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Pathological interactions between hematopoietic stem cells and their niche revealed by mouse models of primary myelofibrosis

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Abstract

Primary myelofibrosis (PMF) belongs to the Philadelphia-negative myeloproliferative neoplasms and is a hematological disorder caused by abnormal function of the hematopoietic stem cells. The disease manifests itself with a plethora of alterations, including anemia, splenomegaly and extramedullary hematopoiesis. Its hallmarks are progressive marrow fibrosis and atypical megakaryocytic hyperplasia, two distinctive features used to clinically monitor disease progression. In an attempt to investigate the role of abnormal megakaryocytopoiesis in the pathogenesis of PMF, several transgenic mouse models have been generated. These models are based either on mutations that interfere with the extrinsic (thrombopoietin and its receptor, MPL) and intrinsic (the GATA1 transcription factor) control of normal megakaryocytopoiesis, or on known genetic lesions associated with the human disease. Here we provide an up-to-date review on the insights into the pathobiology of human PMF achieved by studying these animal models, with particular emphasis on results obtained with *Gata1*^{low} mice.

Keywords

animal model; GATA1; hematopoietic stem cell; megakaryocyte; primary myelofibrosis; transcription factor

Primary myelofibrosis (PMF) is a rare hematological malignancy, with an estimated incidence of 0.3–1.5 cases per 100,000 individuals per year [1]. PMF is a member of a family of diseases named chronic myeloproliferative disorders (MPDs) in 1951 by Dameshek [2] and very recently renamed myeloproliferative neoplasms (MPNs) [3,4]. According to the WHO, chronic MPNs are classified into Philadelphia-positive (Ph⁺) and Philadelphia-negative (Ph⁻) diseases. The first group is represented by chronic myelogenous leukemia (CML). Thanks to the

discovery of the Philadelphia chromosome by Nowell and Hungerford in 1960, CML was the first cancer associated with a specific genomic aberration [5]. The subsequent characterization of the Ph t(9;22)(q34;q11) translocation in 1984 [6] and of the deriving *BCR*—*ABL* fusion gene in 1985 [7] marked the beginning of a new era in understanding the pathobiology of this disease. *BCR*—*ABL* is a powerful oncogene that results from the fusion of the *BCR* on chromosome 22 and the human homolog of the *ABL* tyrosine kinase gene on chromosome 9 [7]. The resulting *BCR*—*ABL* fusion protein displays constitutive tyrosine kinase activity that triggers several intermediate cytosolic signals overcoming the normal signaling of a variety of cytokines, including SCF [8]. The identification of *BCR*—*ABL* led to the important discovery of imatinib, a small molecule that reverses the clinical manifestations of CML by inhibiting the deregulated activity of this oncoprotein [9].

Philadelphia-negative MPNs include three classical MPDs, polycythemia vera (PV), essential thrombocythemia and PMF, as well as chronic neutrophilic leukemia, chronic eosinophilic leukemia/hypereosinophilic syndrome, mast cell diseases and other nonclassifiable MPNs [3, 4]. PMF is characterized by anemia, splenomegaly, leukoerythroblastosis, abnormal trafficking of CD34⁺ stem/progenitor cells in the peripheral blood, multiorgan extramedullary erythropoiesis, especially involving spleen and liver, and destruction of the bone marrow structure due to progressive fibrosis, neoangiogenesis and increased bone deposits (Table 1) [1,2,10–17]. The disease begins with an asymptomatic phase, also defined as prefibrotic, which proceeds with the first manifestations (usually splenomegaly and fatigue) and culminates into the fibrotic stage (Table 1). The symptomatic stage of the disease normally has poor prognosis (life span < 5 years). The principal causes of death include infections, thrombohemorrhagic events and leukemic transformation. In fact, 3.9–20% of PMF patients develop lethal acute myeloid leukemia [18,19]. In addition, Ph⁻ MPNs are clonal stem cell malignancies originating from genetic anomalies that alter the physiological response of the pluripotent hematopoietic stem/progenitor cells to growth factors, thus affecting the balance between proliferation and differentiation in these cells.

Recent studies have highlighted the presence of gain-of-function mutations in the Janus kinase-2 (JAK2), protein (JAK2 V617F, JAK2 T875N and exon 12 mutant alleles) in patients with Ph⁻ MPNs [20–26]. JAK2 is constitutively associated with the receptors for several hematopoietic growth factors, including thrombopoietin (TPO) [27,28], erythropoietin (EPO) [29–31], granulocyte colony-stimulating factor [32] and SCF [33–35], and is, therefore, the first protein to be activated when these receptors are engaged by the corresponding growth factors. The JAK2 V617F substitution replaces valine with phenylalanine in the autoinhibitory domain of the enzyme, leading to its inability to fold back to the off-modus after activation [36,37]. The JAK2 T875N activating mutation replaces a threonine with an asparagine at position 875 of the JAK2 JH1 kinase domain [26]. Both JAK2 V617F and the JAK2 T875N, result in permanent signaling that partially explains the hypersensitivity to cytokines that characterizes hematopoietic cells from MPDs [38,39]. In addition, a set of JAK2 exon 12 mutant alleles have recently been described that induced constitutive activation of JAK—STAT signaling [40]. Furthermore, evidence linking JAK2 signaling with the onset of Ph⁻ MPNs is provided by the identification of the *TEL*—*JAK2* oncogene not only in patients with T- and B-cell acute lymphoblastic leukemia but also in some patients with atypical CML [41–44]. The *TEL*—*JAK2* translocation juxtaposes the oligomerization domain of the Ets-family transcription factor *TEL* with the catalytic domain of JAK2. The corresponding fusion protein is constitutively activated, inducing signaling abnormalities comparable to those that arise due to the *BCR*—*ABL* oncogene [44]. However, although the JAK2 V617F mutation accounts for 90% of PV patients it is found in only 50% of PMF patients [9–14]. Within a minority (between 5–10%) of JAK2 V617F-negative PMF patients, two somatic mutations have been identified in the autoinhibitory loop of the transmembrane domain of MPL (MPL W515L and MPL W515K) [25,45,46]. The W515L, and possibly W515K, substitution leads

to constitutive activation of the JAK—STAT pathway and thereby confers cytokine-independence to hematopoietic progenitor cells (HPCs) [46]. MPL is not only expressed by megakaryocytic cells but also by stem cells and early HPCs. In particular, the interaction between TPO, expressed by the bone marrow stroma, and MPL, expressed by the HPCs, has been shown to play a critical role in maintaining the homeostasis of the stem cell niche [47–50]. Thus the TPO—MPL axis plays an essential role not only in platelet development, but also in the biology of the hematopoietic stem cells [50,51].

In spite of the great body of evidence linking mutations of JAK2 signaling with PMF, the mechanism by which a single mutation can give rise to all the pleiotropic traits of PMF is still poorly understood. It is clear that PMF is the result of multiple genetic alterations accumulated as disease progresses. In fact, although PMF is mainly sporadic, familial predisposition has been observed, indicating the importance of silent mutations that may favor accumulation of JAK2 mutations [52–55]. Furthermore, diverse chromosomal aberrations, such as del(5q), del(13q), del(20q) and trisomy(1q) (Table 2) arise during the transition of the disease to its leukemic stage [56–61]. However, because of their sporadic occurrence these mutations cannot be considered as a diagnostic karyotype. Additional genetic alterations observed in PMF are represented by high levels of expression and/or mutations of *c-KIT*, the gene encoding the SCF receptor, found in HPCs circulating in these patients [62–67]. However, since none of these *c-Kit* mutations are unique to PMF, they may also represent secondary events occurring in an already destabilized stem cell compartment (Table 2).

The similarity between the biochemical defects induced by the JAK2 mutation and those induced by BCR—ABL had raised hope that a JAK2 inhibitor with similar effects to those exerted by imatinib on BCR—ABL might represent an effective therapy for PMF [68]. The importance of finding medications to cure the disease is stressed by the fact that the only cure available to these patients at present is allogeneic bone marrow transplantation [10–16]. Unfortunately, only a minority of PMF patients are eligible for this therapy, either because of difficulties in finding allogeneic matched donors, or for risk considerations usually linked to the advanced age of these patients. Several small-molecules capable of inhibiting the JAK2 kinase (e.g. INCB018424, XL019 and TG101348) [69], and many others that have off-target kinase-inhibitory activity (e.g., MKS-0457, CEP-701 and AT9283) have now been identified (reviewed in [69,70]). The therapeutic efficacy of many of these is being tested in ongoing Phase II clinical studies. However, as predicted on the basis of the importance of JAK2 signaling in hematopoietic cells, the preliminary results of these trials have produced significant evidence showing dose-dependent cytopenia as a major side effect of such drugs. The difficulty of finding a chemical approach to cure PMF highlights the importance of studies in animal models, not only for preclinical evaluation of efficacy and toxicity of JAK2 inhibitors, but also to better understand the pathobiological pathway of PMF in order to identify additional targets for therapy.

Megakaryocyte abnormalities as a ‘cellular signature’ of PMF

Megakaryocyte (MK) development is a tightly regulated process characterized by specific ultrastructural changes that are very similar between mice and men. The transition from promegakaryoblast to MK is associated with a progressive formation of platelet secretory granules (α -granules) and the production of an elaborate system of cisternae and tubules, known as demarcation membrane system (DMS), that spread through the cytoplasm of the cells. The DMS serves both to identify fields, or territories, from which platelets will be released via fragmentation and to increase the membrane surface necessary for the elaboration of proplatelets [71]. The composition of the α -granules is conserved between MKs and platelets. As discovered by Italiano *et al.*, the α -granules of MKs and platelets belongs to two subtypes (Figure 1) [72]. One subtype includes proangiogenic factors, including VEGF and angiotensin

1 (ANG1). The other subtype stores anti-angiogenic molecules, such as endostatin and thrombospondin-1 (TSP-1). Anti-angiogenic α -granules also contain von Willebrand factor (VWF), which is thought to act as a receptor to regulate the release of the α -granules' content in response to thrombin. The corresponding proangiogenic α -granule-specific receptor has not yet been identified. However, the observation that the leukocyte receptor P-selectin and VWF are localized on different α -granules [ZINGARIELLO M *ET AL.*, MANUSCRIPT SUBMITTED], suggests that it may be represented by P-selectin.

Platelets are not only involved in clotting but also in inflammation and wound healing, as they represent efficient long-distance delivery units for several biologically active substances all around the body. The mechanism that mediates the release of the content of the platelet α -granules in the microenvironment in response to injury or inflammation is starting to emerge (Figure 1). It is conceivable that platelets are first trapped to the site of damage by bridges established through the platelet receptor (GPIIb), with plasma VWF and collagen exposed by the vessel injury. Tighter platelet contacts established by direct interaction between collagen and glycoprotein VI allow the platelets to be activated through locally produced thrombin. As part of this activation process, platelets flip-flop the α -granule membranes exposing the molecules present in the inner layer, including VWF, to external agents. In the early phase of the response to injury, α -granule-bound VWF binds thrombin, inducing the release of anti-angiogenic factors. At a later stage, neutrophils recruited to the inflammation sites, by interacting with P-selectin, may induce release of the content of proangiogenic α -granules (FGF, TGF- β , TGF- α osteoprotegerin [OPG] and bone morphogenic protein 4 [BMP4]) (Figure 1) [73–81] inducing tissue repair.

One of the diagnostic features of PMF is the presence of MKs abnormal in both number and morphology in bone marrow biopsies. These alterations include an increased proliferation with delayed maturation, a normal expression level but abnormal localization of P-selectin on the DMS and a reduced expression of VWF (Figure 1). In addition, in spite of the fact that the *GATA1* gene is normal, MKs from PMF patients express reduced levels of GATA1 [82], a transcription factor that controls the expression of many MK-specific genes, hence the reduced levels of VWF expression [83]. The abnormal P-selectin localization on the DMS of the MKs triggers a pathological form of neutrophil emperipolesis within these cells [84,85]. Emperipolesis is defined as the passive passage of a cell through the cytoplasm of another cell that, under normal circumstances, does not alter the biological functions of either of the two cells involved. As a result of their size and of the tubular structure of the cytoplasm, emperipolesis of cells of many types occurs within MKs with a frequency of 1–2% [84]. In PMF patients, however, due to the high amount of P-selectin on the DMS, more than 30% of the MKs are engaged in emperipolesis with as many as three to five neutrophils embedded in each one of them. In addition, the process actively changes the properties of the two cells types that fuse their membranes, triggering the release of proteolytic enzymes by neutrophils and proangiogenic factors by α -granules, first within the cytoplasm of the MKs and then in the microenvironment [85]. These proteolytic enzymes and proangiogenic factors are then responsible for the development of the other traits of the disease, such as fibrosis (TGF- β , FGF), neoangiogenesis (VEGF, ANG1) and osteosclerosis (OPG, BMP4) (Figure 1) [74,76–86].

Mouse models of myelofibrosis

The crucial role played by MK abnormalities in promoting PMF is confirmed by the observation that the phenotype of mice carrying all the genetic mutations in the extrinsic and intrinsic control of MK differentiation, which induce MK alterations similar to those found in PMF, includes the development of myelofibrosis (Figure 2 & Table 3).

The first models established to study the pathobiological mechanism of myelofibrosis were represented by mice with altered TPO/MPL expression (TPO^{high} mice) (Table 3). Upregulation of TPO expression in mice induced MK abnormalities similar to those observed in PMF patients, including reduced levels of GATA1 expression (Figure 3). The mechanism that links high levels of TPO with reduced *Gata1* expression is unclear. The observation that TPO treatment of TPO-responsive cell lines [87] does not alter *Gata1* mRNA expression, while reducing in a time- and concentration-dependent fashion the levels of GATA1 protein (ARM) (Figure 3) [MIGLIACCIO AR, UNPUBLISHED DATA], suggests that TPO may reduce the expression of GATA1 in the MKs from TPO^{high} mice via a post-transcriptional mechanism. It also suggests that constitutively activated elements downstream of TPO signaling, such as MPL and JAK2 mutations, might reduce GATA1 expression in MKs from PMF patients with a similar post-transcriptional mechanism [82]. Additional animal models may be considered as TPO^{high} mice because they mimic reinforced cytokine signal and are represented by mice bearing the mutated JAK2 V617F [88,89] or MPL W515L [46,90] gene, or lacking the *Lnk* gene (*Lnk*^{-/-} mice; *Lnk* encodes a protein that retains MPL in the cell cytoplasm [91–96]). All these animal models develop a disease similar in several ways to that developed by TPO^{high} mice (see later).

A second model of myelofibrosis was generated by deleting the regulatory sequences of the *Gata1* gene that guides its expression in MKs (*Gata1*^{low} mice) (Figure 2) (Table 3) [97]. Since the levels of GATA1 in MKs of TPO^{high} and *Gata1*^{low} mice are comparably low (Figure 3), it is not surprising that the two animal models develop similar MK abnormalities and myelofibrosis. The most important phenotypic difference between the two models is represented by the time required for the disease to manifest itself. TPO^{high} mice rapidly develop a complete myelofibrosis trait, while *Gata1*^{low} mice develop the disease in an orderly sequence of events that span over more than 2 years [97]. For this reason, the two systems are suited to investigating different aspects of the pathobiology of myelofibrosis in mice. The rapidity with which TPO^{high} mice develop the disease makes this model particularly suited for genetic complementation studies such as those that have proved the essential role played by TGF- β and OPG in the disruption of the bone marrow structure. Alternately, the progressive development of myelofibrosis in *Gata1*^{low} mice makes this model specifically suited for studying the pathobiological mechanisms that determine increased stem/progenitor cell trafficking and extramedullary hematopoiesis. Additional genetic mutations that, like the *Gata1*^{low} mutation, target genes that regulate gene expression in MKs (*BACH1*) also induce myelofibrosis in mice [98].

The third most recent mouse model of myelofibrosis has been generated by manipulating genes that control the ability of the marrow microenvironment to support hematopoiesis [99,100]. In this model, development of myelofibrosis is not due to a stem cell defect but rather to a microenvironmental defect. As such, the MKs of these mice are normal. However the microenvironment is not normal and presents many of the alterations characteristic of the microenvironment of PMF patients. This last model highlights the fact that PMF is a disease of abnormal interactions between the stem cells and their niches.

TPO^{high} mouse models

Thrombopoietin is the major cytokine controlling growth, development and turnover of MKs, and is thus responsible for the regulation of platelet homeostasis in the blood [101–107]. This soluble factor is mainly produced by the liver and kidney [103,104] and is strongly induced by drops in platelet numbers or by increases in IL-6 during acute inflammatory stress [105,106]. The first observation that high TPO doses may lead to the development of myelofibrosis was the occasional result of an animal study to monitor the side effects of the long-term administration of recombinant TPO, utilized as a drug to rescue platelet deficiency in individuals with hemorrhagic issues [108]. To date, several approaches have been used to

generate *TPO*^{high} mouse models of myelofibrosis: repeated systemic administration, bone marrow reconstitution with stem cells virally transduced to overexpress the human *TPO* gene and transgenic mice overexpressing the human *TPO* gene in liver cells (Table 3).

Systemic administration—Increasing amounts of a truncated and polyethylene glycol-linked version of the TPO protein (PEG-rHuMGDF) have been injected on a daily basis into BALB/c mice. After 2 weeks all the treated animals developed anemia, splenomegaly and extramedullary hematopoiesis, with more drastic effects at higher TPO concentrations. Reversible bone marrow fibrosis was observed only at the highest TPO doses [108]. Transient increases in marrow MKs and platelet counts were also observed in normal rats injected with PEG-rHuMGDF at suprapharmacologic doses for 5 days. Emerging myelofibrosis normalized by day 20 and was paralleled by increased TGF- β levels in extracellular fluids of bone marrow, and in plasma and platelets [109].

Transplantation approach—Different mouse lines have been generated using bone marrow transplantation. Wild-type HSCs from lethally irradiated recipient mice were replaced with donor HSCs previously transduced to overexpress the *TPO* murine gene, by the use of either adenoviral [110–114] or retroviral vectors [115,116]. In spite of some strain-variability in results observed with adenoviral vectors [110–114], two retrovirally-based *TPO*^{high} models developed myelofibrosis, the severity of which was positively correlated with the levels of TPO expressed in the blood of the animals (30 ng/ml vs 1000 ng/ml). At high plasma levels, mice presented a two-step disease, which strongly resembled the course of the human disorder [115]. The first phase occurred 7–9 weeks after bone marrow transplantation and was characterized by decreased hematocrit and high numbers of platelets and leukocytes in peripheral blood. The bone marrow presented hypoplastic MKs, erythroblasts and granulocytes. The second phase took place 10 weeks after bone marrow transplantation. In this stage the level of blood cells dropped to pancytopenia. Extramedullary hematopoiesis, severe fibrosis in bone marrow and spleen and increased bone deposition were also observed. Finally, all animals died within 7 months after bone marrow replacement [117].

Genetic approach—To date, two transgenic animal models have been generated showing contradictory phenotypes. In one case, expression of the human *TPO* gene was driven by the liver apolipoprotein E enhancer/promoter [117]. These animals, despite increased platelet and tissue MK counts, do not develop bone marrow fibrosis [117]. In the second system, high levels of murine TPO expression were induced by placing the full length *TPO* gene under the control of the mouse IgH-chain promoter [118]. These mice showed a more drastic but still mild phenotype, with anemia, leukocytosis, slightly high TPO levels (increased by 2.3-fold) in plasma, bone marrow fibrosis and osteosclerosis 9 months after birth [118].

The *TPO*^{high} mouse model has been instrumental in formally demonstrating the link between marrow fibrosis and overproduction of TGF- β . In fact, wild-type mice transplanted with TGF- β ^{null} stem cells that had previously been transduced with a retrovirus containing the *TPO* gene, did not develop myelofibrosis. By contrast, animals transplanted with TGF- β ^{+/+} HPCs expressing similar high levels of TPO manifested the disease (*TPO*^{high} model of PMF, see later) (Table 3) [44]. These complementation studies have also clarified the role of OPG overproduction, a recently identified biomarker of PMF patients [119], in the development of myelofibrosis. In these experiments, marrow cells from *Opg*^{-/-} or wild type (*Opg*^{+/+}) littermates were infected with a retrovirus encoding TPO and engrafted into either *Opg*^{-/-} or wild-type mice. The phenotype of the four combinations of graft/host mice (*Opg*^{+/+}/*Opg*^{+/+}, *Opg*^{-/-}/*Opg*^{-/-}, *Opg*^{-/-}/*Opg*^{+/+}, *Opg*^{+/+}/*Opg*^{-/-}) was then analyzed [75]. All four experimental groups showed similarly elevated TPO and TGF- β levels in plasma and developed severe myelofibrosis. In addition, wild-type hosts engrafted with either *Opg*^{+/+} or *Opg*^{-/-} hematopoietic cells developed osteosclerosis associated with increased OPG levels in plasma

and decreased osteoclastogenesis. By contrast, *Opg*^{-/-} hosts did not exhibit osteosclerosis even if transplanted with *TPO*^{high} wild-type stem cells [75]. These experiments highlighted the importance of a reactive microenvironment for development of the entire myelofibrotic trait. On the other hand, Evrard *et al.* showed with a similar transplantation approach that HPCs from mice lacking the *Tsp* gene (*Tsp*^{-/-} mice) expressing high levels of TPO induce myelofibrosis in wild-type mice, indicating that the high levels of TSP similar to those found in PMF patients have no detectable pathological consequences in mice [120].

Biological effects similar to those promoted by TPO overexpression are mimicked in mice by any mutation that activates signaling pathways downstream of this growth factor. Also, ectopic expression of MPL W515L, an active form of the TPO receptor, in a murine bone marrow transplant assay induced a fully penetrant myeloproliferative disease, associated with splenomegaly, extramedullary hematopoiesis, marked thrombocytosis, bone marrow fibrosis and megakaryocytic proliferation (Table 3) [46]. Hematopoietic cells that express the MPL W515L mutation are hypersensitive to stimulation with TPO and present a proliferative advantage in bone marrow and spleen, as assessed by colony-forming assay.

The role of the TPO/MPL/JAK2 signaling in PMF is further supported by the observation that linker of T-cell receptor pathway (*Lnk*^{-/-}) mice, in addition to defective B lymphopoiesis, display many of the manifestations of myelofibrosis, including splenomegaly, hyperplasia of the MK lineage and thrombocytosis due to hyperactivation of TPO signaling, increased number of erythroid cells and hematopoietic progenitors in the spleen [94–95]. *Lnk*, recently designated as SH2B3, is a Src-homology 2 (SH2) domain-containing adaptor protein [91,92]. *Lnk* is highly expressed on HPCs [94,95]. The SH2 domain of LNK negatively regulates translocation to the plasma membrane of several cytokine receptors, including MPL [87,96,121] and the EPO [122] and SCF [123,124] receptor. As a consequence, *Lnk*^{-/-} cells express higher levels of MPL on their surface and respond more readily to TPO [27,119–124]. Given its role in downregulating MPL, LNK may represent a promising target for therapeutic intervention in those MPDs associated with the MPL W515L mutation [125].

The JAK2 mouse models

JAK2 is an important element of the TPO/MPL signaling pathway and it is, therefore, not surprising that the introduction of two activating mutations in this gene (JAK2 V617F, exon 12 mutations and TEL—JAK2), produce similar phenotypes to *TPO*^{high} and MPL W515L mutations in mice (Table 3). In a mouse transplantation model, HPCs overexpressing either JAK2 V617F [88,89] or JAK2K539L (exon 12 mutation) induced a MPD that strongly resembled PV. Recently, two transgenic mouse lines expressing JAK2 V617F in the hematopoietic system under the control of the tissue-specific *vav* promoter have been generated [40]. The line expressing the lower JAK2 V617F level showed a moderate increase in blood cell counts, whereas the line with the higher level of expression displayed marked elevation in blood cellularity and developed a phenotype resembling essential thrombocytopenia and PV. These mice also developed myelofibrosis as they aged with erythroid, megakaryocytic and granulocytic hyperplasia in bone marrow and spleen, as well as splenomegaly and reduced levels of EPO and TPO in the plasma [126].

Since in all of the *JAK2* transgenic mice developed so far the fibrotic phase was preceded by a polycythemic or thrombocythemic phase, these animals more closely represent a model of secondary myelofibrosis rather than PMF. Nevertheless these mice are of great value as preclinical models for screening the *in vivo* efficacy of newly developed JAK2 inhibitors. Notably, the small-molecule JAK inhibitor 1 (Calbiochem, San Diego, CA) also blocks proliferation of MPL W515L transformed 32D cells, suggesting that drugs targeting the JAK—STAT pathway may represent an effective therapy to cure both JAK- and MPL-dependent diseases [46].

Other models of myelofibrosis have been induced in mice by genetic mutations encoding forms of activated kinases found in other human leukemias. In fact, retrovirally transduced murine bone marrow overexpressing the oncogenic TEL—JAK2 fusion gene promotes a mixed myelo- and lympho-proliferative disorder when transplanted in irradiated syngenic hosts [42,43]. Furthermore, CD34⁺ cells obtained from the human umbilical cord and expressing TEL—JAK2 rapidly induce myelofibrosis *in vivo* in a nonobese diabetic/severe combined immunodeficiency xenotransplantation model [127]. However, since myelofibrosis is not observed in patients harboring this translocation, the genomic rearrangement is unlikely to be responsible for the human disease. These results may instead reflect the biological functions acquired by TEL when fused with a tyrosin kinase in mice. In agreement with this hypothesis, also the TEL—PDGF-βR (the β chain of the PDGF receptor [PDGF-βR] has tyrosine kinase activity) fusion gene, the result of the t(5;12)(q33;p13) translocation associated with chronic myelomonocytic leukemia [128], introduced into whole bone marrow by retroviral transduction also caused a rapidly fatal MPD in mice. TEL—PDGF-βR-transplanted mice developed leuko cytosis, splenomegaly, extramedullary hematopoiesis and bone marrow fibrosis (Table 3) [129,130].

Gata1^{low} mouse models

GATA1 is a gene localized both in mice and in humans on the X chromosome that encodes a transcription factor necessary for the development of hematopoietic cells of many lineages, including erythroid, megakaryocytic, mast, eosinophilic and dendritic cells [131–135]. *Gata1* was first identified as one of the factors up regulated in response to erythroid differentiation induced in murine erythroleukemic cells by dimethylsulfoxide by differential display [135]. GATA1 binds the promoter region of many erythroid-specific genes, such as *EPO* and *GATA1* itself [136,137], and its crucial role for erythroid maturation was first demonstrated by the observation that the germinal knockout of this gene in mice results in fatal anemia before birth [138]. GATA1 belongs to a family of zinc finger transcription factors that share a well conserved common architecture. This similarity ensures redundancy, with different proteins being interchangeable in regulating the same function [139]. It is conceivable that accurate control of gene expression, rather than the functional structure of individual proteins, drives the biological specificity of the different family members [137,139–142]. The lineage specificity of *GATA1* expression is ensured by the complex promoter region through a specific activation of the chromatin configuration of the locus. The *Gata1* locus, in fact, contains two untranslated first exons: the proximal exon, mostly used by erythroid cells and MKs, named IE exon, and the distal exon, named the IT exon, which was first identified because it is used as a transcription start site by Sertoli cells of the testis [141,142]. The hypomorphic *Gata1^{low}* (or *Gata1^{neoHS}*) mutation is a targeted deletion that replaces approximately 8 kb of the upstream region, which includes the IT promoter and the DNaseI hypersensitivity site (HS1), with a neomycin-resistance cassette. This construct was used to generate mutant embryonic stem cells, which were then injected into C57BL/6 blastocysts. *Gata1^{low}* pups are severely anemic and thrombocytopenic and more than 95% of them die around birth [143], highlighting the importance of HS1 for appropriate *Gata1* expression in MKs and erythroid cells. Anemia was due to a high apoptotic rate of the erythroblasts, linked to low GATA1 expression (~20% of wild-type expression levels) [143]. Thrombocytopenia was also caused by reduced levels of GATA1 protein in MKs, which, therefore, remained immature and released abnormally large platelets [144,145]. Surprisingly when the mutation was transferred in the CD1 background, perinatal mortality was lost [146]. CD1 mutant mice were still born anemic but recovered from anemia within the first month by establishing extramedullary erythropoiesis in the spleen and developing splenomegaly. Severe thrombocytopenia, however, remained a life-long trait, with platelet counts at 30% of the physiological value. Thrombocytopenia was associated with MK hyperproliferation and retarded maturation in the spleen and bone marrow. By 18 months *Gata1^{low}* mice showed marked myelofibrosis with

collagen deposition and new bone formation, reduction of bone marrow cavity and extramedullary hematopoiesis in the liver (Figure 4) [97,146].

By contrast, in the DBA/2 background, the mutation induced a milder myelofibrosis picture, while a similar mutation in BALB/C mice induced thrombocytopenia and osteopetrosis, but not myelofibrosis, and the mice died of megakaryocytic leukemia at an old age (Figure 5) [METCALF D, PERS. COMM.]. The different penetrance of the myelofibrotic trait induced by the hypomorphic *Gata1*^{low} mutation in different mouse strains suggests the existence of gene modifiers that cooperate with the hypomorphic mutation in determining the mouse phenotype. The observation that the severity of the phenotype is positively correlated with the ability of the strains to recruit the spleen as an erythropoietic site to recover from phenylhydrazine-induced hemolytic anemia [146] suggests that at least one of these gene modifiers is represented by sf-Stk. The activation of spleen erythropoiesis in response to stress is controlled by a truncated form of the Stk receptor, sf-Stk, transcribed from an alternative GATA1-responsive promoter located inside the Fv2 locus. The presence of sf-Stk also confirms the susceptibility to infection by the Friend leukemia virus (FLV) [147,148]. Strains that are susceptible to FLV infection, such as BALB/C, express a truncated version of sf-Stk, while C57B/6 mice, which harbor a point mutation destroying the GATA1 binding site, are unable to express sf-Stk and are resistant to FLV infection (Figure 5) [147,148]. Alternatively, CD1 and DBA/2 mice that express levels of sf-Stk intermediate between those expressed by C57BL/6 (null) and BALB/C (maximal) mice, present a consequent variegation in the extent of FLV susceptibility (Figure 5) [147].

MKs of *Gata1*^{low} mice present all the abnormalities of the MKs observed in PMF patients

As in PMF patients, MKs from *GATA1*^{low} mice are hyperplastic and immature, showing altered structure of β -granules, low or un detectable expression of VWF, abnormal P-selectin distribution associated with increased and abnormal emperipolesis of neutrophils, and augmented levels of para-apoptosis (Figure 6) [146,149,150]. Moreover, thrombocytopenia is observed in all of the strains examined, while anemia is greatly dependent on the genetic background. Therefore, the hypomorphic mutation is linked directly to the MK abnormalities observed in PMF patients with the reduced GATA1 protein in these cells (Figure 3) [146, 150]. In spite of the fact that the *GATA1* locus in PMF patients is normal, a large number of MKs in the bone marrow of these patients are *GATA1*-negative (Figure 5) [82]. It is interesting that, in contrast to the results observed in normal mice, TPO treatment increased the levels of GATA1 expression in MKs of *Gata1*^{low} mice and protected these animals from developing myelofibrosis [76], indicating that the levels of GATA1 expressed by the MKs may represent a druggable target for treatment of the human disease.

Genetic models that target additional transcription factors that regulate MK development are also indirectly linked to the *Gata1*^{low} model of PMF. Broad complex—tramtrack—bric-a-brac domain and cap'n'collar (CNC) homolog 1 (BACH1) are transcriptional repressors closely related to the *Drosophila* nuclear regulatory protein CNC [151,152]. It is located on chromosome 21q22.1 within the Down syndrome-associated gene region [153,154] and is expressed in MKs as well as in erythroblasts [155]. BACH1 heterodimerizes and other members of the CNC family (e.g., the p45 subunit of the nuclear factor—erythroid 2 [NF—E2]) and musculoaponeurotic fibrosarcoma (Maf) proteins to bind Maf-recognition element (MARE) [151,152,156], present themselves in the promoter region of several erythroid and megakaryocytic genes; including the globin genes and genes encoding enzymes involved in the biosynthesis of heme [157–160]. The functions of CNC proteins in MKs have been well documented in animal studies. Homozygous disruption of the mouse p45 subunit results in defective megakaryopoiesis and profound thrombocytopenia, leading to postnatal death [161]. In addition, BACH1 overexpression in erythroid cells and MKs phenocopies p45-mutant

animals [92]. Transgenic mice bearing human *BACH1* under the control of GATA1 regulatory sequences present significant reductions in the numbers of circulating platelets due to arrest in MK maturation. The MK abnormalities observed in these mutants are similar to those observed in *Gata1*^{low} mice with aberrant morphology, characterized by polyploidy, disorganized DMS, reduced granules and increased neutrophil emperipoiesis (Table 3). *BACH1* transgenic animals develop myelofibrosis with reticulin fibers, which are detectable as early as 5 weeks of age [92].

Occurrence of osteosclerosis in *Gata1*^{low} mice

New bone formation is the result of a balanced positive and negative effect exerted by osteoblasts and osteoclasts, respectively [162]. The accelerated bone deposition or impaired bone resorption both lead to osteosclerosis, a pathological increase of bone density [163]. All the *Gata1*^{low} mice developed osteosclerosis by 1 month of age, although the degree of osteogenesis is greatly dependent upon the genetic background (Figure 2) [146,164]. In these mice, new bone formation is mainly due to increased osteoblast proliferation while the number of osteoclasts remains normal. Increased osteoblast proliferation is probably promoted by the altered MKs through the establishment of direct cell—cell contact and the release of bioactive molecules, such as OPG and BMP4 [146,164].

***Gata1*^{low} mutation promotes neo-angiogenesis**

Similar to all tumors, PMF is characterized by abnormal angiogenesis [17]. A myelofibrotic bone marrow displays marked new vessel formations with increased pericyte coverage [17, 165]. Mice with low GATA1 expression present the same abnormal vessel formation observed in humans, with increased mean vessel perimeter, raised microvascular density and pathological branching [166,167]. The higher level of VEGF, which is a major pericyte-attractant, found in the extracellular fluid of these mice suggests that deregulation of pericyte recruitment may contribute to the abnormal angiogenic process induced by the hypomorphic *Gata1*^{low} mutation (Figure 2) [167].

***Gata1*^{low} mice present increased stem/progenitor cell trafficking & extramedullary hematopoiesis in liver**

A tuned HSC/HPC trafficking is responsible for establishing and maintaining normal hematopoiesis. During development, HPCs migrate via the blood from the fetal liver to the newly formed cavities in the bone marrow, by a process that involves reprogramming of the expression of the receptors that ensure the contact with the microenvironment. Although limited HPC trafficking via the blood among different niches persists throughout adult life, HPC trafficking is thought to be regulated by the interaction between the chemokine stromal cell-derived factor (SDF)-1, expressed by stromal cells of many types, with its receptor CXCR4, expressed on the surface of the HPC [168,169]. SDF-1 is not only produced by the liver but also by several stromal cells, including endothelial cells, in the bone marrow [170, 171]. In addition MKs and platelets express CXCR4, therefore, these cells sequester SDF-1 from the microenvironment and store this factor in their cytoplasm to release it upon appropriate stimulation [172,173]. The importance of the interaction between SDF-1 and CXCR4 for appropriate HPC trapping within their niches [168,169] was proved by the observation that deletion of either the *SDF-1* or *CXCR4* gene in mice resulted in dramatic decreases in the numbers of HPCs contained in the bone marrow with a corresponding increase in the number of circulating HPCs [168,174]. Also, experimental increases of SDF-1 in the blood and the decrease of this factor in bone marrow both result in increased HPC mobilization [175,176]. Similarly, downregulation of CXCR4 expression in the HPCs results in the increased mobilization of these cells [177].

Primary myelofibrosis is accompanied by increased (up to 200-fold) levels of HSCs/HPCs, as well as endothelial progenitor cells, in blood [1,178–180]. It is conceivable that these displaced stem cells will then leave the blood to colonize niches in the spleen and the liver where normal HPCs would not survive. Evidence for this hypothesis is provided by the observation that fibroblasts isolated from the spleen of PMF patients sustains proliferation of autologous CD34⁺ cells but not that of CD34⁺ cells from normal donors [181,182]. A similar increase in HPC trafficking and development of extramedullary hematopoiesis has been described in *Gata1*^{low} mice (Figure 4) [146].

Given the central role of the SDF-1—CXCR4 axis in stem/progenitor cell trafficking, several investigators have analyzed whether increased HPC trafficking and extramedullary hematopoiesis observed in *Gata1*^{low} mice and PMF patients might be related to abnormalities in SDF-1—CXCR4 expression. Not surprisingly, very similar SDF-1—CXCR4 levels were detected in the two systems. Both PMF patients and *Gata1*^{low} mice had modestly increased levels of SDF-1 in blood, highly increased SDF-1 immunostaining in bone marrow and reduced levels of CXCR4 expression in stem/progenitor cells [179,183,184].

It is unlikely that increased levels of SDF-1 in the blood may play a major role in an increased stem/progenitor cell mobilization in PMF and myelofibrosis because of their modest nature (twofold) and of the fact that similar increases are detectable in MPD patients, such as those with PV, who do not experience increased HPC mobilization. On the other hand, dramatic increases in SDF-1 immunostaining were evident in bone marrow sections from *Gata1*^{low} mice and in marrow biopsy of PMF patients (Figure 7). In both cases a strong reactivity toward the SDF-1 antibody was associated with both MKs and endothelial cells (Figure 7) [183].

Augmented levels of SDF-1 in bone marrow should, in theory, promote a stronger interaction between the HPCs and their niche and inhibit egression of the cells from the bone marrow. However, because of the pathological process of MKs emperipoiesis observed in both the *Gata1*^{low} mice and PMF patients, both bone marrow microenvironments are rich in neutrophil proteases that might cleave and inactivate SDF-1. Therefore, in spite of high SDF-1 staining, the microenvironment of the mutant mice, as well as that of the PMF patients, might be deprived of active SDF-1. Since it is not possible to discriminate between bioactive and proteolytically degraded SDF-1 by immuno staining, it is not possible to assess whether reduced levels of active SDF-1 in the bone marrow are responsible for increased stem cell trafficking in PMF patients and *Gata1*^{low} mice [183].

Alternatively, HPCs from both PMF patients and *Gata1*^{low} mice express reduced levels of CXCR4 protein and mRNA [179,183,184]. In the case of PMF patients it has been possible to demonstrate that reduced levels of CXCR4 mRNA are associated with hyper-methylation of the promoter of the *CXCR4* gene [184], linking abnormal CXCR4 expression directly to an intrinsic property of the PMF HPC clone [179,185]. It is possible that the decreased CXCR4 expression may impair the HPC affinity for the marrow niche, determining, at least in part, the increased HPC trafficking observed in PMF.

Another feature of myelofibrosis common both to PMF patients and mice, that may contribute to the increased HPC trafficking, is represented by the augmented number of HPCs associated with this disease [179,180]. In the case of *Gata1*^{low} mice, the augmented number of HPCs may be a direct result of the reduced GATA1 expression in these cells. In fact, the fate of hematopoietic progenitor cells is regulated by a balance between the levels of GATA2 and GATA1 expression. When the GATA2 levels are higher than those of GATA1, the cells proliferate, while high GATA1 levels favor maturation [186,187]. Since GATA1, in association with Friend of GATA1 (FOG-1) suppresses GATA2 expression by binding to regulatory sequences on the GATA2 promoter [188,189], *Gata1*^{low} HPCs are impaired in their

ability to suppress GATA2 expression and so remain in a proliferative state for a longer time [91,146].

To summarize, extramedullary hematopoiesis, which is associated with PMF in humans and myelofibrosis in mice, may partly be the result of increased HPC trafficking, due to reduced CXCR4 expression in the HPCs and the destruction of a supportive bone marrow microenvironment caused by fibrosis, and the increased numbers of progenitor cells.

Leukemic transformation in the *Gata1*^{low} mouse model

In humans, leukemic transformation is frequently associated with PMF progression; by contrast, *Gata1*^{low} mice do not develop leukemia. Exceptions were represented by the *Gata1*^{low} BALB/C mice, which die of megakaryoblastic leukemia at old age [METCALF D, PERS. COMM.], and by the most stringent *Gata10.5* mouse model. In this last model, replacement of the IE exon promoter by a neomycin resistance gene induces drops in *Gata1* expression down to 5% of the levels observed in wild-type cells [190]. These animals do not have the time to develop myelofibrosis because they die of erythroblastic leukemia a few months after birth (Figure 5) (Table 3) [190].

Alternatively, an amino-terminal truncated form of GATA1 (sGATA1), a hypomorphic GATA1 protein which is tenfold less active than the full length one, is detectable in megakaryocytic leukemia associated with Down syndrome in humans [191]. Down syndrome is caused by trisomy of the human chromosome 21, the affected children display a 500-fold increased risk of developing megakaryocytic leukemia associated with myelofibrosis [192]. In order to clarify the respective roles of sGATA1 and trisomy 21 in the development of leukemia in these patients the phenotypes of the mouse models carrying the two genetic abnormalities have been analyzed. *sGATA1* mice develop a transient hyperproliferation of a unique fetal-specific progenitor cell, highlighting the possible tumor-suppressor role of *GATA1* during fetal development in mice [193]. Unexpectedly, overexpression of the chromosome 21 mouse ortholog (Ts65Dn strain) induced a phenotype that strongly resembled MPD (Table 3). All of the Ts65Dn mice, in fact, presented morphological alteration and accumulation of MKs in bone marrow and the spleen, a profound thrombocytosis and a developed myelofibrosis [194].

Microenvironment-defective mouse models

Hematopoiesis depends on an appropriate response of progenitor/stem cells to extrinsic signals provided by the bone marrow microenvironment. Walkley *et al.* generated two mouse models that demonstrate that the myeloproliferation may result from perturbed interaction between HSCs and their niche. Indeed, germline deletion of retinoic acid receptor subtype- γ (*RAR γ*) gene [99] or the conditional knockout of the retinoblastoma (Rb) protein in the murine hematopoietic compartment [100] resulted in a myeloproliferative syndrome. RARs are nuclear hormone receptors that act as transcriptional repressors in the free form and as transcriptional activators when bound to their ligand [195]. *RAR γ ^{-/-}* mice develop age dependent myeloproliferation, associated with extramedullary hematopoiesis, splenomegaly and loss of trabecular bone [99]. The syndrome partially involves elevated levels of TNF- α expression, since the hematologic defects are mostly rescued when knockout mice are transplanted with TNF- α ^{-/-} hematopoietic cells [99]. On the other hand, Rb is a negative cell cycle regulator, which has been extensively studied for its relevance as a tumor suppressor [196,197]. Inactivation of Rb in hematopoietic cells by crossing the interferon-inducible *Mx*—*Cre* transgenes and *Rb*^{fl/fl} animals resulted in profound myeloproliferation, with loss of HSCs from the bone marrow due to the migration to extramedullary sites [100]. Neither the *RAR γ ^{-/-}* nor the *Mx*—*Cre*⁺ *Rb*^{fl/fl} phenotypes are rescued by the transplantation with wild-type HSCs or induced by transplantation of the mutated HSC into wild-type mice, indicating that the myeloproliferation is the result of an aberrant niche [99,100].

Expert commentary

Hematopoiesis is strictly dependent on the establishment of appropriate interactions between stem cells and their niches in the marrow. The quantitative stem cell assays and the numerous genetic tools developed in recent years have elucidated the nature of these interactions in mice. Much work remains to be done, however, to validate these discoveries in humans. PMF is a disease in which the interactions between the stem cells and their niches in the marrow are disrupted. Therefore, the comparison of the biological properties of the stem cells and of the marrow microenvironment between PMF patients and normal donors provides the necessary tool to understand how the hematopoietic process is regulated in humans. It is anticipated that in the next few years many investigators will study PMF as a disease model to understand the relationship between stem cells and their niches in humans.

Five-year view

In spite of the numerous important discoveries made in recent years on the pathobiology of PMF, the primary molecular defect leading to development of this disease is still obscure and allogeneic stem cell transplantation remains the only curative therapy. Given that not all patients are eligible for this procedure and that the transplant-related mortality is relatively high, development of alternative and safer therapeutic approaches for this disease remains a priority. In the search for the ultimate druggable target to cure PMF, several animal models of the disease have been generated. These models provide invaluable tools to better understand the pathobiology of such a complex disease. Although mutations of the *GATA1* gene have not been discovered in PMF patients yet, the MKs from these patients are characterized by reduced GATA1 content. Therefore, these cells phenocopy MK alterations induced by the presence of the hypomorphic *Gata1*^{low} mutation in mice. Owing to the similarities between the MK abnormalities observed in PMF patients and those induced by the *Gata1*^{low} mutation, *Gata1*^{low} mice are of inestimable value for dissecting the mechanisms that underlie the development of myelofibrosis, in particular those responsible for traits such as abnormal stem cell trafficking and development of extramedullary hematopoiesis, which are very difficult to study in humans. In addition, the affinities between the MK abnormalities found in *Gata1*^{low} and *TPO*^{high} (another animal model of myelofibrosis) mice and those observed in PMF patients allow us to predict that PMF may be the result of multiple gene lesions occurring on a single pathobiological pathway that regulates the interaction between the stem cells and their niches. In summary, the crucial link between MK abnormalities, alterations of the microenvironment and PMF developments discovered thanks to the murine models of the disease suggests that agents that restore MK development in PMF patients might also be able to cure the disease.

Key issues

- Primary myelofibrosis (PMF) is a chronic myeloproliferative disease caused by a clonal stem cell defect and is characterized by progressive bone marrow fibrosis, splenomegaly, extramedullary hematopoiesis, osteosclerosis and pancytopenia. The most frequent (50% of cases) genetic mutation associated with PMF is the JAK2 V617F mutation. Nevertheless, the role of the biochemical abnormalities associated with the presence of this mutation in the etiology of PMF has not yet been fully characterized.
- In spite of the fact that the genetic cause of PMF is still unclear, PMF is characterized by distinctive abnormalities in megakaryocyte (MK) development. These abnormalities are represented by reduced levels of expression of the transcription factor GATA1 and of those MK-specific genes, such as von Willebrand factor, whose expression is regulated by these transcription factors. In addition, P-selectin, although expressed at normal levels in MKs from PMF

patients, is delocalized on the DMS region of these cells. Such delocalization induces a pathological process of neutrophil emperipolesis, with consequent para-apoptosis of the MKs and the release of growth factors stored in these cells into the bone marrow microenvironment.

- Any mutation in mice that induces abnormalities in MK development similar to those observed in PMF also induces myelofibrosis, a syndrome similar to PMF. These mutations define a pathobiological pathway that links the extrinsic (thrombopoietin [TPO]) to the intrinsic (GATA1) control of MK development. Only some of these mutations have been identified in PMF patients or in other hematological malignancies so far.
- A number of different animal models are presently available to study the pathobiological pathway of myelofibrosis. Each of these models has appropriate applications. Models that generate the disease within a few months, such as *TPO^{high}* mice, are useful for gene complementation studies with the aim to analyze factors downstream of the primary lesion that contribute to the establishment of the myelofibrotic trait. Those generated by introducing mutations in mice found in PMF patients, such as JAK2 V617F mice, are more suited for preclinical studies aimed at identifying drugs that may cure PMF by inhibiting the biological activity of JAK2 V617F. Interestingly, mice such as *Gata1^{low}* mice that develop the disease according to well-defined sequential phases are more suited to dissect the hierarchy of events occurring during disease progression and to define stage-specific strategies to cure the PMF.
- The most intriguing of all the abnormalities observed in PMF is increased stem/progenitor cell trafficking and the development of extramedullary hematopoiesis in multiple organs, including the spleen and the liver. A *vis-à-vis* comparison of the abnormalities observed in PMF patients and in *Gata1^{low}* mice highlights the fact that this complex trait is likely a consequence of both a stem cell defect (reduced expression of the adhesion receptor CXCR4) and of a microenvironment defect (increased growth factor milieu induced by MK abnormalities). This concept paves the way to identify strategies to cure PMF based on a better understanding of the interactions between the stem cells and their niches and how this interaction is regulated by MKs.

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Figure 1. Role of MKs and platelets in the control of tissue repair in normal individuals (left) and in a patient with primary myelofibrosis (PMF; right)

Under normal conditions, mature MKs extend their protrusions through the endothelial cells of the blood vessels of the bone marrow, shedding platelets in the blood flux. MKs and platelets both contain the same two types of α -granules: those that contain proangiogenic factors (and an adhesion receptor still to be identified, probably represented by P-selectin) and those containing anti-angiogenic factors (and VWF as adhesion receptor). Upon injury, platelets are recruited to the site of damage and activated, flip-flopping their membranes and exposing the adhesion receptors on the α -granules. VWF stored in the anti-angiogenic α -granules then becomes capable of binding thrombin, triggering the dismissal of anti-angiogenic factors in the microenvironment. Later on, probably triggered by the interaction between P-selectin and neutrophils recruited in the mean time at the site of injury, proangiogenic α -granules also release their content, stimulating proliferation of multiple types of cell. Thus, sequential and concerted releases of anti- and pro-angiogenic growth factors at the inflammation site is first responsible for stilling the hemorrhage and then promotes tissue repair. Probably due to their reduced GATA1 content, MKs from PMF patients remain immature shedding reduced numbers of platelets in the bloodstream. These cells accumulate in the marrow, and, lacking VWF expression, respond poorly to stimuli that would normally induce the release of anti-angiogenic factors. Moreover, abnormal localization of P-selectin on the DMS results in the pathological process of emperipoiesis, with neutrophils increasing the release of the content of stimulatory α -granules in the bone marrow microenvironment. This release stimulates stromal cells of all types (fibroblasts, endothelial cells and osteoblasts), inducing fibrosis, neo-angiogenesis and osteosclerosis, respectively.

ANG1: Angiopoietin 1; DMS: Demarcation membrane system; MK: Megakaryocyte; OPG: Osteoprogesterin; VWF: Von Willebrand factor.

Figure 2. Unifying pathobiological pathways leading to the abnormal MK development in mouse models of myelofibrosis and in primary myelofibrosis (PMF) patients

TPO exerts the major extrinsic control on megakaryocytopoiesis. By interfering with TPO signaling it is possible to reproduce in mice many of the MK alterations observed in PMF patients, including reduced levels of *GATA1* expression. In addition to the MK abnormalities, mice either overexpressed TPO or exhibited hyperactive TPO signals that led to myelofibrosis. Hyperactive TPO signals can be represented by activating mutations of MPL or of its downstream effector JAK2, or even genetic deletion of the gene (*Lnk*) encoding the protein that inhibits MPL transport to the cell surface. On the other hand, downregulation of *GATA1* and *BACH1* expression, two genes encoding transcription factors exerting an intrinsic control on MK development, induced MK abnormalities in mice similar to those observed in PMF and led to the development of myelofibrosis. The major consequence of the blockade in MK development induced by all these mutations is that the P-selectin is delocalized on the demarcation membrane system, which triggers an abnormal process of neutrophil emperipolesis that increases the proteolytic and growth factor milieu of the microenvironment. MK: Megakaryocyte; MPL: Monophosphoryl lipid; TPO: Thrombopoietin; VWF: von Willebrand factor.

Figure 3. Megakaryocytes (MKs) from *TPO^{high}* and *Gata1^{low}* mice express similar reduced levels of GATA1

(A) Spleen sections from *TPO^{high}*, wild-type and *GATA1^{low}* mice stained with an anti-GATA1 antibody. Similar numbers of clusters of MKs negative for GATA1 staining are present in sections from *TPO^{high}* and *Gata1^{low}* mice. By contrast, the isolated MKs observed in the section of wild-type mice are positive for GATA1. (B) Effect of TPO stimulation on the levels of GATA1 protein expressed by a TPO-dependent MK cell line (32DTPO) [83]. 32D cells were stimulated with different amounts of TPO for the indicated time periods. Untreated GM979 cells were used as control. Protein lysates were electrophoretically separated on SDS PAGE followed by western blotting and immunostained with antibodies against either GATA1 or FOG1 (as control). Equal amount of protein loading was monitored by β-tubulin immunostaining. As shown, the observed decrease in GATA1 signal upon TPO stimulation is dose dependent and probably due to post-transcriptional events, since comparable levels of *GATA1* mRNA were detected in all the experimental points investigated (ARM, results not shown).

(C) MKs from wild-type and *Gata1^{low}* mice were isolated by sorting and the expression of *GATA1* mRNA measured by quantitative reverse transcriptase PCR. As expected, the presence of the hypomorphic *Gata1^{low}* mutation significantly reduced the *GATA1* mRNA levels in MKs. TPO: Thrombopoietin.

Modified with permission from [76].

Figure 4. *Gata1^{low}* mice (CD1 background) develop MF, a disease that mimicks the progression of human primary MF

At birth *Gata1^{low}* mice are anemic and thrombocytopenic. In the pre-MF stage (1–6 months of age) mice recover from the anemia by developing extramedullary hematopoiesis in the spleen and splenomegaly. In this phase increased osteogenesis is also observed. In the early-MF phase (8–12 months) mice develop bone marrow fibrosis and neovascularization of the bone marrow stroma. By 15 months of age, animals show an increased mobilization of hematopoietic progenitor cells and extramedullary hematopoiesis in the liver. Nevertheless, the life expectancy of *Gata1^{low}* mice remains normal [97].

MF: Myelofibrosis.

Figure 5. Correlation between *Sf-Stk* expression and background-dependent variegation of the phenotype induced by the *Gata1*^{low} mutation

The left panel shows the difference in expressions of *Sf-Stk* mRNA in the fetal liver of BALB/C and C57BL/6 mice by northern blotting [147]. The *Sf-Stk* mRNA is transcribed from an alternative promoter within the *Stk* gene under the control of a GATA1 binding site mutated in C57BL/6 mice. The erythroid cells from these mice are, therefore, incapable of expressing Sf-Stk and are resistant to FLV infection. By contrast, CD1, DBA/2 and BALB/c all contain the GATA1 binding site and their livers express Sf-Stk. The lowest levels of expression are observed in CD1 mice, while maximal levels of expression are observed in BALB/c mice (as indicated by the shade of the arrow). These levels of expression correlate with the susceptibility of the different strains to FLV infection. On the right, phenotypes of the different *Gata1*^{low} strains are indicated. The severity of the phenotype of these mutants correlates with the intrinsic susceptibility to FLV infection of the single strain (the higher the susceptibility, the lower the lethality) [146].

FLV: Friend leukemia virus.

Modified with permission from [147].

Figure 6. Ultrastructural analyses of megakaryocytes (MKs) from the spleen of wild-type (A,B) and *Gata1*^{low} (C,D) mice

(A) The arrow indicates an isolated MK in the semi-thin section from a wild-type spleen that was then further analyzed by transmission electron microscopy (TEM) in (B). The ultrastructural morphology (cytoplasm rich in α -granules and platelets territories) characterizes this cell as a stage III MK. (C) The arrow and arrowhead indicates a cluster of MKs in a semi-thin spleen section from *Gata1*^{low} mice further analyzed by TEM in (D). Two populations of mutant MK were identified by TEM, one with normal (arrow) and the other with high (arrowhead) electron density of the nucleus and cytoplasm. High electron-dense MKs present signs of cytoplasmic damage (swollen mitochondria with disrupted crests, numerous empty vacuoles and occasional remnants of demarcation membrane system). Their extensive heterochromatic nuclear organization and the shrunk and degraded appearance of their cytoplasm are indications of para-apoptosis, a terminal deoxynucleotide transferase-mediated dUTP nick-end labeling-negative process of cell death.

Magnification 100x in (A) and (C), and 3000x in (B) and (D).

Figure 7. Increased stromal cell-derived factor (SDF)-1 immunostaining of marrow sections from *Gata1*^{low} mice with age and the marrow biopsies from PMF patients

(A) SDF-1 immunostaining of representative sections from the marrow of the young (7 months) and old (10–12 months) wild-type (top panels) and *Gata1*^{low} (bottom panels) littermates are presented as indicated. Slides were counterstained with hematoxylin—eosin. (B) SDF-1 immunohistochemistry of marrow biopsies from a normal donor (upper panel) and a PMF patient (bottom panel) are shown. Bone marrow biopsy sections were stained with a monoclonal anti-SDF-1 antibody and then counterstained with Light Green (200x magnification).

PMF: Primary myelofibrosis.

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Table 1

Clinical manifestations of primary myelofibrosis.

Prefibrotic stage	Fibrotic stage
Non-bone marrow	
Mild to absent splenomegaly	Marked splenomegaly
Mild anemia	Marked anemia
Mild thrombocytosis	Pancytopenia with tear-drop poikilocytes
	Extramedullary hematopoiesis
Bone marrow	
Hypercellularity	Hypocellularity
Megakaryocytic hyperplasia	Megakaryocytic hyperproliferation and abnormal differentiation
Mild fibrosis	Marked fibrosis
	Osteosclerosis
	Neoangiogenesis
	May evolve to leukemia

Table 2

Genetic abnormalities associated with primary myelofibrosis.

Mutation	Frequency (%)	Ref.
JAK2	50	[20–26]
del(5q); del(13q); del(20q); trisomy(1q)	35	[56–61]
TET2	15	[198]
MPL	5–10	[25,45,46]
c-Kit	<1	[62–67]
Familial predisposition	<1	[52–55]

Table 3

Available mouse models for primary myelofibrosis.

Mouse model	Mutated in primary myelofibrosis patients	Mouse phenotype/application	Ref.
<i>TPO^{high}</i> Systemic administration Transplantation Transgenic expression	No	Decrease in hematocrit, pancytopenia, extramedullary hematopoiesis, osteosclerosis, fibrosis/genetic complementation studies	[76,108,109] [111–117] [118,119]
MPL W515L	Yes	Splenomegaly, extramedullary hematopoiesis, thrombocytosis, megakaryocytic hypoplasia, bone marrow fibrosis/ preclinical testing of JAK2 inhibitors	[46]
JAK2 V617F Transplantation Transgenic expression	Yes	Polycythemia vera-like disease, erythroid, megakaryocytic, and granulocytic hyperplasia in the bone marrow and spleen, splenomegaly and reduced level of erythropoietin and thrombopoietin in the plasma/preclinical testing of JAK2 inhibitors	[88,89]
<i>Lnk^{-/-}</i>	No	Splenomegaly, leukocytosis, thrombocytosis, MK hyperplasia, extramedullary hematopoiesis, increased response of HPCs to cytokines	[91–96]
Osteoprotegerin	No	Osteopetrosis, splenomegaly and extramedullary hematopoiesis	[75,119]
TEL—JAK2	No (ALL, CML)	Myelo- and lympho-proliferative disorder. Myelofibrosis	[42,43,127]
TEL—PDGFR	No	Hyperproliferation of neutrophils, abnormal MKs, splenomegaly, extramedullary hematopoiesis	[128–130]
<i>GATA1^{low}</i>	No	Severe anemia and thrombocytopenia, extramedullary hematopoiesis, MK hyperproliferation and abnormal differentiation, bone marrow fibrosis/pathobiology of HPC trafficking and extramedullary hematopoiesis	[97,146,149,150]
<i>GATA1^{0.5}</i>	No	Erythroblastic leukemia, no myelofibrosis	[190]
BACH1	No	Aberrant MKs, thrombocytopenia, myelofibrosis	[92]
Trisomy 21	Yes	Accumulation of abnormal MKs in bone marrow and spleen, profound thrombocytosis and myelofibrosis. Abnormal HPC compartment	[194]
<i>RAR^{γ-/-}</i>	No	Age-dependent myeloproliferation, extramedullary hematopoiesis, splenomegaly, bone loss	[99]
Mx-Cre Rb ^{fl/fl}	No	Myeloproliferation, HSCs mobilization, extramedullary hematopoiesis	[100]
Op/opFlt1 ^{TK-/-}	No	Bone marrow fibrosis due to osteoclast deficiency, extramedullary hematopoiesis	[199]

ALL: Acute lymphoblastic leukemia; CML: Chronic myeloid leukemia; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; MK: Megakaryocyte; MPL: Monophosphoryl lipid.