immunodeficient mice to create in vivo models, may be due to the absence of functional HSC.
- Studies of inherited thrombocytopenia (Table 1). Many human inherited thrombocytopenia are
related to mutations of genes coding for Rho-GTPase effectors such as MYH9, a member of the ARP2/3 complex, WASP, and DIAPH1 [73]. Studies of the physiopathological mechanisms leading to thrombocytopenia using in vitro techniques including iPSC have permitted a precise characterization of the role of these genes in human megakaryopoiesis.

Therefore, it is important to combine several approaches such as mouse models and in vitro techniques, more particularly with human cells to better understand the role of Rho-GTPase on MK biology.

**Rho-GTPases and their effectors as regulators of MK polyploidization**

The mechanism of endomitosis and polyploidization, as shown by time-lapse microscopy, is an abortive mitosis with a defect in late cytokinesis [14,15]. During transition from the 2N to the 4N stage, the two daughter cells are nearly separated, but after rapid regression of the furrow the two daughter cells fuse to give back one mononucleated cell. The mechanism of this cytokinesis defect is related to a local reduction of RHOA activation. In addition to be present at low level in the cleavage furrow, RHOA is not significantly activated as demonstrated using a FRET-based RHOA biosensor [79]. The defect in RHOA activation has been linked to a low expression of two GEFs, GEF-H1 and ECT2 (Figures 1 and 2). The down-regulation of GEF-H1 is indispensable for the first (2N-4N) endomitotic cycle, thus for the switch from a proliferative stage to polyploidization, whereas low expression of ECT2 is necessary for subsequent polyploidization (>4N) [79]. There is evidence that the GEF-H1 (ARHGGEF2) expression in MKs is regulated by the MKL1/SRF pathway. Indeed, in Mkl1-deficient mice, Arhgef2 is upregulated with a decrease in MK polyploidization, which is mainly related to an excess of 2N and 4N cells [79].

RHOA activation induces the accumulation of F actin at the cleavage furrow by the formin/profilin

**Table 1.** Genes mutated in inherited thrombocytopenia and encoding for up- and down-stream molecules of Rho-GTPase pathways.

| RHOA effectors | Macrothrombocytopenia | Microthrombocytopenia |
|---------------|-----------------------|-----------------------|
| CDC42 effectors | MYH9, DIAPH1 | WASP, ADAP, ARPC1B |
| Cytoskeleton components | FLNA, ACTN1, TUBB1, TPM4 | |
| Receptor and signalling molecules | GP1BA, GP1BB, GP9, ITGA2, ITGB3, SRC, TRPM7 | PTPRJ |

machine that subsequently recruits myosin II. Myosin is activated by phosphorylation of MLC2 through ROCK and citron kinase. During MK differentiation, two types of myosin II are synthesized: myosin IIA (MYH9) and myosin IIB (MYH10). Myosin IIA requires more F actin accumulation than myosin IIB to be recruited at the cleavage furrow [80]. Thus, in MK precursors, the myosin IIB is almost the only myosin II to be recruited at the cleavage furrow [81]. However, during further MK differentiation, MYH10 (the heavy chain of myosin IIB) is silenced by a transcriptional complex containing RUNX1, which leads to a marked defect of contractile forces in the cleavage furrow potentially explaining the abortive cytokinesis [81].

PAK2, a downstream regulator of CDC42 and RAC1/RAC2, has been suggested to restrain the endomitosis of MKs. The cultured murine wild type and Pak2 deficient MKs are mainly different within the 2N ploidy class while the distribution of higher polyploid MKs is extremely close between the two models, with a normal modal ploidy at 16N [82]. Thus, PAK2 may regulate the switch from proliferation to endomitosis, but not directly the polyploidization itself. Aurora A could be the target of PAK2 as Pak2 KO decreases Aurora A phosphorylation and Aurora A inhibition increases polyploidization [82,83].

However, endomitosis cannot be summed up to an abnormal cytokinesis, but also requires a defect in both karyokinesis to avoid the generation of a multinucleated cell and the tetraploid checkpoint to allow further DNA replication after the 4N stage [16,84]. It is unknown whether Rho-GTPases are also involved in these two additional defects.

**Rho-GTPases and their effectors as regulators of MK proplatelet formation**

The important role of Rho-GTPases in platelet production was initially suspected following the identification of MYH9 and WASP mutations in two inherited thrombocytopenia, respectively (Table 1). It is now established that many inherited thrombocytopenia with altered platelet size are related to mutations in genes encoding direct Rho-GTPase effectors or molecules of the Rho-GTPase pathways. While macrothrombocytopenia are rather related to aberrant RHOA activation, mutations in CDC42 effectors lead to microthrombocytopenia. The implication of Rho-GTPase pathways in platelet formation was further confirmed using KO mice and in in vitro experiments.
RHOA and proplatelet formation

The role of RHOA in proplatelet formation has been demonstrated by both in vivo and in vitro experiments. Ptf4-Cre-induced RhoA deficiency leads a moderate macrothrombocytopenia (around 400,000 platelet/μL, 50% of the normal value) [85,86]. The decreased platelet life span in these mice was not sufficient to explain the thrombocytopenia. Moreover, mice displayed an increased number of MKs with a high ploidy and normal or increased proplatelet formation but a nonsynchronous polarization of the DMS was observed during MK maturation. Finally, the thrombocytopenia was suggested to be mostly related to an increased MK migration with abnormal proplatelet formation in the sinusoids (see next chapter) [87].

In mouse and human, the in vitro use of a dominant-negative form of RHOA or of the inactivating TAT-C3 exoenzyme increases proplatelet formation whereas an active form inhibits it [1,188]. Furthermore, the inhibition of PKCe induces RHOA activation, resulting in a defective formation of proplatelets [89]. Similarly, proteasome defects (pharmacological inhibition or genetic deletion) or Filamin A (FLNa) ablation results in the inhibition of proplatelet formation as a consequence of an inappropriate RHOA activity [74,90]. Additionally, the interaction of MKs with collagen I induces an integrin α2β1- and/or GPVI-dependent activation of RHOA preventing the premature formation of platelets in the BM [91,92]. However, the double constitutive Gp6 and Itga2 (encoding subunit alpha2 of α2β1 integrin) KO mice have no defect in platelet production [93]. The dysregulation of this pathway due to a gain-of-function mutation in GP1BA involved in the Platelet-type von Willebrand disease results in proplatelet formation and an ectopic release of platelets in the BM itself instead of inside the sinusoids [94].

RhoA has three main effectors directly or indirectly involved in proplatelet formation (Figures 1 and 2).

ROCK

In vitro selective pharmacological inhibition of ROCK 1 and 2 with Y27632 leads to the same phenotype as RHOA inhibition, i.e., an increase in proplatelet formation in both human and mouse MK culture [1]. Moreover, in vitro ROCK inhibition rescues the defect of proplatelet formation induced by activated RHOA as a consequence of a FLNa ablation or proteasome defects suggesting that ROCK is the main effector of RHOA activation [74,90]. In contrast, the constitutive genetic deletion of Rock1 has no effect on platelet production and platelet size [95] whereas specific MK/platelet Rock2 ablation leads to a very moderate macrothrombocytopenia (83% of normal platelet count) [96]. In contrast to RhoA deficient MKs, the phenotype observed when inhibiting ROCKs is much weaker, maybe due to a redundancy between the two ROCKs or to the role of other RHOA effectors in platelet production. Overall, the in vitro inhibition of RHOA by small molecules, TAT-C3 or a dominant-negative form increases proplatelet formation, and suggested that RhoA or Rock ablation in MKs would lead to a thrombocytosis. Thus, the presence of a macrothrombocytopenia after RhoA or Rock2 MKs ablation in vivo underscores that proplatelet formation is not the only determinant of the blood platelet level. Indeed, the RHO/ROCK pathway may play some opposite roles in the different processes that lead to platelet production and clearance. It will be important to study the space-time activation of RHOA and ROCK during MK differentiation to better understand their precise role in the different steps of platelet production.

The in vitro inhibition of proplatelet formation by ROCK is related to an increased myosin IIA (MYH9) activity through the phosphorylation of MLC2 [97]. ROCK induces the phosphorylation of MLC2 either directly or indirectly through inhibition of the myosin phosphatase. The role of myosin II is underscored by the fact that inhibition of its activity by blebbistatin leads to an increased proplatelet formation [88,98,99]. Based on in vitro experiments, it is considered that myosin IIA and actomyosin are not directly involved in proplatelet formation itself, which is driven by microtubules, but rather play a role in the branching process of proplatelets that increases platelet production [29]. However, they clearly play an important upstream role by regulating the cortical contractile forces, DMS relocalization and MK polarization required for efficient proplatelet formation. In addition, actomyosin forces may be required at the beginning of proplatelet formation induced by the shear forces and for proplatelet and platelet fission from proplatelets [99]. Furthermore, in BM microtubules are dispensable in the driving forces of proplatelets, while myosin IIA may play a more important role in their growth [34].

Myh9 deletion in the MK lineage leads to a macrothrombocytopenia associated with a disorganized DMS [100,101] that induces an increased proplatelet formation in vitro in standard liquid culture and inversely a defect in BM environment in vitro [100,102]. In vivo, Myh9−/− compared to wt MKs rapidly elongate longer and thinner proplatelets. In human, the heterozygous MYH9 mutations localized either in the rod or motor domain of myosin
IIA result in a macrothrombocytopenia [73]. (Table 1) However, these mutations do not lead to a totally similar phenotype as Myh9 KO. Indeed, in contrast to Myh9 KO mice, in vitro proplatelet formation is markedly decreased and abnormal in patients with a MYH9-related disorder or in Myh9 knock-in mice [88,97]. Surprisingly, the defect in proplatelet formation is rescued by blebbistatin and thus by the inhibition of myosin IIA activity [88]. There is evidence that these mutations confer to MKs an abnormal myosin IIA activity with some of them leading to a myosin IIA hypercontractility and others to a defect in contractility [103]. Thus, thrombocytopenia is due to complex mechanisms including defects in MK migration, which was not described for Myh9-deficient MKs, proplatelet formation and fission [88,97,99,103].

The crucial role of actomyosin in proplatelet formation has also been well documented when studying heterozygous mutations in the ACTN1 gene encoding for α-actinin (Table 1). The α-actinin dimers cross-link actin filaments with myosin to generate contractile forces [104,105]. Additionally, heterozygous mutation in the TPM4 gene encoding a tropomyosin 4 that binds to and stabilizes actin filaments also result in macrothrombocytopenia [106]. (Table 1)

**DIAPH1**

DIAPH1, usually called mDia1, has been described as one of the main effectors of RHOA in the regulation of actin stress fiber formation. It acts on both accelerating actin filament elongation and increasing microtubules stability via post-translational modifications. The coordination of these two processes allows a cross-talk between the two types of cytoskeleton. Dia1 constitutive KO mice develop a mixed myeloproliferative/myelodysplastic syndrome with a predominance of the granulocytic and myeloid lineages and an extramedullary hematopoesis that partially mimics the 5q- syndrome in human (DIAPH1 is localized in 5q) [107]. Abnormalities in the number and the function of platelets were not initially described although these mice may present an increased platelet size [70]. **DIAPH1** knock down (KD) in human cultured MKs leads to a marked increase in proplatelet formation with a decreased F actin polymerization and a surprising increased microtubule stability [69]. **DIAPH1** KD and ROCK inhibition cooperate to increase proplatelet formation suggesting that RHOA regulates proplatelet production by both effectors. As expected, a constitutive active form of DIAPH1 inhibits proplatelet formation of human cultured MKs [69]. In agreement with this in vitro approach, **DIAPH1** gain-of-function mutations, which can be associated with hearing loss and a moderate neutropenia, were subsequently found in a rare form of inherited macrothrombocytopenia [108,109]. (Table 1)

Interestingly, numerous small size MKs with a hypolobulated nucleus are observed suggesting that **DIAPH1** gain-of-function mutations inhibits ploidization.109 The R1213* variant found in the first described patients induces an increased stability of microtubules in contrast to what was observed with another constitutive active form [69,108]. The disorganization of microtubules in patients with TUBB1 germline mutations also leads to macrothrombocytopenia [110].

### The SRF transcriptional pathway

RHOA regulates the transcription of numerous genes in MKs. MKL1 (MRTFA) is the main cofactor of SRF linking transcription to the cellular G-actin levels. A recurrent translocation (t:1;22) leading to the RBM15-MKL1 (also called OTT–MAL) fusion protein was identified in a pediatric acute megakaryoblastic leukemia [111,112]. This fusion protein is a constitutive activator of SRF, independently of the G-actin level [113]. Among the MRTF family members, only MKL1 and, at lower level, MKL2 are expressed in MKs [114]. SRF must be associated either to ternary factors regulated by the MAPK pathway or to the MRTF family members to be transcriptionally active. Actin polymerization induces MKL1/2 localization in the nucleus either by allowing their shuttle from the cytoplasm and/or by retaining them in the nucleus [115]. Three effectors of the Rho-GTPase family are directly involved in the regulation of actin polymerization: ROCK, DIAPH1 and LIMKs, making a link between the regulation of actin cytoskeleton and transcription. Furthermore, many SRF target genes encode components of the actin cytoskeleton and thus Rho-GTPases can regulate the expression of their own targets.

A specific deletion of Srf in the MK lineage leads to a moderate macrothrombocytopenia (50% of normal platelet number) while an interferon-inducible Srf KO in hematopoietic cells leads to a more severe thrombocytopenia [116,117]. The thrombocytopenia is associated with an increased number of MKs with an abnormal cytoskeleton organization and a defect in DMS development and granule distribution. The decrease in proplatelet formation and a MK fragmentation in the marrow suggests a defect in MK migration [117]. Constitutive Mkl1 KO mice have a milder phenotype with also a defect in proplatelet formation whereas mice with a conditional Mkl2 KO in the MK lineage have no significant phenotype [114,117,118]. However the double Mkl1/2 KO mice develop a more
profound thrombocytopenia close to what was observed in Srf KO mice [114]. It has been suggested that the effects of MKL1 and MKL2 might be mediated by both a Rho-dependent and -independent mechanisms because more genes are deregulated in the double KO than in the Srf KO MKs [114].

In human, the nuclear localization of MKL1 is also regulated by the Rho-GTPase pathway and its KD by a shRNA leads to a major defect in MK maturation with an abnormal cytoskeleton, an abnormal distribution of α-granules and a disorganized DMS leading to defects in proplatelet formation and migration, a phenotype close to KO mice [119]. MKL1 KD leads to the deregulation of around 600 genes including many genes encoding cytoskeleton components and regulators. MYL9 encoding for MLC2 is found among the most deregulated genes and its KD also leads to a defect in proplatelet formation in cultured human MKs suggesting that it is an important target of SRF/MKL1 during MK terminal maturation [119].

**CDC42 and RAC and proplatelet formation**

Overall, CDC42 and RAC1 play an inverse role than RHOA on proplatelet formation and cell migration.

The role of CDC42 and RAC on MK differentiation was investigated in mouse using a KO strategy. The loss of Rac1 or Rac2 in all haematopoietic cells or in MK/platelets modifies neither the platelet count nor the platelet size [120,121] whereas Cdc42 MK/platelet ablation induces a moderate macrothrombocytopenia (50% of normal platelet count) [122,123]. In addition, a constitutive ablation of RhoG, an atypical member of the Rac subfamily of the Rho family, does not alter proplatelet formation [124]. As expected, proplatelet formation is not affected in Rac1 KO mice whereas it is slightly decreased in Cdc42 KO mice due to a decrease in membrane invagination and MK cytoplasmic organization [87,123]. Interestingly, the MK/platelet-specific double Rac1/Cdc42 KO induces an important macrothrombocytopenia (around 25% of normal platelet count) associated with the quasi-absence of proplatelet formation and of the peripheral MK cytoplasmic zone [123]. In addition, the half-life of platelets is decreased. Thus, there is a clear redundancy between Rac1 and CDC42 with the latest being more important. The deficit in proplatelet formation was attributed to a defect in tubulin organization due to coflin inactivation [123] that may be related to RHOA activation [87] and to a downregulation of IQGAP1 [123].

The in vitro pharmacological inhibition of CDC42 in murine MKs induced a stronger phenotype than Cdc42 KO resembling the phenotype of the double Rac1/Cdc42 KO with a quasi-absence of DMS [22]. One possible hypothesis is that the CDC42 inhibitors used (CASIN and ML141) may also inhibit other members of the CDC42 family such as TC10 expressed in the MK/platelet lineage. It is also possible that in mice the Cdc42 KO might be compensated by other Rho-GTPases. This last study points out the role of CDC42 in the development of DMS, more particularly in the invagination of the membrane, and of F actin in the normal repartition of DMS [22]. A similar observation was made on human MKs using either pharmacological inhibition by CASIN or transduction of a dominant negative form of CDC42. In addition, a constitutive active form of CDC42 increases proplatelet formation. This important role of CDC42 in platelet formation in human is strongly supported by the identification of a deleterious de novo heterozygous mutation in CDC42 in a propositus with a macrothrombocytopenia ranging from to 50,000 to 90,000 platelets per μL of blood [125].

There is increasing evidence that CDC42 acts downstream of GPIb in MKS presumably associated with FLNa and F-BAR proteins such as PACSIN2 and CIP4 [126,127]. This complex is necessary to induce the first invagination of the plasma membrane to form a pre-DMS (Figure 2).

Two major effector pathways of CDC42 and RAC appear to be involved in proplatelet formation (Figures 1 and 2):

**WASP and WAVE and the activation of the ARP2/3 complex**

The WASP gene located on the X chromosome was initially discovered as being responsible of the Wiskott Aldrich syndrome (WAS) [128]. WASP mutations induce two disorders: one characterized by a microthrombocytopenia without clinical immunodeficiency called XLT (X-linked thrombocytopenia) and the WAS characterized by a profound microthrombocytopenia, an immunodeficiency and eczema [129]. The differences between the two disorders are related to the level of residual WASP protein, and thus to the level of functional activity. Thrombocytopenia results from a mild decrease in the protein whereas a nearly total absence of the protein causes a profound immunodeficiency [130]. So, the MK/platelet lineage is extremely sensitive to the level of WASP. However, the complex mechanism responsible for the thrombocytopenia remains partially understood. Furthermore, constitutive Wasp ablation in mouse only resulted in a mild thrombocytopenia with normal size platelets associated with an immune deficiency [68]. In human, mutations in
**WIPF1** encoding WIP (WASP-Interacting protein) that binds and stabilizes WASP, induces a disorder resembling WAS [131]. Constitutive Wip null mice do not exhibit a thrombocytopenia, but an immune deficiency [132]. In vitro studies have shown that MKs derived from WAS patients display normal proplatelet formation and generate platelets of normal size in vitro [133]. Indeed, the small size of platelets seems to be a secondary event as suggested by the fact that splenectomy in human partially corrects the platelet both size and count [130]. Thus, the microthrombocytopenia may be the consequence of an altered platelet membrane with a further fragmentation in the circulation, or an increase in the phagocytic properties of spleen macrophages. In agreement with this hypothesis, the platelet half-life is decreased in WAS patients. In mice, a similar decrease in platelet half-life is observed suggesting that the thrombocytopenia might be in part related to an increase in platelet clearance [134]. There is evidence that the platelet membrane is abnormal with misarranged hyperstable microtubules that may be responsible of the microthrombocytopenia [135]. Surprisingly, the Wasp KO mice present an increased number of MKs with aberrant fragmentation inside BM suggesting a premature release of platelets that may be related to the absence of podosome formation [42] (see next chapter). A similar phenotype is found in mice with a specific ablation of Profilin 1 (*Pfli1*) in the MK lineage, PFN1 being an actin regulating protein that binds monomeric G actin, promotes actin elongation and interacts with WASP [136]. One of the major role of WASP is to activate the ARP2/3 complex that induces F actin branching. The ARP2/3 complex is composed of ARP2 and ARP3 and 3 associated proteins (ARPC2, 3 and 4). In human, a complete homozygous loss-of-function mutation in **ARPC1B** has been shown to induce a profound microthrombocytopenia with platelets very similar to WASP null platelets whereas mutations leading to the presence of a residual protein induce only changes in platelet size (microplatelets) without thrombocytopenia [137]. However, in contrast to what was observed for WASP, **ARPC1B**-null MKs differentiating from an iPSC-derived continuous cell line (imMKCL) present a decreased proplatelet formation [137]. In mouse, genetic deletion of the Arp2/3 complex (Arpc2 KO) in the MK lineage also leads to a moderate microthrombocytopenia (around one third of the normal platelet count) with an abnormal tubulin ring, a decreased half-life and a premature release in the bone marrow, a phenotype close to Wasp null mice, but more severe [138]. In addition, a normal proplatelet formation was observed. Another study using an ARP2/3 inhibitor shows an increase in proplatelet formation in vitro [139]. Thus it is possible that the ARP2/
3 complex activation may not be directly implicated in the proplatelet formation. There is evidence of a cross-talk between the ARP2/3 complex and HDAC6 for the regulation of tubulin stability and the function of cortactin, an actin binding protein that simultaneously binds F actin, the ARP2/3 complex and N-WASP [72,140]. Inhibition of HDAC6 in human, but not murine MKs inhibits proplatelet formation and this effect is surprisingly not mediated by α-tubulin acetylation, but by cortactin acetylation [71,72].

MKs not only express WASP, but also the ubiquitous N-WASP reaching one fifth of WASP expression level [141]. The two proteins are highly homologous, have many identical, but also some specific functions [142]. Surprisingly, N-WASP KD, but not of WASP KD by an shRNA strategy leads to a decrease in proplatelet formation with the quasi-absence of DMS and the presence of large vacuoles in cultured human MKs [141]. This defect in proplatelet formation is in part attributed to the activation of the RHOA pathway suggesting that, in contrast to WASP, N-WASP is involved in the cross-talk between the CDC42 and the RHO pathways [141]. Another possible mechanism could be that F-BAR protein CIP4 (CDC42 interacting protein 4) is necessary for membrane invagination and DMS development associates with N-WASP, but not WASP [127].

The ARP2/3 complex can be also regulated by the WAVE/SCAR family, which is activated by RAC through IRSp53. The three forms of WAVE (WAVE1-3) are expressed in platelets and MKs, but WAVE3 at very low level [143]. Constitutive WAVE1 KO is lethal after birth, but transplantation of Wave1−/− foetal liver cells into irradiated recipient mice restores a normal haematopoiesis with normal platelet counts and MKs, demonstrating that WAVE1 is dispensable for MK differentiation [144]. However, WAVE1 has an important function in platelet activation. In contrast to WAVE1 KO, Wave2 KO ES cells have a defect in in vitro MK terminal differentiation with a defect in proplatelet formation and platelet production [144]. It is unknown if the adult megakaryopoiesis is similarly affected. Indeed, the effect of WASP ablation was different between primary adult MKs and MKs derived from iPSCs [133,145]. It remains to be determined whether the effect of WAVE2 is mediated through the ARP2/3 activation or by another mechanism.

**PAK2**

CDC42 and RAC also activate the kinase activity of the members of the PAK family. In the group I, the phosphorylation of PAK1/2/3 and the expression of PAK2 increase during human MK differentiation. An inducible Mx-Cre Pak2 KO leads to a moderate macrothrombocytopenia (50% of the normal platelet count) related to an increase in platelet clearance and a defect in proplatelet formation [82]. In contrast, a constitutive Pak1 KO had no effects on MK differentiation and platelet count [82]. Another study showed that Cdc42 regulates Pak2 phosphorylation in cultured murine MKs and that chemical PAK inhibition impairs DMS development and polarization leading to a defect in proplatelet formation [22]. These two studies underscore the major role of the CDC42/PAK pathway in F actin distribution necessary for DMS development in mouse MKs. In contrast to mouse MKs, our group did not find any major effect of chemical inhibition of PAK or of PAK2 KO on maturation, DMS development and proplatelet formation as well as on endomitosis of cultured human MKs (Debili et al., in preparation). Therefore, the role of PAKs on megakaryopoiesis deserves further investigation.

PAK2 phosphorylates to activate its major target LIMK1 that in turn phosphorylates and inactivates coflin. MKs and platelets express two members of this F actin severing protein family, N-COFILIN/COFILIN 1 (COF1) and ADF [146]. Mice with Ptf-Cre-induced Cof1 deficiency have a mild macrothrombocytopenia whereas a constitutive Adf KO has no effect on MK maturation [146]. The double Adf/Cof1 KO leads to a marked defect in MK maturation with altered DMS development and proplatelet formation [146]. Similarly, the double Twinfilin1 (Twf1)/Cof1 KO in the MK lineage induces a severe macrothrombocytopenia with defects in proplatelet and podosome formation [147].

LIMK1 is the LIMK, which is expressed at the highest level in platelets, but LIMK2 can be also detected [148,149]. Constitutive Limk1 and Limk2α KO mice have no defect in platelet count and volume [150]. However, it has been shown that RHO activation in severe von Willebrand disease-type 2B associated with a macrothrombocytopenia induces the ROCK/LIMK1/COFILIN pathway and LIMK chemical inhibition rescues the proplatelet formation defect observed in vitro [151]. The opposite result is observed in MK lineage deficient phosphoinositide-dependent kinase 1 (Pdk1) mice, where the macrothrombocytopenia has been attributed to the abrogated PAK/LIMK1 pathway leading to an increased COFILIN activity, as previously suggested for Pak2 KO mice [33,82]. However, a recent study shows that PDK1 ablation or inhibition leads to the inactivation of the PI3K/PDK1/AKT pathway and more particularly to the overall inhibition of protein translation [152]. The defect in proplatelet formation might be the consequence of the
downregulation of MARCKS (a PKC substrate) due to the translational defect [152]. MARCKS expression increases during proplatelet formation and interacts with the ARP2/3 complex and microtubules [139].

In addition, the PAK family has other substrates such as AURORA A involved in MK differentiation and podization, and MLC2 and FLNa involved in proplatelet formation.

**Rho-GTPases and their effectors as regulators of MK migration in the marrow and of proplatelet elongation through the endothelial barrier**

For efficient platelet biogenesis, the last steps of MK differentiation take place in close proximity of the BM sinusoids. It is rare to see the entire MK transmigration at the end of maturation and rather proplatelets elongate through the endothelium barrier in order to release platelets in the blood circulation (Figure 2).

CDC42 is the main Rho-GTPase involved in transmigration of MKs across the endothelial barrier and its activity is negatively regulated by RHOA [87]. A deletion of Cdc42 in MK/platelet induces a mislocalization of MKs in the BM with only rare MKs in contact with BM sinusoids. RhoA deletion induces a high activation of Cdc42 and the transmigration of MKs into the BM sinusoids where their subsequent fragmentation takes place. In contrast, Cdc42+/−/RhoA−/− MKs are localized close to the sinusoids and do not transmigrate [87]. The activation of CDC42 and RHOA occurs downstream of the GPIbα ectodomain [87]. It has been suggested that the association between GPIbα and FLNa is required to regulate CDC42 activity during MK transmigration. In contrast, FLNa/β3 but not FLNa/GPIbα interaction was shown to be crucial for proplatelet formation during *in vitro* human MK differentiation, since the absence of FLNa/β3 interaction leads to the increased in both αIIbβ3 and RHOA activities [74]. The absence of the GPIb complex at the MK/platelet membrane due to mutations in genes encoding component of this complex is responsible for the Bernard Soulier syndrome and gain-of-function mutations in *ITGA2B* and *ITGB3* genes encoding components of αIIbβ3 complex induce autosomal dominant thrombocytopenia, both diseases are characterized by the presence of macro platelets [73]. Further studies are necessary to examine whether RHOA/CDC42 deregulation is responsible for these two diseases.

CDC42 and RAC control the polarized migration of MKs towards sinusoids inside the BM environment as a result of the chemokine CXCL12 interaction with its receptor CXCR4. This migration plays an important role in the platelet production as the forced redistribution of MKs by increased expression of CXCR4 after VEGFA stimulation leads to an increased thrombopoiesis [153]. Moreover, the residual number of platelets in constitutive *Mpl* or *Thypo* KO mice has been attributed to the localization of the MKs close to the sinusoids [154] although other growth factors such as erythropoietin may be responsible for this residual platelet production [155]. However, the loss of interaction between CXCR4 and CXCL12 and/or signalling is required for MK transmigration [156,157].

WASP and the ARP2/3 complex activation are considered to be the main effectors of the CDC42-regulated polarized migration. In addition, WASP is absolutely required for the formation of podosomes by MKs [42]. Podosomes are essential both *in vitro* and *in vivo* to degrade the extra cellular matrix and the basement membrane allowing MK transmigration and/or the extension of proplatelets in the sinusoids [43,44].

Interestingly, deletion in five different genes leads to the same phenotype: WASP (human), WIP (human), genes of the ARP2/3 complex (human and mice), *PFN1* (mice), ADAP responsible of the autosomal-recessive small-platelet thrombocytopenia syndrome (human and mice) (Table 1) [73]. The common phenotype is usually characterized by a microthrombocytopenia with a premature release of platelets in the marrow and a short platelet half-life due to their destruction, mainly in the spleen that may spare the microplatelets that may more easily circulate in the spleen circulation. All these five genes are involved in the CDC42-WASP-ARP2/3 signalling pathway playing a major role in MK transmigration and in microtubule stability that might be responsible of the decreased platelet size and short life. Recently, homozygous loss-of-function mutations in PTPrJ responsible of a defect in CXCL12 dependent MK migration were described in an inherited microthrombocytopenia [103,158]. However, PTPrJ is essentially a regulator of the SRC signalling pathway [159,160]. Interestingly no thrombocytopenia was observed in mice with either a constitutive Ptprj or a conditional KO [160]. This further underscores that mouse models are not always predictive of what occurs in human MK/platelet diseases.

RHOA also plays a direct role in MK migration by regulating the activity of myosin IIA. The thrombocytopenia of the MYH9-related disorder is in part due to a defect of MK migration towards BM sinusoids [103].

In conclusion, the Rho-GTPases act as regulators of the cytoskeleton and of transcription (Figure 1) and play a central role in late stages of megakaryopoiesis that require the regulation of F-actin and a crosstalk with microtubules to develop a normal DMS distribution, MK migration in the BM, proplatelet formation and proplatelet elongation through the
endothelium barrier or MK transmigration in the circulation (Figure 2). This important role is underscored by the fact that most human inherited thrombocytopenia associated with changes in platelet size are related to mutations in genes involved in the Rho-GTPase pathways (Table 1). In addition, the Rho-GTPases play an important role in platelet function (not developed in this review). However, many unsolved questions remain on how the Rho-GTPase are precisely regulated during differentiation, whether other Rho-GTPases than RHOA and CDC42 might also be involved in MK differentiation and the basis of the differences observed between mouse models and some human inherited thrombocytopenia. A deeper knowledge of the role of Rho-GTPases during MK differentiation may be important to understand the pathogenesis of human inherited thrombocytopenia and to develop new therapies for thrombocytopenia and thrombocytosis as well as to optimize the platelet production from cultured MKs.

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