REVIEW ARTICLE

The contribution of mouse models to the understanding of constitutional thrombocytopenia

Catherine Léon,[1,2,3,4] Arnaud Dupuis,[1,2,3,4] Christian Gachet,[1,2,3,4] and François Lanza[1,2,3,4]

[1]UMR_S949, INSERM, Strasbourg; [2]Etablissement Français du Sang-Alsace (EFS-Alsace), Strasbourg; [3]Université de Strasbourg; and [4]Fédération de Médecine Translationnelle de Strasbourg (FMTS), France

Haematologica 2016
Volume 101(8):896-908

ABSTRACT

Constitutional thrombocytopenias result from platelet production abnormalities of hereditary origin. Long misdiagnosed and poorly studied, knowledge about these rare diseases has increased considerably over the last twenty years due to improved technology for the identification of mutations, as well as an improvement in obtaining megakaryocyte culture from patient hematopoietic stem cells. Simultaneously, the manipulation of mouse genes (transgenesis, total or conditional inactivation, introduction of point mutations, random chemical mutagenesis) have helped to generate disease models that have contributed greatly to deciphering patient clinical and laboratory features. Most of the thrombocytopenias for which the mutated genes have been identified now have a murine model counterpart. This review focuses on the contribution that these mouse models have brought to the understanding of hereditary thrombocytopenias with respect to what was known in humans. Animal models have either i) provided novel information on the molecular and cellular pathways that were missing from the patient studies; ii) improved our understanding of the mechanisms of thrombocytopoiesis; iii) been instrumental in structure-function studies of the mutated gene products; and iv) been an invaluable tool as preclinical models to test new drugs or develop gene therapies. At present, the genetic determinants of thrombocytopenia remain unknown in almost half of all cases. Currently available high-speed sequencing techniques will identify new candidate genes, which will in turn allow the generation of murine models to confirm and further study the abnormal phenotype. In a complementary manner, programs of random mutagenesis in mice should also identify new candidate genes involved in thrombocytopenia.

Correspondence:
catherine.leon@efs.sante.fr

Received: March 16, 2016.
Accepted: May 4, 2016.
Pre-published: No prepublication

doi:10.3324/haematol.2015.139394

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/101/8/896

Article summary

- Constitutional thrombocytopenias of hereditary origin have long been poorly studied due to the difficulties of obtaining invasive marrow samples.
- Genetic engineering has allowed the generation of numerous mouse models for all these pathologies, providing invaluable information to understanding these diseases and serving as preclinical models to test new therapies.

Constitutional thrombocytopenias result from genetic mutations affecting platelet production. These rare diseases are still underdiagnosed, especially in adults, because they remain little-known and have a highly variable expression. Autoimmune thrombocytopenia is still often wrongly diagnosed, thereby leading to the inadequate management of patients, with occasionally inappropriate splenectomy. The diagnosis of congenital thrombocytopenia relies on cytological and functional platelet analyses performed almost exclusively in specialized laboratories. Furthermore, about 50% of thrombocytopenias, associated or not with a thrombopathy, still remain of unknown origin.[1] In cases where platelet studies orient the diagnosis to a known disease, the detection of mutations in the suspected genes can

confirm the pathology. Our understanding of the pathophysiological mechanisms leading to congenital thrombocytopenia has long been based only on the observation of the megakaryocytes present in bone marrow. The need for invasive marrow samples and the rarity of these cells (less than 1% of bone marrow cells) have for a long time hampered such studies. Although obtaining culture of megakaryocytes from circulating hematopoietic progenitors is now possible, it remains confined to research laboratories, and only a few patients have been investigated in this manner. The recent development of the genetic reprogramming of iPS cells and their megakaryocytic differentiation has enabled some progress, but these *in vitro* systems are imperfect and do not faithfully reproduce all the steps leading to the formation of platelets. The development of tools to genetically manipulate mice now allows us to generate *in vivo* models mimicking these various pathologies, enabling the assessment of the impact of mutations on platelet production and function.

Targeted mutagenesis and transgenesis now offer a wide range of such models. "Total knockout mice" are generated by the inactivation of the gene in the whole organism. "Conditional knockout mice" permit inactivation of a gene in the megakaryocytic line tissue, or at a specific stage of development through the use of cre recombinase expressed under the control of the promoter of interest. The Mx-cre system has been most widely employed to excise a portion of DNA at a selected developmental stage. Almost all recombination in the megakaryocytic lineage has been obtained by using the Pf4-Cre system developed by Radek Skoda's group.[2,3] And finally, "knock-in mice" allow the introduction of point mutations or insertions/deletions through homologous recombination at the locus of interest. These models faithfully reproduce the mutations present in humans and represent the best approach to mimic the pathology. In addition to this genetic recombination toolkit, several chemical mutagenesis programs based on the treatment of gametes with N-nitroso-N-ethylurea followed by systematic phenotypic screening have been established to increase the frequency of mutations potentially targeting the hematopoietic system.[4-6] This approach might allow one to direct the screening of novel genes in patients with unidentified congenital thrombocytopenias.

Mouse models thus represent an essential tool to deepen our understanding of the mechanisms involved in platelet formation. The objective of this review is to focus on the contribution which mouse models have made to the elucidation and treatment of these diseases. We will briefly discuss the steps and key points of platelet formation, with emphasis on the roles played by proteins whose mutations are responsible for congenital thrombocytopenia. We will then describe the various constitutional thrombocytopenias where the contribution of animal models has been essential for their elucidation and/or treatment. For a more detailed description of the human pathologies, the reader may refer to three excellent reviews[7-9] and the OMIM database (input numbers in Table 1).

Platelet information

Megakaryocyte differentiation and maturation

In humans, as in mice, blood platelets are mainly formed from multipotent hematopoietic stem cells (HSCs) located within the bone marrow. The process is complex and involves a series of steps of proliferation and differentiation of progenitors and maturation of megakaryocytes. During their maturation, the megakaryocytic progenitors undergo several endomitotic cycles resulting in the formation of giant (>50 microns) and polyploid cells (up to 128N). In parallel, the cytoplasm considerably enlarges and a network of extremely complex and structured internal membranes develops. This network, called the demarcation membrane system (DMS), serves as a reserve for the membranes of long projections called proplatelets, which after release into the circulation form preplatelets, before being remodeled to give platelets (Figure 1). Cytoskeletal proteins play a key role in the extension of proplatelets and platelet formation, as in the case of the microtubule polymerization and sliding which condition the elongation of proplatelets and the generation of discoid platelets. Actomyosin also plays a crucial role in megakaryocyte differentiation and proplatelet formation. Several congenital thrombocytopenias result from mutations in genes directly or indirectly related to the cytoskeleton[10] (Table 1).

Regulation of megakaryocytopoiesis

Megakaryocytopoiesis is regulated through various steps controlling the proliferation of progenitor cells, their commitment and differentiation into mature megakaryocytes and the release of platelets. This involves the concerted action of a number of factors, including cytokines and growth factors, of which thrombopoietin (TPO) plays a major role, and a number of transcription factors (Figure 2). Many of these actors are the sites of mutations responsible for thrombocytopenia (Table 1). TPO acts through the MPL receptor, leading to the modulation of the expression of genes involved at different points of the process of megakaryocytic differentiation/maturation. It plays a part especially in the survival of HSCs and their differentiation and commitment to the megakaryocytic pathway. It is therefore not surprising that patients with mutations in the TPO-MPL pathway, according to whether these mutations cause loss or gain of function, develop particularly severe thrombocytopenia or thrombocytosis.

Transcription factors play an important role throughout megakaryopoiesis in regulating both the commitment of progenitor cells to their lineages and the transcriptional program determining megakaryocyte maturation. Megakaryocytes derive from a bipotent megakaryocyte-erythroid progenitor (MEP) through the coordinated activation of specific megakaryocytic genes and selective inactivation of transcription factors of the erythroid lineage (Figure 2), although recent data propose the existence of megakaryocyte-biased progenitors.[11] Some key transcription factors mutated in congenital thrombocytopenia include: GATA1, a zinc finger protein which forms a complex with the common factor FOG1 (Friend of GATA1). A number of thrombocytopenias result from mutations in the *GATA1* gene, while a point mutation in the GATA1 binding site in the promoter of the gene encoding GPIbβ (*GPIBB*) causes a form of Bernard-Soulier syndrome. RUNX1 (AML1), a RUNT family transcription factor which acts with its cofactor CBFB (core-binding factor, β subunit), and plays an important part in megakaryocytic differentiation through its role in regulating the expression of cytoskeletal proteins and platelet components. Some mutations in RUNX1 lead to thrombocytopenia associated with a high risk of developing leukemia and myelodys-

Table 1.

Disease (abbreviation)	Transmission #N°OMIM	Gene (chromosome)	Human Symptoms (platelet number±SD) (x10⁹/L)	Mouse models	Mouse phenotype
Anomalies of the cytoskeleton					
MYH9 related diseases (MYH9-RD)	AD #600208 #155100 #152640 #605249 #153650	*MYH9* (22q12-13)	Giant platelets (35±25.9) Leukocyte inclusions and/or nephropathy, cataract, hearing loss	1. Total inactivation[13,14] 2. Tissue-specific inactivation [15] 3. Knock-in [20,112]	1. Embryonic lethal (E7.5) 2. Defective megakaryocytes/ macrothrombocytopenia 3. Recapitulate human phenotype
Bernard-Soulier syndrome (BSS)	#231200	*GP1BA* (17p13) *GP1BB* (22q11)		1. Total inactivation *Gp1ba*[23] 2. Total inactivation *Gp1bb*[24,25]	1. Defective proplatelet formation and
- Biallelic (AR) -Monoallelic (AD)	AR AD	*GP9* (3q21)	Giant platelets (41±34.6) Large platelets (87±30.2)	3. Knock-in *Gp1ba* with IL4R extracellular part[28]	2. macrothrombocytopenia 3. Partial recovery of the WT phenotype
Wiskott-Aldrich syndrome (WAS) (THC1)	X-linked #301000 #313900	*WAS* (Xp11)	Small platelets (61±64.7) Severe immunodeficiency	1. Total inactivation[35-37] 2. Knock-in Y293[113]	1. Thrombocytopenia with normal sized platelets 2. Mimic most features of complete WASp-deficiency
Thrombocytopenia linked to filamin A	X-linked #none	*FLNA* (Xq28)	Large platelets (34±12.7)	Tissue-specific inactivation[47]	Macrothrombocytopenia Increased platelet clearance
Thrombocytopenia linked to tubulin 1	AD #nd	*TUBB1* (6p21.3)	Giant platelets (82±44.7)	Total inactivation[48]	Macrothrombocytopenia with defective marginal band
Thrombocytopenia linked to α-actinin	AD #615193	*ACTN1* (14q24.1)	Large platelets (87±31.7)	No mouse model	-
Thrombocytopenia linked to DIAPH1	AD #none	*DIAPH1* (5q31)	Large platelets (range 69-147)[114] Mild neutropenia Severe early onset hearing loss	Total inactivation[115]	No thrombocytopenia Normal platelets
Thrombocytopenia linked to PRKACG	AR #616176	*PRKACG* (9q21.11)	Thrombocytopenia with giant platelets (range 5-8)[116] Decreased level of FLNA Impaired platelet activation	No mouse model	-
Mutations of the TPO-cMPL pathway					
Congenital amegakaryocytic thrombocytopenia (CAMT)	AR #604498	*MPL* (1p34)	Normal sized platelets (13±4.7) Evolution to medullary aplasia	Total inactivation[51,52] Tissue-specific inactivation[61]	Severe thrombocytopenia
Mutations of transcription factors					
GATA1-related diseases (GATA1-RD)	X-linked #300367 #314050	*GATA1* (Xp11)	Large platelets (24±9.8) Anemia	1. Total inactivation[65] 2. Knock-in in the promoter region (Gata^{low})[68]	1. Embryonic lethal (E10.5-11.5) 2. Severe thrombocytopenia due to defective MK maturation
Congenital thrombocytopenia with radio-ulnar synostosis (CTRUS)	AD #605432	*HOXA11* (7p15-14)	Normal sized platelets (30) radio-ulnar synostosis ± other defects	Total inactivation[73,74]	Abnormal
Familial platelet disorder and predisposition to acute myeloid leukaemia (FPD/AML)	AD #601399	*RUNX1* (21q22)	Normal sized platelets (103±35.9) Risk of leukemia or MDS	1. Total inactivation[78,79] 2. Conditional inactivation (Mx-Cre)[80-83] 3. Conditional inactivation (Pf4-cre)[87]	1. Embryonic lethal (E11.5-12.5) 2. Defective MK and lymphocyte maturation 3. Defective MK maturation
Paris-Trousseau thrombocytopenia (TCTP) Jacobsen syndrome (JBS)	AD #188025 #600588 #147791	Deletion (11q23-ter) including *FLI-1*	Normal sized platelets (49±9.9)	1. Total inactivation Fli1[89] 2. Knock-in with truncated Fli-1[92]	1. Embryonic lethal (E11.5) 2. Decreased viability Thrombocytopenia Functional platelet defects
Thrombocytopenia linked to GFI1B	AD #187900	*GFI1B* (9q34.13)	Large platelets (97±24.7) Decrease in α-granules number	1. Total inactivation[95] 2. Conditional inactivation (Mx-cre)[96] 3. Conditional inactivation (inducible by doxycycline)[96]	1. Embryonic lethal (E14.5) 2. Expansion of functional HSC no longer quiescent 3. Lethal within 3 weeks Decreased Hb level and platelets
Thrombocytopenia linked to ETV6 ; THC5	AD #616216	*ETV6* (12p13.2)	Normal sized platelets (range 44-132) Erythrocyte macrocytosis Predisposition to leukemia	Total inactivation[102]	Embryonic lethal (E10.5-11.5)

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Other mutations

Gray platelet syndrome	AR #139090	*NBEAL2* (3p21.1)	Thrombocytopenia which worsens with age (55±21.3) Myelofibrosis and splenomegaly	Total inactivation[104-107]	Macrothrombocytopenia Absence of α-granules in platelets
Thrombocytopenia linked to ANKRD26 / Familial thrombocytopenia (THC2)	AD #313900	*ANKRD26* (10p2)	Normal sized platelets (43±28.4) Risk of leukemia or MDS	Total inactivation[109,110,117]	Obesity, gigantism due to hyperphagia No thrombocytopenia
Thrombocytopenia with absent radii (TAR)	AR #274000	*RBM8A* (1q21.1)	Thrombocytopenia which normalizes in adults (19) Normal sized platelets Bilateral radial aplasia±other malformations		No mouse model -
Thrombocytopenia linked to CYCS	AD #612004	*CYCS* (7p15.3)	Thrombocytopenia (109x10⁹/L) (range 73–167)[118,119]	Knock-in mouse expressing a mutant Cyt c[120]	Embryonic or perinatal death
Thrombocytopenia linked to ITGA2B/ITGB3	AD	*ITG2B/ITGB3* (17q21.31/17q21.32)	Macrothrombocytopenia[121]	No mouse model	-

plasia. FLI-1, a factor of the ETS family (E26 transformation-specific or E-twenty-six), whose binding site is present in most megakaryocytic promoters. Deletions in the *FLI-1* gene are responsible for Paris-Trousseau syndrome and Jacobsen's disease. And finally, *HOXA11*, a member of the family of homeobox genes mutated in patients with radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT). The genes responsible for several forms of thrombocytopenia have been identified, and mouse models exist for most of the diseases listed to date (Table 1).

Changes affecting the cytoskeleton

MYH9-related diseases

The *MYH9* gene encodes the heavy chain of non-muscle myosin IIA (NMMHC-IIA, non-muscle myosin heavy chain IIA) and mutations in this gene are responsible for several syndromes, including May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Epstein syndrome and some forms of Alport syndrome. Since the early 2000s, when the gene was identified, these diseases have been collectively renamed "MYH9 syndrome" and are the most common causes of constitutional thrombocytopenia. This is an autosomal dominant disease characterized by thrombocytopenia, platelet macrocytosis and the presence or absence of Döhle-like inclusions in leukocytes. Depending on the type of mutation, the macrothrombocytopenia may be accompanied by the gradual onset of kidney failure, cataracts and/or sensorineural hearing loss. A genotype-phenotype comparison has enabled a correlation of the presence of mutations in the N-terminal motor domain with the likelihood of developing non-hematological events and the severity of thrombocytopenia. The latter is also directly correlated with the severity of hemorrhagic manifestations, which are weak in most cases but require transfusion in a minority of patients.[12] There are three isoforms of non-muscle myosin type II (IIA, IIB and IIC), but only myosin IIA persists in the late stages of megakaryocytic differentiation and in platelets, explaining the congenital platelet phenotype. During megakaryocytic differentiation, the expression of myosin IIB is normally repressed by the RUNX1 transcription factor. The persistence of myosin IIB thus characterizes thrombocytopenic patients with mutations in *RUNX1*.

Several mouse models have been developed to study MYH9-related diseases and the possible role of myosin IIA in their different manifestations. Homozygous mice with complete inactivation of the *Myh9* gene (knockout) are not viable and die at the E7.5 embryonic stage despite the presence of myosin IIB, confirming the specific functions of the different isoforms of myosin II.[13,14] Selective inactivation of the gene in the murine platelet lineage reproduces the macrothrombocytopenia observed in patients.[15] Platelet shape changes and clot retractions are abolished, resulting in a decreased stability of platelet thrombi *in vivo*. This model has also helped to answer a number of questions concerning platelet abnormalities in MYH9 patients. Although based on *in vitro* experiments with human MYH9-RD or *Myh9*-/- progenitors, a possible ectopic platelet release within the extravascular bone marrow compartment was proposed, which could account for thrombocytopenia;[16,17] no such observation has been reported *in vivo* using mouse models. On the contrary, a lower viability of megakaryocytes in the marrow and defective DMS formation prevent *in situ* normal proplatelet extension,[18] which accounts for the thrombocytopenia. Less expectedly, these mice have helped to identify the role of myosin in the distribution of platelet granules. Finally, a preclinical study of the long-term effects of romiplostim, a second-generation thrombopoietic agent, showed increased myelofibrosis secondary to the treatment of myosin IIA-deficient mice.[19]

The most recent development by two independent teams of "knock-in" mice recreating mutations present in patients now allows us to study the human disease more closely.[20,21] In both cases, the heterozygous mice display not only macrothrombocytopenia but also non-platelet events, namely intracytoplasmic aggregates of myosin in leukocytes, kidney failure, hearing loss and cataracts.

However, the genotype-phenotype correlation observed in humans is not found in these murine models. Despite this difference, these mouse strains are still excellent models which can be used to study the mechanisms altered in the patients' platelets and the pathophysiology of the renal, auditory and visual defects.

Bernard-Soulier syndrome

Bernard-Soulier syndrome (BSS) is in its classical form an autosomal recessive disease characterized by thrombocytopenia and the presence of giant platelets. There is also a dominant monoallelic form, usually more moderate, where the patients display mild thrombocytopenia and large platelets, but little or no bleeding. The disease results from mutations in genes encoding one of the subunits of the platelet GPIb-V-IX complex (*GP1BA*, *GP1BB* or *GP9*). The GPIb-V-IX complex is the receptor for von Willebrand factor (vWF), which plays a key role in the early phases of platelet adhesion to injured subendothelium. In the classical biallelic forms, these mutations lead to a substantial absence of surface expression of the complex. The resultant concomitance of macrothrombocytopenia and a functional defect in platelets explains the severity of the disease. The monoallelic form leads to a reduction of about 40% in expression of the GPIb-V-IX complex, and platelet function is little or not affected.[22]

Whereas the absence of the GPIb-V-IX complex explains the functional defect in platelets, the mechanisms underlying the macrothrombocytopenia are not fully understood. Various BSS mouse models have been developed and have helped to explore potential mechanisms involving cytoskeletal proteins. Among these models, complete inactivation of the gene encoding GPIbα[23] or GPIbβ[24,25] reproduces the bleeding phenotype, macrothrombocytopenia and failure to extend proplatelets. The decrease in proplatelet formation may partly result from a lack of maturation of megakaryocytes, as shown especially by abnormalities in the development of the DMS observed *in situ*.[23,24,26,27] A third murine model has been generated by inserting a transgene corresponding to a chimeric receptor comprising an extracellular portion of the interleukin-4 receptor fused to the transmembrane and intracellular portions of GPIbα.[28] After crossing with GPIbα-deficient mice, the complex is expressed on the plasma membrane. The number of circulating platelets is increased and platelet size is reduced as compared to GpIbα-deficient animals. These findings point to an important role of the intracellular domain of GPIbα in regulating the number and size of platelets, presumably through interaction with the actin cytoskeleton *via* filamin A binding. Defects in structuration or dynamics of the microtubule cytoskeleton could also account for the macrothrombocytopenia. Thus, absence of the GPIb-V-IX complex results in an increase in the number of microtubule coils in proplatelets and in the marginal band of circulating platelets.[27] Finally, knockout of the *Gp5* gene has shown that this subunit is not necessary for the expression of other subunits of the GPIb-V-IX complex or for its main functions, including the production of platelets. This probably explains the lack of BSS patients related to a defect in GPV.[29]

BSS mouse models have also proved useful in preclinical studies. GPIbα-deficient mice display resistance to throm-

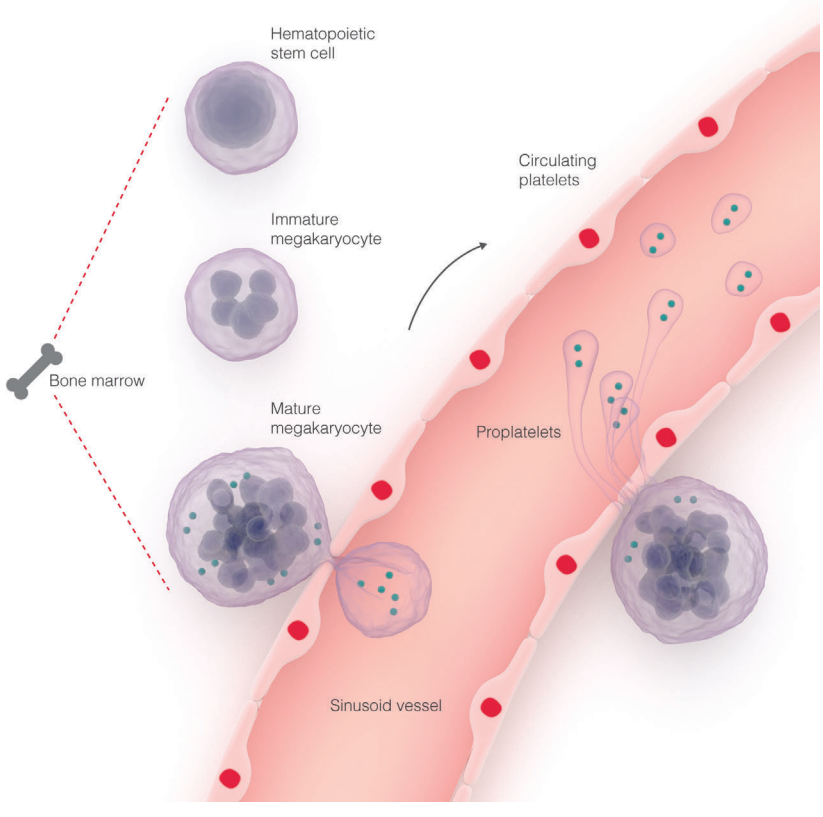

Figure 1. Main stages of megakaryocytopoiesis. After differentiation from the hematopoietic stem cells, megakaryocytes undergo a series of endomitoses leading to polyploid giant cells. The cytoplasm also undergoes a maturation phase during which the granules are synthesized together with a complex network of internal membranes. In the final stages, the megakaryocytes extend cytoplasmic projections in the sinusoid vessel to release platelets. (Drawing by Fabien Pertuy).

bosis in several *in vitro* and *in vivo* models, while their platelet procoagulant activity is greatly reduced.[27,30,31] Moreover, GPIbα-deficient mice and transgenic animals expressing human GPIbα have provided proof of the concept that a genetic platelet disease can be cured by gene therapy.[32,33]

Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT)

Wiskott-Aldrich syndrome (WAS) and moderate so-called "X-linked thrombocytopenia" (XLT) result from mutations in the WAS gene, which is located on the X chromosome and codes for the protein WASp (Wiskott-Aldrich syndrome protein). Nonsense mutations, deletions, insertions or complex mutations lead to a truncated, absent or non-functional protein and cause severe WAS, a condition characterized by thrombocytopenia associated with the progressive onset of severe immune deficiency, eczema and an increase in the incidence of autoimmune and malignant diseases. Missense mutations generally result in XLT syndrome. WASp is expressed exclusively in hematopoietic cells and plays a key role in regulating the intracellular cytoskeleton. Its absence leads to pronounced microcytic thrombocytopenia (typically less than 10% of the normal platelet count) accompanied by severe bleeding events, the most serious being intracranial, gastrointestinal or oral, which are major causes of morbidity and mortality.[34]

It was long thought that thrombocytopenia resulted essentially from a decreased platelet life span, this being less than 24 hours in patients, and comparable when WAS platelets are transfused into normal control recipients. The number of megakaryocytes in the bone marrow of patients is normal or even increased, ruling out the possibility of a platelet production defect. Finally, WAS megakaryocytes differentiated *in vitro* from $CD34^+$ cells exhibit normal ultrastructure and proplatelet formation capacity. Since the platelets produced *in vitro* are of normal size, the microcytosis observed in patients could result from abnormal platelet cytoskeleton reorganization in the circulation.

Mouse models shed new light on the role of WASp and the effects of its absence on platelet formation. Total inactivation reproduces the thrombocytopenia, which is moderate to severe depending on the genetic background and the individual, although microcytosis is absent.[35-37]

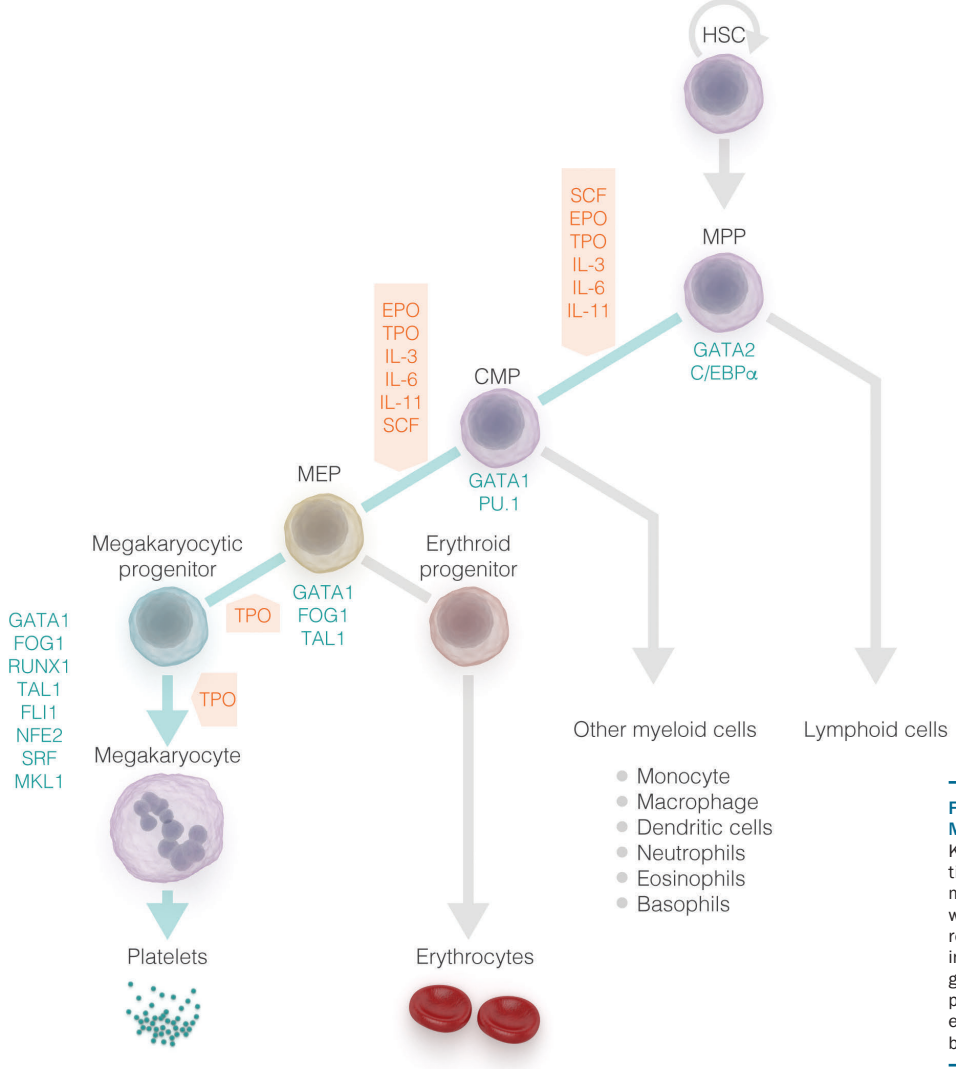

Figure 2. Hematopoiesis and Megakaryocytic commitment. Key cytokines (red) and transcription factors (blue) controlling megakaryocytopoiesis, some of which are the targets of mutations responsible for thrombocytopenia in humans. MPP: multipotent progenitor; CMP: common myeloid progenitor; MEP: megakaryocyte-erythrocyte progenitor. (Drawing by Fabien Pertuy).

Absence of WASp resulted in ectopic platelet release.[37] By relaying the signalling induced by collagen I and favoring the migration of megakaryocytes to sinusoids, WASp plays a key role in preventing the premature release of platelets in the bone marrow compartment.[37] In addition, this model proved to be useful in showing that the increased platelet consumption resulted from both intrinsic platelet factors and extrinsic factors, due to an increased susceptibility of these mice to develop platelet antibodies with subsequent opsonization and phagocytosis of platelets.[35,36] The development of "knock-in" mouse models has allowed one to explore the mechanisms governing the activation and stability of WASp, including the important role of phosphorylation at the Y293 single site (the murine equivalent of the Y291 site in human WASp). This phosphorylation site is critically involved in the activation of WASp in numerous cellular responses including proliferation, phagocytosis and especially the assembly of adhesion and chemotaxis structures.[38]

Finally, these murine models have been successfully used for ten years to conduct preclinical trials evaluating somatic gene therapy as an alternative to transplantation. In fact, despite the advances in their diagnosis, the prognosis of WAS patients remains poor. While transfusion therapy in the event of severe hemorrhage together with splenectomy may suffice in moderate cases, allogeneic HSC grafts remain the treatment of choice for WAS/XLT syndromes. HSC transduction trials have been carried out using murine retroviral or lentiviral vectors encoding WASp, and followed by transplantation into WAS-deficient mouse models. The results show a very high transduction efficiency, long-term engraftment of the cells and transgene expression in the lymphoid and myeloid lineages. Transplantation corrects the immune deficiency, while reducing the production of autoantibodies.[39-44] These advances, together with improvements in the transcription vectors and promoters, have permitted the evaluation of gene therapy trials to treat some of these patients.[45,46]

FNLA-related thrombocytopenia

Filamin A, encoded by the gene *FLNA*, is an actin-binding protein belonging to the family of filamins historically called ABP-280 (actin-binding protein 280). The filamins are large dimeric molecules ensuring cross-linking and stabilization of the filamentous actin network and its anchorage to transmembrane glycoproteins, including the GPIb-V-IX complex and integrin αIIbβ3, and also serve as a protein scaffold for different signalling intermediates. Monoallelic mutations in the *FLNA* gene lead to various defects including brain, bone and cardiovascular abnormalities. At the platelet level, macrocytosis is observed with or without thrombocytopenia.[8,9] These observations are consistent with data obtained in a mouse model lacking filamin A in the platelet lineage, which is characterized by severe macrothrombocytopenia and decreased expression of the GPIb-IX complex on the platelet surface.[47] Thrombocytopenia results from both rapid clearance of the platelets appearing most vulnerable and ineffective platelet production. The proplatelets are formed prematurely and release large cell fragments. These are then further fragmented into platelets, which are subject to microvesiculation and rapidly cleared from the circulation. The microvesiculation is due to a reduced stability of the cell membranes, possibly caused by instability of the submembranous actin network.[47]

Thrombocytopenia related to β1 tubulin

The β1 tubulin isoform is predominantly expressed in blood platelets. Mutations in the *TUBB1* gene have been described in two families but have not yet been reproduced in animals. These patients exhibit macrothrombocytopenia associated with a decrease in the amount of β1 tubulin in platelets. The mutations make the protein unstable and unable to be incorporated into microtubules, leading to a decreased number of megakaryocytes forming proplatelets. The importance of this particular tubulin isoform has been demonstrated by inactivation of the *Tubb1* gene in mice.[48] As in patients, the mice display macrothrombocytopenia resulting from defective proplatelet formation, despite compensatory overexpression of *Tubb2* and *Tubb5*. The lack of phenotype observed in heterozygous mice suggests that the phenotype of patients could be related to a dominant negative effect. A dominant negative effect due to another mutation causing instability of microtubules has been reported in dogs presenting β1 tubulin mutations.[49]

Changes affecting the cMPL-TPO pathway

Congenital amegakaryocytic thrombocytopenia (CAMT)

Congenital amegakaryocytic thrombocytopenia (CAMT) is the best known form of amegakaryocytic thrombocytopenia. It is an autosomal recessive disease caused by mutations in the *MPL* gene encoding the TPO receptor. This receptor is present on HSCs, megakaryocytes and platelets and activates different signalling pathways, in particular Jak2/STAT, Ras/MPK and PI3K. The thrombocytopenia is severe at birth and progresses to aplastic anemia during the first year of life, inevitably requiring HSC transplantation.[50] There is a clear correlation between genotype and phenotype as a non-functional protein causes profound thrombocytopenia, while patients with residual activity of the receptor are less severely affected. Conversely, *MPL* mutations resulting in constitutive activation of the receptor or reduced clearance of TPO, as likewise mutations in the THPO gene leading to increased gene translation, have been identified in some familial forms of essential thrombocythemia.

Mice deficient in Mpl partially reproduce the clinical picture. As in humans, the thrombocytopenia is severe, although pancytopenia is not observed despite a decrease in the numbers of hematopoietic progenitor cells of different lineages.[51,52] Subsequent studies of this model have demonstrated the role of TPO-MPL signalling in the regulation of HSCs in adult mice[53] and in the establishment of definitive hematopoiesis during embryonic development.[54,55] On the other hand, the presence of residual functional platelets in MPL-deficient animals point to the existence of other factors involved in platelet formation, and these mice have proved an extremely useful model to identify such factors. Various studies have shown that the interleukins IL-3, IL-5, IL-6, IL-11 and IL-17 do not participate in the residual thrombopoiesis.[56-59] In contrast, the chemokine SDF-1 and FGF-4 promote thrombopoiesis independently of TPO, allowing the interaction of megakaryocytic progenitors with the vascular niche.[60] Recently, the inactivation of *MPL* specifically in the murine megakaryocytic lineage has revealed that the TPO-MPL axis plays its key role at the level of bipotent megakaryocytic precursors. Thus, the MPL receptor is not essential for megakaryocyte prolif-

eration, maturation or platelet production, but is crucial to control the local concentrations and therefore the availability of TPO in the bone marrow microenvironment, and hence to avoid myeloproliferation through over-stimulation of progenitors.[61] Finally, as in other pathologies, mice invalidated for the *MPL* gene have proved a tool of choice for the development of gene therapy and the validation of novel vectors.[62,63]

Changes affecting transcription factors

GATA 1-related thrombocytopenia

There are two types of GATA1-related thrombocytopenia, dyserythropoietic anemia with thrombocytopenia (DAT) and X-linked thrombocytopenia with beta-thalassemia (XLTT). In both cases GATA1 mutations are responsible for dysmegakaryopoiesis and dyserythropoiesis with varying degrees of anemia and macrothrombocytopenia, the phenotype being less severe in XLTT. GATA1 is a transcription factor controlling the expression of numerous genes involved in megakaryocytopoiesis (*GPIBB*, *PF4*, *MPL*, *NFE2* etc.) and genes of the erythroid lineage (*HBB*, *ALAS1*, *BCL2L1*).[64] GATA1 contains two zinc finger domains, one N-terminal (Nf) and the other C-terminal (Cf). The Nf domain interacts with both FOG1 and DNA. Mutations responsible for XLTT affect DNA binding but not the interaction with FOG1, while mutations causing DAT affect FOG1 binding.

Complete gene inactivation is lethal at the E10.5-11.5 embryonic stage. Studies of chimeric mice derived from Gata1-deficient ES cells have shown an increased number of megakaryocytes in the fetal liver.[65] Similar observations have been made in a mouse model with a point mutation induced by ENU at the initiation codon (*Gata1*Plt13), mimicking the mutations present in some patients.[66] The further development of transgenic mice carrying the *Gata1* promoter sequence and reporter gene has allowed for the identification of the promoter regions and the specificity of GATA1 expression during embryonic development.[67] Targeted replacement of a long sequence upstream of the *Gata1* locus including the distal promoter has been used to generate a viable murine lineage (*Gata1low*), with a specific lack of GATA1 expression in megakaryocytes.[68] These mice display severe thrombocytopenia resulting from a defect in megakaryocyte maturation. The cells exhibit low ploidy and an underdeveloped DMS, leading to a failure to extend proplatelets and defective granule formation.[69] These defects are associated with a reduction in the transcription of genes coding for key proteins (GP1Bα, GP1Bβ, PF4, MPL, NFE2) during megakaryocyte maturation. The absence of GATA1 also causes deregulation of the cell cycle with hyperproliferation of the mutant megakaryocytes. With age, *Gata1low* mice then develop a hematological clinical picture resembling idiopathic myelofibrosis in several respects,[70] and therefore represent an excellent model to study this disease.[71]

Radioulnar Synostosis with amegakaryocytosis thrombocytopenia (RUSAT)

This rare congenital disorder is characterized by severe amegakaryocytic thrombocytopenia which is present at birth and associated with proximal fusion of the radius and ulna. The thrombocytopenia does not improve with age and the children can also develop myeloid failure requiring bone marrow transplantation.[72] All these symptoms allow for the distinction of the disease from CAMT and TAR syndrome (thrombocytopenia with absent radii), and in most cases mutations in the *HOXA11* gene have been identified. *HOXA11* is a member of the HOX family of homeobox genes which code for transcription factors. These proteins play important roles during embryonic development, where their expression is strongly regulated in space and time. At the hematopoietic level, HOXA11 is expressed only in the earliest precursors. Mouse models with *Hoxa11* inactivation have been developed but do not permit the study of the mechanisms involved in platelet formation. Heterozygous and homozygous *Hoxa11*-deficient mice have malformations in the lower and hind limbs, consistent with the observations in patients, but thrombocytopenia has never been reported.[73,74] At present, it is not known whether the megakaryocytopoietic defects present in patients result from a dominant negative effect of the point mutations or not.[75]

Familial platelet disorder with a predisposition for acute myeloid leukemia (FPD/AML)

Familial platelet disorder with a predisposition for acute myeloid leukemia (FPD/AML) is the consequence of mutations in the *RUNX1* gene (also known as *AML1* or *CBFA2*). Somatic mutations in *RUNX1* are also responsible for a non-inherited form of AML and for chronic myelomonocytic leukemia.[76] *RUNX1* encodes the α subunit of the CBF (core binding factor) transcription complex.[77] The heterodimerization of CBFα with CBFβ increases its affinity for DNA and protects it from proteolytic degradation. The transcription factor CBF regulates the expression of many specific hematopoietic genes and is essential for the establishment of definitive hematopoiesis.

Most of the mutations identified in patients lead to haploinsufficiency, although some variants may act through a dominant negative effect. The patients exhibit thrombocytopenia, sometimes accompanied by a functional defect in platelet aggregation in response to collagen, together with an increased risk of developing acute myeloid leukemia or myelodysplastic syndromes (40% of cases).

Complete inactivation of *Runx1* in mice is lethal at the E11.5-12.5 embryonic stage due to intracranial hemorrhages. These mice have nevertheless been useful to show that *Runx1* is required for the establishment of definitive hematopoiesis during embryonic development.[78,79] Conditional gene ablation by crossing with mice expressing cre recombinase under the control of the *Mx1* promoter allows inactivation of the gene in adult mice. In this case, the loss of RUNX1 in adulthood does not result in a total loss of hematopoiesis.[80-83] However, megakaryocyte and lymphocyte maturation are inhibited, consistent with a role of *Runx1* in the maturation of these cells. Furthermore, the fraction of immature progenitors is increased since *Runx1* negatively regulates HSC quiescence.[84] It has been reported that hematopoietic progenitors from FPD/AML patients have an increased clonogenic potential, and in some cases abnormal self-renewal capacity.[85] The expansion of these immature cells observed in RUNX1-deficient mice could thus be relevant to the pathogenesis of human hematological tumors associated with a lack of RUNX1.[86]

More recently, inactivating *Runx1* during megakaryocytic maturation by crossing floxed mice with Pf4-cre mice has demonstrated the importance of this factor in

megakaryocyte maturation and allowed a genomic analysis of RUNX1-controlled gene expression during the late stages of megakaryocytopoiesis, again providing useful information for our understanding of FPD/AML.[87]

Paris-Trousseau and Jacobsen syndromes (FLI1)

Paris-Trousseau syndrome and Jacobsen syndrome, with dominant transmission, result from a partial deletion of the long arm of chromosome 11 (11q23-ter). About 200 cases have been described with twice as many women affected as men. The distinction between the two syndromes is based on the severity of the disease. In all cases thrombocytopenia due to a defect in megakaryocyte maturation is present, with many immature, microcytic, hypolobulated and dystrophic cells.[88] The patients also suffer from facial dysmorphia. Additional symptoms are observed in Jacobsen syndrome including cardiac, renal, gastrointestinal and genital abnormalities and an intellectual deficit. The variability of the symptoms is probably correlated with the type of deletion, which is indeed highly variable in size, up to 20 Mb. The cleavage site is generally located in or near the sub-band 11q23.3 and the deletion usually extends to the telomere. This region covers in particular two genes encoding the ETS family of transcription factors, *ETS1* and *FLI1*.

The study of a mouse model with inactivation of the *Ets1* gene has demonstrated that this transcription factor is not critical for megakaryopoiesis, whereas differentiation of the lymphoid lineage is strongly disrupted.[89] In contrast, studies of mice with *Fli1* inactivation have shown that this protein plays a key role in the human syndromes through its involvement in megakaryocytic gene transactivation. Complete inactivation of *Fli1* results in embryonic lethality at the E11.5 stage, following intracranial hemorrhage.[90] The absence of FLI1 affects both vasculogenesis and megakaryopoiesis. As in patients, the megakaryocytes display morphological abnormalities with characteristics of immature cells. In this study heterozygous *Fli1*+/- mice showed no abnormal phenotype, which raised the question of the mechanisms through which the haploinsufficiency in patients might lead to the various symptoms. Another recent study compared *FLI1*+/- and [*Ets1*+/+; *Fli1*+/-] animals. While mild thrombocytopenia and craniofacial abnormalities were found in *Fli1*+/- mice, some symptoms were more severe in [*Ets1*+/-; *Fli1*+/-]animals, suggesting partial functional redundancy of the two transcription factors. Hence, at least some of the patients' symptoms may result from hemizygosity.[91] Finally, mutant mice expressing a truncated form of the molecule lacking the C-terminal regulatory domain have been generated to study the role of functional FLI1 domains.[92] Homozygous mice have a decreased viability (30% survive into adulthood) and present thrombocytopenia associated with functional defects due in particular to a decrease in megakaryocytic gene expression (*Mpl*, *Itga2b*, *Gp5*, *Gp9*, *Pf4*, *Nfe2*, *Mafg* and *Rab27b*).

Thrombocytopenia associated with GFI1B

A nonsense mutation was very recently discovered in the *GFI1B* gene in a family with an autosomal dominant form of Gray platelet syndrome.[93] GFI1B (growth factor independent 1B) is a transcription factor containing a SNAG repressor domain (Snail GFI1) in the N-terminal conserved domains and 6 zinc fingers. The SNAG domain plays the role of a transcriptional repressor. GFI1B is expressed in hematopoietic cells and especially in erythroid cells, megakaryocytes and their common progenitors (MEPs). The generation of a reporter mouse line expressing GFP (green fluorescent protein) at the *Gfi1b* locus (*Gfi1b*+/GFP animals) showed that the expression of this protein during hematopoiesis is inversely proportional to the expression of another protein of the same family, GFI.[94]

In humans, the mutation results in a truncated protein lacking the last 44 amino acids, which has a dominant negative effect with respect to the normal protein. Similarly as in autosomal recessive Gray platelet syndrome (see below), thrombocytopenia is associated with large platelets displaying partial or total absence of the α-granules, depending on the patient. Moderate to severe bleeding has been reported, which may be attributed in part to a decrease in the expression of GPIbα. Within the bone marrow, the development of stage I myelofibrosis has been observed with an increased number of pleomorphic megakaryocytes containing only a few granules. Megakaryocytes and platelets retain strong expression of the CD34+ stem cell marker.

Studies in mice have demonstrated the role of GFI1B as a transcriptional repressor acting during embryonic and adult erythropoiesis and megakaryopoiesis. Total gene deletion causes the arrest of embryonic development at around E14.5 due to the delayed maturation of primitive erythrocytes and a failure to produce definitive anucleate erythrocytes, accompanied by a lack of differentiation in the megakaryocytic lineage.[95] Heterozygous animals develop normally, indicating that haploinsufficiency is not responsible for the human disease, in agreement with a potential dominant negative effect of the human deletion. Moreover, the generation of conditional knockout mice in which cre recombinase is under the control of the inducible Mx1 promoter has demonstrated that this protein is critically involved in regulating the dormancy and proliferation of adult HSCs.[96] Specific inactivation of *Gfi1b* in erythrocytes [by crossing with mice expressing cre recombinase under the control of the *Epor* (erythropoietin receptor) promoter] has revealed the involvement of GFI1B in the differentiation of pro-erythroblasts into mature erythrocytes, and the extinction of globin gene expression in embryonic and adult cells.[97] Finally, by crossing with mice expressing cre recombinase under the control of an inducible doxycycline promoter, it was possible to confirm the role of GFI1B as a transcriptional repressor of adult erythropoiesis and thrombopoiesis.[98] During megakaryopoiesis in adults, GFI1B acts as a repressor mainly after the completion of endomitoses but before the onset of cytoplasmic maturation.

Thrombocytopenia associated with ETV6

Very recently, a few families with variable thrombocytopenia have been found to harbor germline mutations in *ETV6* (Ets-variant 6, also known as TEL). *ETV6* encodes a transcriptional repressor of the ETS family, and was initially identified as a tumor suppressor through its role in childhood leukemia resulting from somatic translocations. In addition to autosomal dominant thrombocytopenia, the patients present a high erythrocyte mean corpuscular volume, pointing to a defect affecting megakaryocytic-erythroid precursors. Mild to moderate bleeding has been reported, with an increased susceptibility to develop acute lymphoblastic leukemia. It has been proposed that the

mutations affect megakaryocyte development with an increased presence of small and immature cells, possibly due to abnormal cytoskeleton organization.[99-101] Total inactivation of mouse *Etv6* has long been known to be embryonically lethal on account of yolk sac angiogenic defects.[102] While ETV6 appears to be dispensable for fetal liver hematopoiesis, it is required for bone marrow hematopoiesis and hematopoietic stem cell maintenance. Heterozygous loss of one *Etv6* allele does not affect hematopoiesis, suggesting a dominant negative effect in patients, which has now been confirmed through *in vitro* studies.

Other mutations

Gray platelet syndrome

The name Gray platelet syndrome derives from the gray appearance of platelets on a blood smear stained with May-Grünwald-Giemsa, which is due to the absence of α-granules. This syndrome is characterized by macrothrombocytopenia associated with myelofibrosis and splenomegaly.[103] The autosomal recessive disease is due to a biallelic mutation in the *NBEAL2* (neurobeachin-like 2) gene, the patients being either homozygous or compound heterozygous with a resultant loss of function of the protein. Studies in patients have shown that the number of megakaryocytes is normal while thrombocytopenia increases with age. A decreased platelet survival, together with the progressive development of myelofibrosis, probably due to the premature release of growth factors, may explain these observations.

Three mouse models with knockout of *Nbeal2* have been generated recently.[104-107] While all three reproduce the macrothrombocytopenia and lack of α-granules in platelets, they display differences which are worth noting. The study from Kahr *et al.*,[107] (Model 1), reports splenomegaly, like the human pathology, but no myelofibrosis. Ultrastructural examination of megakaryocytes from these mice show abnormalities in the development of the DMS and in the maturation of α-granules, with an abundance of immature cells. Cultured megakaryocytes are hypopolyploid and extend fewer proplatelets, while von Willebrand factor is abnormally located at the cell periphery and can be secreted. All these features suggest that the normal development of α-granules contributes to the maturation of megakaryocytes. The mouse model reported by Deppermann *et al.*,[105] (Model 2), exhibits little or no splenomegaly, even in aged mice, and the demarcation membranes develop normally in megakaryocytes. α-granules are absent, but there is an increase in the numbers of mitochondria and vacuoles. Since proplatelet formation and platelet survival are normal, the authors propose that the thrombocytopenia results from defects in the terminal stages of platelet release. Finally, the mouse line developed by Guerrero *et al.*,[106] (Model 3), displays both splenomegaly and myelofibrosis. *In situ*, the megakaryocytes are smaller and generally less polylobed, while ultrastructural images reveal a slightly more rudimentary DMS. The proplatelet formation visualized by the adhesion of megakaryocytes cultured *in vitro* to a fibrinogen surface is similar between these animals and those of Model 2. Surprisingly, mature α-granules are present in megakaryocytes *in situ* and in the proplatelet buds of megakaryocytes differentiated *in vitro*. The authors suggest that the lack of α-granules in platelets results from an abnormal retention of the granules, rather than from their abnormal production.

In all three genotypes the deletion is total and Models 1 and 2 share the same origin. Hence the discrepancies, at least between the first two models, could arise from a difference in strain or from heterogeneity between mice, especially as the numbers of animals used in these studies were relatively low. Moreover, we cannot exclude that the differences with respect to the third model result from the way in which the inactivation was performed. Although these discrepancies do not allow one to conclude the exact role of NBEAL2 in the formation of platelet α-granules and thrombocytopenia, on the basis of the present data it has been possible to formulate a number of hypotheses. The studies need to be continued to determine the actual mechanisms responsible for the human diseases, and how they depend on the type of mutation.

Thrombocytopenia associated with ANKRD26 / familial thrombocytopenia 2

Familial thrombocytopenia 2 (THC2) with a predisposition for leukemia is a rare form of autosomal dominant thrombocytopenia. Whereas the platelets are morphologically and functionally normal, the thrombocytopenia is moderate to severe and leads to moderate bleeding. A platelet production defect has been proposed in view of the fact that the bone marrow of patients displays dysmegakaryopoiesis and micromegakaryocytosis. This disease has recently been associated with point mutations in the non-coding 5' part of the *ANKRD26* (ankyrin repeat domain 26) gene.[108] ANKRD26 encodes a 192 kDa protein abundantly expressed in various tissues such as the brain, liver and adipose tissue, skeletal muscle and hematopoietic tissue. Interestingly, *Ankrd26*-deficient mice develop obesity and gigantism associated with hyperphagia but do not exhibit thrombocytopenia, suggesting that haploinsufficiency is not responsible for the thrombocytopenia observed in patients.[109,110] Consistent with these observations, recent data have shown that the 5 'UTR mutations affect binding of the RUNX1 and FLI1 transcription factors, resulting in the absence of the repression of the *ANKRD26* gene which normally occurs in the late stages of megakaryopoiesis.[111] This abnormal overexpression increases ANKRD26 signalling *via* the TPO/cMPL axis, causing abnormal signalling through the ERK/MAPK pathway and consequently defective proplatelet formation. Hence, mouse overexpression of ANKRD26 is anticipated to better recapitulate the pathology.

Conclusions

Recent years have seen major advances in our understanding of the normal and pathological mechanisms of thrombopoiesis. The contribution of animal models in this area has been important to date, not so much to identify genes implicated in thrombocytopenia, but rather to elucidate the mechanisms involved through their use in structure-function studies in mutated genotypes, and as preclinical models to test new drugs or develop gene therapies.

At present, the cause of thrombocytopenia is unknown in 40% to 50% of cases. The new high-speed sequencing techniques, in particular the sequencing of complete genomes or exomes, now allow us to identify candidate genes. These new candidates will be the targets of many

future murine models. Complementarily, programs of random mutagenesis in mice should enable us to identify other candidate genes involved in thrombocytopenia, prior to their investigation in patients.

The ideal animal model remains the "knock-in" mouse, with identical reproduction of the human mutation when this is known, provided the gene and its function are conserved between mouse and man. The recent possibility of generating induced pluripotent stem cells (iPS) now permits the production of mutated hematopoietic stem cells from fibroblasts of patients. Xenotransplantation of these stem cells into immunodeficient (NOD/SCID/γc) mice may be expected to mimic or reproduce the human pathology, even when the gene responsible is unknown. This strategy, already employed for hematological malignancies, will allow us to study the abnormal mechanisms in the cells of interest (megakaryocytes and platelets) and evaluate new therapeutic approaches.

Sometimes, however, the mouse model does not reproduce the entire human phenotype and can even raise new questions, as in the case of Nbeal2-deficient mice. Hence the *in vitro* culture and differentiation of human megakaryocytes remains absolutely essential and fully complementary to studies in mice. Indeed, it is the confrontation of data obtained in animals and humans, *in vivo* and *in vitro*, which has and will enable major advances in our understanding of thrombocytopenia, and likewise in our knowledge of the normal mechanisms of platelet production.

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