Modeling myeloproliferative neoplasms: From mutations to mouse models and back again

Review

Modeling myeloproliferative neoplasms: ~~f~~From mutations to mouse models and back again

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Abstract

Myeloproliferative neoplasms (MPNs) are defined according to the 2008 World Health Organization (WHO) classification and the recent 2016 revision. Over the years, several genetic lesions have been associated with the development of MPNs, with important consequences for identifying unique biomarkers associated with specific neoplasms and for developing targeted therapies. Defining the genotype-phenotype relationship in MPNs is essential to identify driver somatic mutations that promote MPN development and maintenance in order to develop curative targeted therapies. While studies with human samples can identify putative driver mutations, murine models are mandatory to demonstrate the causative role of mutations and for pre-clinical testing of specific therapeutic interventions. This review focuses on MPN mouse models specifically developed to assess the pathogenetic roles of gene mutations found in human patients, as well as murine MPN-like phenotypes identified in genetically modified mice.

Keywords: ~~m~~Mouse modeling; ~~m~~Myeloproliferative neoplasms; JAK2; MPL; ~~c~~Calreticulin

~~1.~~1 Introduction

Myeloproliferative neoplasms (MPNs) comprise a group of clonal hematopoietic stem cell (HSC) disorders characterized by excessive proliferation of at least one of erythroid, megakaryocytic and myeloid lineages, retaining the ability of terminal differentiation. Thus, these disorders present with an increased number of erythrocytes, platelets and granulocytes. The 2008 World Health Organization (WHO) classification described these diseases according to proliferative features or dysplastic characteristics [1,2]. In particular, three major groups have been identified, namely i) myeloproliferative neoplasms (MPN), ii) overlapping myelodysplastic/myeloproliferative syndromes (MDS/MPN) and iii) myeloid/lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA/PDGFRB or FGFR1 (Table 1). Philadelphia positive Chronic Myeloid Leukemia is classified as an MPN disorder, but is generally considered as a separate entity, due to its unique biological properties and responses to therapy. Philadelphia negative MPN includes polycythemia vera (PV), essential thrombocytosis (ET), primary (idiopathic) myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), mastocytosis and unclassifiable MPNs. MDS/MPN syndromes include chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), atypical Chronic Myeloid Leukemia (aCML) [3] and unclassifiable MDS/MPN [1,2]. A 2016 revised classification has

been recently published, in which molecular criteria play an increasingly important role [4,5]. Over the years, several genetic lesions have been identified and associated with specific MPN diseases, as extensively reviewed [6–11]. Generally, most MPNs are characterized by aberrant activation of tyrosine kinases or direct signal transduction effectors, which are able to promote and sustain proliferation of myeloid progenitor cells. Among the most common genetic aberrations, JAK2 mutations represent a hallmark of several MPNs and confer cytokine hypersensitivity and cytokine-independent growth to hemopoietic cells [12,13]. The most common JAK2 mutation is V617F, which is found in about 95% of cases of PV, 50% of ET, 58% of PMF and about 50% of refractory anemia with ringed sideroblasts associated with thrombocytosis. Furthermore, several JAK2 V617F negative PV patients bear JAK2 mutations within the exon 12 [14]. The thrombopoietin tyrosine kinase receptor, MPL, is aberrantly activated due to W515L or W515K mutations, which are found in about 10% of PMF and a small proportion of ET patients [15]. The Stem Cell Factor (SCF) receptor, c-KIT, is mutated (D816F) in systemic mastocytosis (SM) [16], while the PDGF-receptor A and B genes are aberrantly activated or translocated (i.e. FIP1L1-PDGFRA) in a specific subgroup of MPNs [17].

Table 1 2008 World Health Organization classification of myeloid neoplasia.

alt-text: Table 1

2008 WHO classification of myeloid neoplasia
1. Myeloproliferative neoplasms (MPN)
1.1 Chronic myelogenous leukemia, BCR-ABL-positive (CML)
1.2 Polycythemia vera (PV)
1.3 Essential thrombocythemia (ET)
1.4 Primary myelofibrosis (PMF)
1.5 Chronic neutrophilic leukemia (CNL)
1.6 Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
1.7. Mast cell disease (MCD)
1.8 MPN, unclassifiable
2. MDS/MPN
2.1 Chronic myelomonocytic leukemia (CMML)
2.2 Juvenile myelomonocytic leukemia (JMML)
2.3 Atypical chronic myeloid leukemia, BCR-ABL-negative (aCML)
3. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB and FGFR1
4. Myelodysplastic syndromes (MDS)
5. Acute myeloid leukemia (AML)

More recently, in addition to tyrosine kinase mutations, other mutations have been recurrently found in MPNs. In particular, the calcium binding protein calreticulin was found to be mutated in ET and PMF [18–20]; SETBP1 was mutated in aCML patients [21,22]; the receptor for colony-stimulating factor 3 (CSF3R) was mutated in CNL [23,24]; and ETNK1 was found to be mutated in a small portion of aCML patients [25]. Identifying these mutations has led to an important step forward for the diagnosis and the biological assessment of MPNs. Also, mutations in genes involved in epigenetic regulations, such as TET2, DNMT3A, ASXL1, EZH2 and IDH1/2 [26], were found to play a role in MPN pathogenesis even if mutations of these genes are generally considered insufficient per se to drive a cancer phenotype [8,27,28]. Each of these mutations can be considered as specific diagnostic markers, in particular for the unclassifiable MPN forms. Furthermore, gene mutations may reveal the complexity of the pathogenetic mechanisms of MPN development, maintenance and targeting with specific inhibitors.

While studies with human samples are mostly correlative (i.e. identifying an association between a putative driver somatic mutation with a phenotype), the only approach for attributing a pathogenetic role in

cancer development and maintenance to a particularly mutation is the development of specific mouse models. This review focuses on MPN mouse models that have been generated to demonstrate the causal role of MPN-associated somatic mutations (Table 2). Finally, it should also be noted that analyzing the phenotype of specific knock-out models has sometimes been unexpectedly associated with the development of myeloproliferative disorders (MPDs). Therefore, this approach (i.e. from the mouse model to human genetics) has resulted in the identification of new genes with a role in human MPN. Thus, this review will also summarize the '*mouse-to-human*' strategy to identify novel candidate genes in MPN pathogenesis.

Table 2 From human pathology to mouse models.

Summary of mouse models developed to assess the contribution of a given mutation in the MPN pathogenesis.

alt-text: Table 2

Human pathology	Mutated gene	Mouse model	Mouse phenotype	References
PV, ET, MF	JAK2	Mice transplanted with BM cells transduced with retrovirus coding for the human JAK V617F	PV, but not ET or PMF	[29,34–38]
PV, ET, MF	JAK2	Transgenic mice carrying the human JAK2 V617F cDNA under the control of the vav gene promoter	PV, ET and BM fibrosis in aged mice	[39]
PV, ET, MF	JAK2	Transgenic mouse carrying the mouse Jak2 V617F coding sequence under the control of H-2Kb MHC class I promoter	First transgenic mouse line: PV or ET. Second transgenic line: PMF	[40]
PV, ET, MF	JAK2	Transgenic mouse expressing an inducible human JAK2 V617F under the control of the Vav promoter	ET	[41]
PV, ET, MF	JAK2	Transgenic mouse expressing an inducible human JAK2 V617F under the control of the MX1 promoter	PV	[41]
PV, ET, MF	Jak2	Knock-in mouse for Jak2 V617F	PV	[42]
PV, ET, MF	Jak2	Knock-in mouse in which mouse Jak2 V617F is constitutively expressed under the control of the endogenous Jak2 promoter	PV	[43]
PV, ET, MF	Jak2	Knock-in mouse in which mouse Jak2 V617F is conditionally expressed under the endogenous Jak2 promoter in the early embryo	PV	[44]
PV, ET, MF	JAK2	Knock-in mouse in which the human mutant protein is conditionally expressed under the mouse Jak2 endogenous promoter	TE, only 10% of mice display PV	[45]
PMF, ET, MF	MPL W515L	Mice transplanted with BM cells transduced with retrovirus coding for the MPL W515L mutant receptor	ET, thrombocytosis, splenomegaly and increased fibrosis	[46]
ET, MF	Calreticulin del52 and ins5	Mice transplanted with BM cells transduced with retrovirus coding for the human calreticulin del52 and ins5	ET	[52]
aCML and CNL	CSF3R-T618I	Mice transplanted with BM cells transduced with retrovirus coding for the human CSF3R-T618I	lethal myeloproliferative disorder in 3 months	[53]
SM	c-Kit D814V	Subcutaneous injection in nude mice of Ba/F3 cells transduced with retrovirus coding c-Kit D814V	Large tumors and lethal leukemia in two weeks	[57]
SM	c-KIT D816V	Intravenous injection of Ba/F3 cells carrying the human c-Kit D816V mutation	Lethal leukemia in 3 months	[58]
ISM	c-KIT D816V	*Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice transplanted with bone marrow cells carrying c-KIT D816V	Focal accumulation of mast cells in different organs	[59]
ASM/MCL	c-Kit D814Y	Retro-orbital injection of the murine mastocytoma cell line P815 cells in into DBA/2 syngeneic mice	Decreased platelets, leukocytosis, malignant mast cells in the blood and lethality in 9 days	[60]
SM	c-KIT D816V	Transgenic mice with the human c-KIT D816V under the control of a mast cell	Mastocytosis with different clinical signs (from indolent mast cell	[61]

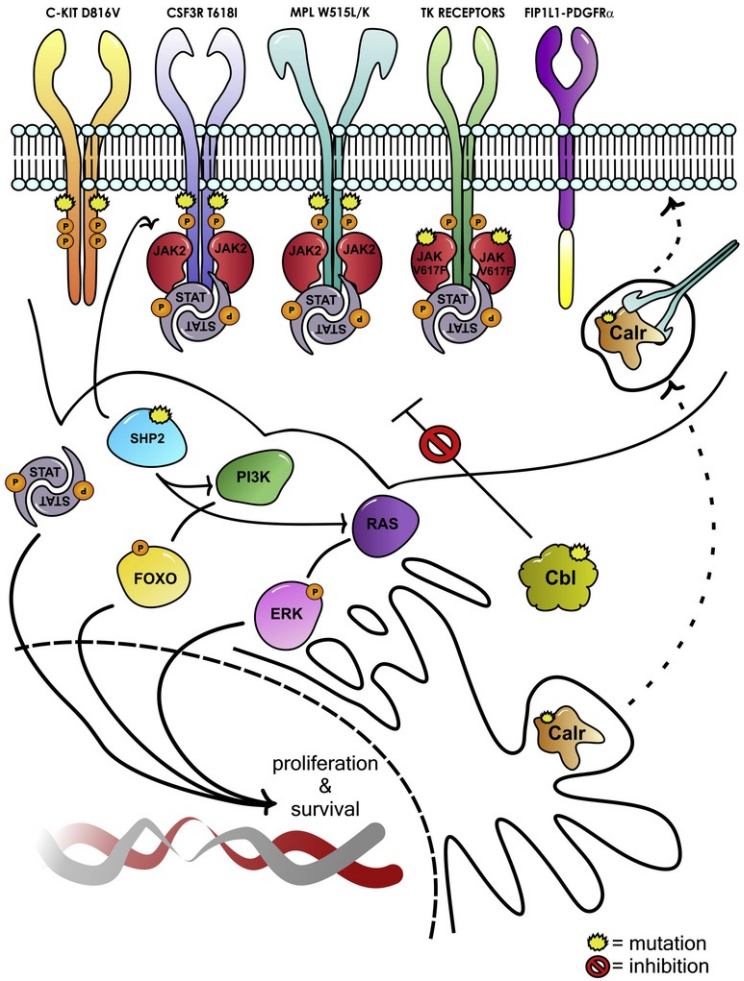
		specific promoter (chymase promoter)	hyperplasia to invasive mast cell tumors)	
SM	c-Kit D814V	*KitD814Vflox* bacterial artificial chromosome transgenic mice x *Mx1*-Cre	Severe mastocytosis associated with other hematopoietic neoplasms and intestinal inflammation	[62]
Mild SM	c-Kit D814V	*KitD814Vflox* bacterial artificial chromosome transgenic mice x Mcpt5-Cre	Mastocytosis	[62]
Mainly JMML, CMML and MF	Cbl	Cbl and Cbl-b double knock-out in the HSC	Early-onset lethal MPD	[69]
SM and myeloid and lymphoid neoplasms with eosinophilia	FIP1L1-PDGFRα	CD2-IL-5Tg mice transplanted with BM cells transduced with retrovirus coding for the human FIP1L1-PDGFRα	HES	[72]
JMML	PTPN11-E76K	Conditional *Ptpn11 E76K*/+/*Mx1-Cre*$^{+}$ mice	MPD evolving in AML	[79]
JMML	PTPN11-D61Y and E76K	Mice transplanted with BM cells transduced with retrovirus coding for Shp2 D61Y and E76K mutants	Fatal JMML-like disorder	[80]

~~2.~~2 Murine models of MPN-associated somatic mutations

~~2.1.~~2.1 JAK2 mutations

~~*2.1.1.*~~*2.1.1 JAK2 V617F*

The JAK2 V617F mutation has been found in most patients suffering from PV and in about 50% of patients with ET or PMF. As mentioned before, these pathologies show relevant clinical differences, for example, PV patients mainly display erythroid hyperproliferation, while megakaryocytic hyperplasia tends to be prevalent in ET and PMF. Interestingly, this mutation is present in homozygosity in 30% of PV patients [29], while the homozygous mutation is less common in ET and PMF [30], suggesting a quantitative effect in determining the pathological phenotype. The JAK2 V617F mutation is detectable in HSCs [31] and progenitor cells in PV patients [32]. The expression of JAK2 V617F in cell lines constitutively activates STAT5, AKT and ERK signaling pathways, transforming cytokine-dependent cells into becoming cytokine independent (Fig. 1) [29,30]. A number of different strategies have been established in recent years to model MPNs driven by JAK2 mutations in mice (for an extensive review see [33]). Interestingly, different models may recapitulate the features of different MPNs. Mice transplanted with bone marrow (BM) cells transduced with a retrovirus coding for the human JAK2 V617F mutant were shown to develop PV, but not ET or PMF [29,34–38], and displayed erythrocytosis, splenomegaly, low erythropoietin levels, leukocytosis and neutrophilia, with little or no effect on platelet counts. Transplanted mice subsequently developed anemia, together with fibrosis in the BM and in the spleen, resembling the “spent” phase of human PV. However, there was a phenotypic variability depending on the genetic background of the mice. Notably, in transplant models, JAK2 V617F expression is 10 to 40 times greater than the normal endogenous protein. Different levels of transgene expression due to different viral integration sites could also account for specific disease features in this mouse model.



~~Figure 1~~Fig. 1 Signal transduction in myeloproliferative neoplasms (MPNs).

MPNs are associated with the presence of constitutively active tyrosine kinases or downstream effectors that lead to the activation of various pathways, including PI3K, JAK/STAT and MAPK, which are responsible for proliferation and survival. Representative MPN-associated mutations are shown.

alt-text: Fig. 1

Transgenic mice carrying the human JAK2 V617F mutant cDNA including the 3′ untranslated region under the transcriptional control of the *vav* gene promoter have been generated by pronuclear injection. Different lines of transgenic mice show features of MPN with varying degrees of severity. The transgenic line expressing a lower level of mutated JAK2 showed a moderate elevation of red cells, but there was no PV or ET phenotype. Conversely, a second line of transgenic mice expressing higher levels of mutated JAK2 developed PV and ET and BM fibrosis in older mice, demonstrating a relationship between JAK2 V617F expression levels and the severity of the pathology [39].

A second transgenic mouse model was produced using the mouse Jak2 V617F coding sequence under the control of the H-2Kb MHC class I promoter. Half of the first transgenic mouse line showed features of human PV or ET, while the second transgenic line recapitulated all the feature of PMF [40]. It is unknown why distinct phenotypes are manifested according to different transgenic lines, but they may be related to the number of transgene copies integrated into the mouse genome as well as the particular integration sites.

Furthermore, Tiedt et al. produced a transgenic mouse expressing JAK2 V617F in an inducible manner. A construct carrying a bacterial artificial chromosome containing the 5′ regulatory region and the first 12 exons of the

human JAK2 gene, followed by a cDNA fragment encoding mutated JAK2 exons 13–25 plus a polyadenylation signal from SV40, placed in the inverse orientation and flanked by antiparallel lox sites, was used to generate a transgenic line carrying nine copies of the transgene [41]. Expression of full-length wild type or mutated JAK2 was induced by crossing these mice with transgenic mice carrying the Cre recombinase coding gene under the control of the Vav or Mx1 promoter. Due to diverse possible recombination events between the lox sites flanking multiple transgene copies, various JAK2 V617F expression levels were obtained in response to different Cre activity. In particular, JAK2 V617F/VavCre mice expressed lower levels of JAK2 V617F than the endogenous wild type JAK2. These mice displayed thrombocytosis, moderate neutrophilia and unchanged hematocrit, resembling ET. Conversely, JAK2 V617F/Mx1Cre mice displayed high transgene copies and showed increased erythrocytosis as well as thrombocytosis, similar to PV, suggesting a dependence on the levels of mutant JAK2 in determining a particular phenotype [41].

Mouse Jak2 V617F heterozygous and homozygous knock-in model reproducing the main features of human PV have been generated [42]. These mice show increase in hematocrit, hemoglobin, red blood cells, leukocytes and thrombocytes, splenomegaly and reduced serum erythropoietin levels, with homozygous mice showing a worsening in pathological signs and an accelerated BM fibrosis compared to heterozygous mice.

A second knock-in mouse model was generated by Marty et al. in which the murine Jak2 V617F was constitutively expressed under the control of the endogenous JAK2 promoter. Similarly to the previous mouse model by Akada et al., these mice showed a clear PV phenotype. They also displayed increased megakaryopoiesis and spleen hyperplasia, followed by fibrosis around 9 months of age [43].

A third knock-in mouse model was generated in which the murine JAK2 V617F was conditionally expressed under the endogenous JAK2 promoter when mice were crossed with E2A transgenic Cre mice, which drives Cre expression in the early mouse embryo [44]. These heterozygous mice all develop a lethal MPD similar to human PV with a median survival of 146 days. The disease is characterized by elevated hematocrit, splenomegaly and splenic extramedullary erythropoiesis without thrombocythemia, differences in megakaryocytes ploidy and BM fibrosis [44].

A fourth JAK2 V617F mouse model, in which the human mutant protein was conditionally expressed under the mouse JAK2 endogenous promoter in adult mice, mainly developed ET phenotypes, and only 10% of the mice showed erythrocytosis or BM fibrosis after 26 weeks [45], resembling human PV.

~~2.1.2.~~2.1.2 *JAK2 exon 12 mutations*

In addition to the more common JAK2 V617F mutation, a JAK2 exon 12 heterozygous mutation has been described in patients affected by PV or idiopathic erythrocytosis [14]. Similarly to JAK2 V617F, JAK2 exon 12 mutations are gain-of-function mutations inducing constitutive activation of JAK2, STAT5 and ERK1/2. Hematopoietic cell lines carrying exon 12 mutations acquire the ability to proliferate in the absence of cytokines [14]. The Ba/F3 hematological cell line infected with a retrovirus carrying the murine Jak2 K539L mutant transplanted into lethally irradiated BALB/c mice develop erythrocytosis, leukocytosis and modest thrombocytosis, with a lower leukocyte and platelet count compared to mice transplanted with Ba/F3 carrying the JAK2 V617F mutation [14].

The extensive efforts to study and model JAK2 mutations in mice have demonstrated that mutant JAK2 is sufficient to induce PV in vivo and has led to the hypothesis that high levels of JAK2 mutant protein often cause PV, while more physiological expression levels generate ET-like phenotypes [33]. Moreover, all three knock-in mouse models expressing the mouse Jak2 V617F caused a PV phenotype, while the mouse model expressing the human JAK2 mutant showed an ET phenotype, suggesting differences in mouse and human JAK2 mutant activity, or in the impact of the mutant receptors in an endogenous context [33].

~~2.2.~~2.2 MPL

Somatic mutations in the thrombopoietin receptor MPL (MPL W515L/K) have been found in approximately 5% of patients affected by primary myelofibrosis, in 1% of ET patients and in 10% of patients with post-ET myelofibrosis, but not in patients with other MPDs [15,46]. Similarly to JAK2 mutations, the expression of MPL mutants transforms cytokine-dependent hematopoietic cells into becoming cytokine independent, and induces constitutive activation of JAK-STAT signaling (Fig. 1).

A mouse model was developed by transducing BM cells with a vector coding for the MPL W515L mutant receptor followed by transplantation into lethally irradiated mice [46]. These mice developed MPD with a short latency, characterized by thrombocytosis, splenomegaly and increased fibrosis.

~~2.3.~~2.3 Calreticulin

It has been recently found that most patients affected by ET or MF without JAK2 or MPL mutations carry a mutated calreticulin coding gene. Calreticulin is a chaperone protein located in the endoplasmic reticulum (ER) and involved in regulating intracellular Ca^{2+} homeostasis and the Ca^{2+} capacity of the ER. Moreover, calreticulin plays a role in folding and quality control of newly synthesized glycoproteins [19,47]. Interestingly, calreticulin mutations are insertions or deletions that occur in the last exon of the gene, where they cause a specific frameshift that generates a common aberrant terminal peptide and disrupts the acidic Ca^{2+} binding c-terminus of the protein and the KDEL ER retention sequence [9]. However, a mutant lacking the entire exon 9 does not cause ET, indicating that the mutated c-terminal polypeptide plays an active role in inducing megakaryocyte proliferation. Recently, it has been proposed

that the oncogenic feature of the mutant calreticulin is due to the electrostatic change of the c-terminal of the protein by substitution of negatively charged amino acids with positively charged ones. This structural modification allows calreticulin to bind MPL in the ER and to be exported to the cell surface where it induces MPL ligand-independent activation of the JAK2/STAT/PI3K and MAPK pathways [48–51] (Fig. 1). The two most common calreticulin mutations, present in 85% of cases, are a 52-bp deletion (del52) and a 5-bp insertion (ins5). The first mutation is more frequent in myelofibrosis, while the second is predominantly associated with ET. Using retroviral mouse models, it has been demonstrated that calreticulin mutations cause ET in the mice [48,52]. However, calreticulin del52 induces a higher level of megakaryocyte proliferation compared with calreticulin ins5, and causes myelofibrosis and osteosclerosis post-ET [52]. Moreover, the two mutations impact differently on HSC populations, with the calreticulin del52 mutation being advantageous to HSCs [52].

~~2.4.~~2.4 CSF3R-T618I

Colony-stimulating factor 3 receptor (CSF3R) is involved in the control of granulocyte maturation and growth. Upon stimulation, CSF3R promotes the activation of the JAK-STAT pathway and the SRC family member LYN. Recently, it was found that CSF3R was mutated in patients with chronic neutrophilic leukemia (CNL) and aCML [23,24], and in subsequent analyses, CSF3R mutations were predominantly associated with CNL patients. Two classes of CSF3R mutations have been identified, namely truncation of the cytoplasmic domain and membrane proximal point mutations. While truncation mutations are mostly associated with congenital neutropenia, membrane proximal mutations are associated with both CNL and aCML. These mutations (and in particular T618I) constitutively activate JAK and SRC signaling allowing the identification of putative targets with therapeutic relevance in these kinases [53] (Fig. 1). The proof-of-concept that CSF3R mutations play an essential role in CNL development was demonstrated with the transplantation of CSF3R-T618I-infected cells in mice [53]. In this model, mutant CSF3R stem cells cause the development of a lethal MPN resembling human CNL.

~~2.5.~~2.5 c-KIT

c-KIT is a tyrosine kinase receptor involved in the activation of signaling pathways involved in cell survival, proliferation and migration. c-KIT plays a physiological function in normal hematopoiesis, in gametocyte development, intestinal motility, inflammation and vasculogenesis. c-KIT mutations have been involved in a number of human tumors, including gastrointestinal stromal tumors, melanomas, small cell lung carcinoma, systemic mastocytosis (SM) and acute myeloid leukemia [54]. Activated mutations of c-KIT are found in about 40% of patients with core–binding factor acute myeloid leukemia and in almost all adult patients with SM. SM is a complex disease caused by clonal proliferation of abnormal mast cells accumulating in the skin, BM and other organs. The disease has different clinical presentations, from cutaneous and indolent forms to aggressive SM and mast cell leukemia [55]. The most common c-KIT mutation in hematological neoplasms is D816V, an activating mutation occurring in the tyrosine kinase domain leading to constitutive autophophorylation (Fig. 1). Interestingly, the c-KIT D816V mutation is present in patients with different SM clinical phenotypes, suggesting that further mutations may account for disease progression [56].

Different mouse models have been generated to evaluate the ability of c-KIT mutations to drive tumorigenesis, to determine the molecular basis of the disease and to test drug efficacy.

In early studies, murine c-Kit D814V, corresponding to the human D816V, was shown to induce IL-3 independent growth in pro-B-type Ba/F3 cells and myeloid FDC-P1 cells. Moreover, subcutaneous injection of Ba/F3 cells in nude mice induced the formation of large tumors in 2 weeks, and rapidly caused a lethal leukemia with mutant cells infiltrating the BM, spleen and liver [57]. When injected intravenously into nude mice, Ba/F3 cells carrying the human c-KIT D816V mutation invade the BM, spleen and lymph nodes and induce a leukemia-like disease with a latency of 3 months [58]. However, Mayerhofer et al. demonstrated that doxycycline-mediated inducible expression of c-KIT D816V in Ba/F3 cells does not induce IL-3-independent growth or tumorigenicity in nude mice. Moreover, C57BL/6 mice deficient for Rag2 and the common γ-chain, which were transplanted with BM cells transduced with a retrovirus coding for the human c-KIT D816V, did not develop an aggressive disease, but a focal accumulation of mast cells in different organs, resembling indolent SM [59].

A simple mouse model of aggressive SM and mast cell leukemia has been developed by Demehri et al., by retro-orbital injection of the murine mastocytoma cell line P815 carrying the c-Kit D814Y mutation into DBA/2 syngeneic mice. After 8 days, injected mice manifested a decreased platelet count, leukocytosis and the appearance of malignant mast cells in the blood, and rapidly died [60].

Transgenic mice in which the human c-KIT D816V is expressed under the control of a mast cell specific promoter have been produced and characterized. These mice develop a disease similar to human mastocytosis, presenting various clinical signs of the human disease, from indolent mast cell hyperplasia to invasive mast cell tumors [61]. A conditional mouse model has also been generated, in which expression of the c-Kit D814V mutant is driven by the c-Kit promoter after Cre recombinase induction with poly (I:C) injection. Mutant c-Kit expression was shown to cause a severe form of mastocytosis frequently associated with other hematopoietic neoplasms and intestinal inflammation [62]. Conditional expression of c-Kit D814V, specifically in mature mast cells taking advantage of the Mcpt5Cre mouse transgenic line, induces mastocytosis with slow kinetics, probably due to downregulation of c-Kit D814V transgene expression in differentiated mast cells [62].

In addition to murine models, a zebrafish model has also been generated that expresses the human c-KIT D816V under the control of the ubiquitous zebrafish beta-actin promoter. Around 60% of adult transgenic fish manifest a myeloproliferative disease with mast cell expansion in the kidney marrow, which is functionally similar to the BM in mammals. Zebrafish carrying the human c-KIT D816V may represent a useful tool for high throughput in vivo drug screening.

~~2.6.~~2.6 Cbl

The family of Casitas B-lineage Lymphoma (Cbl) RING finger ubiquitin ligases is composed of three homologous proteins in mammals, namely Cbl, Cbl-b and Cbl-c. The first two members are enriched in the hematopoietic compartment, while Cbl-c is specifically expressed in epithelial cells. Cbl proteins are highly related E3 ubiquitin ligases that have different ubiquitin-associated domains; they are involved in tyrosine kinase receptor turnover, leading to inhibition of signal transduction (Fig. 1). Moreover, Cbl also binds to intracellular signaling proteins, which are consequently targeted for degradation, further inhibiting intracellular signaling [7,63].

Mutations in the *Cbl* coding gene have been reported by several authors in patients affected by myeloid malignancies, and in particular in MPNs. Most mutations were found in patients affected by JMML (19%), CMML (5–17%) and MF (6%) [64–68]. Mutations in the *Cbl*-b and *Cbl*-c coding genes, however, are rarely detected [68].

Naramura et al. crossed Cbl-b null mice carrying a conditional allele for Cbl with an MMTV-Cre transgenic mouse, targeting the Cre recombinase in the mammary gland and in other districts, including the early HSC compartment. Cbl and Cbl-b double knock-out in the HSC compartment induces an early-onset lethal myeloproliferative disease in mice similar to human MPD. This disease is characterized by anemia and myeloid expansion in the BM and in the periphery, and is fully penetrant and transplantable [69]. The MPN that develops likely depends on the expansion of the HSC compartment and myeloid, granulocyte/macrophage and lymphoid progenitors [70].

~~2.7.~~2.7 FIP1L1-PDGFRα

According to the 2008 WHO classification of MPN, the translocation coding for FIP1L1-PDGFRα is recurrently found in a specific subgroup of MPN disorders (Table 1), and accounts for the second most frequent lesion in SM. This translocation causes constitutive activation of the PDGF-receptor [17,71] (Fig. 1). Transduction of BM stem cells/progenitor cells with a vector bearing this fusion gene has been shown to generate a CML-like disease that did not, however, resemble the features of hyper eosinophilic syndrome (HES). However, the authors demonstrated that transplantation of FIP1L1-PDGFRα-infected cells in transgenic mice with aberrant expression of IL-5 caused a significant increase in the aberrant proliferation of eosinophils. This work suggests that, while FIP1L1-PDGFRα is associated with the development of a CML-like MPN, it is not sufficient for promoting HES development and requires additional lesions [72].

~~2.8.~~2.8 SHP2

In addition to mutations that activate tyrosine kinases, MPNs have also been associated with aberrant regulation of phosphatases, which modulate the signal transduction of various tyrosine kinase receptors [73].

SH2-containing tyrosine phosphatase 2 (SHP2) is a ubiquitously expressed protein tyrosine phosphatase encoded by the PTPN11 gene, and plays a crucial role in organism development and homeostasis [74]. Although protein phosphatases are usually considered to be negative regulators of signal transduction, SHP2 exerts a positive role in sustaining tyrosine receptor signaling, due to dephosphorylation and inactivation of signaling inhibitors [75]. From a molecular point of view, SHP2 plays a role in activating the Ras-MEK, the PI3K-AKT and the JAK-STAT pathways, in some cellular contexts [74,75]. In hematopoiesis, SHP2 has been shown to play an essential role by regulating HSC self-renewal, as documented by two independent mouse models, (see review [76]). Germline gain-of-function mutations in the *PTPN11* gene were discovered in 50% of patients with Noonan syndrome, a rare genetic disorder causing craniofacial and skeletal abnormalities, cardiac defects and short stature. Notably, these patients are predisposed to develop JMML. The involvement of SHP2 in sporadic hematological cancers, particularly in MPNs, depends on either gain-of-function point mutations or overexpression. In particular, SHP2 is mutated in about 35% of JMML cases, and more rarely in other myeloid neoplasms; SHP2 has also been shown to be aberrantly regulated as a downstream effector of mutated KIT in SM [76].

To recapitulate the phenotype of PTPN11 mutations, various mouse models have been developed, as recently reviewed [76]. In a knock-in mouse model for the Noonan syndrome-associated mutation Shp2 D61G, viable heterozygous mice displayed a transplantable MPD, in addition to skeletal and cardiac abnormalities [77,78]. The pathogenesis of this disease depends on the accelerated HSC cycling due to enhanced intracellular proliferative signaling [78].

Conditional knock-in of the most common mutation (PTPN11-E76K) associated with JMML was produced using different Cre transgenic mice. While global expression of the Shp2 E76K mutant during early embryogenesis causes embryonic lethality at 11.5 days, the presence of the mutation in the HSC compartment induces an MPD that progresses to an accelerated phase and evolves into AML, but also into T cell acute lymphoblastic leukemia/lymphoma or B cell acute leukemia [79].

In another approach, the SHP2 D61Y and E76K mutants, typically found in JMML and other neoplasms, were transduced into primary mouse BM cells using retroviral vectors, followed by transplantation into lethally irradiated recipients. SHP2 mutants caused progressive leukocytosis in transplanted mice within 3 months, with some mice also showing lymphocytosis [80]. From these models, SHP2 was attributed to play a role in leukemogenesis and particularly in the modulation of HSCs.

More recently, it has also been shown that SHP2 plays an essential role in mediating the oncogenic signal of mutant c-KIT in SM [81]. Notably, treatment of primary human KITD816V-CD34^{+} cells with the SHP2 inhibitor II-B08

was shown to inhibit their growth and to prolong the survival of mice transplanted with myeloid 32D expressing the KITD814V mutant [81].

~~2.9.~~2.9 Epigenetic regulators

Various genes belonging to the family of epigenetic regulators, such as TET2, DNMT3A, ASXL1, EZH2 and IDH1/2, have been found recurrently mutated in myeloid leukemia and in about 5–30% of MPNs [9,26,82].These mutations have been in general described as passenger mutations [8,27,28], however, mouse model phenotypes and recent evidences with human samples [82] suggest that their role is much more complex and that they may also be directly involved in MPN pathogenesis.

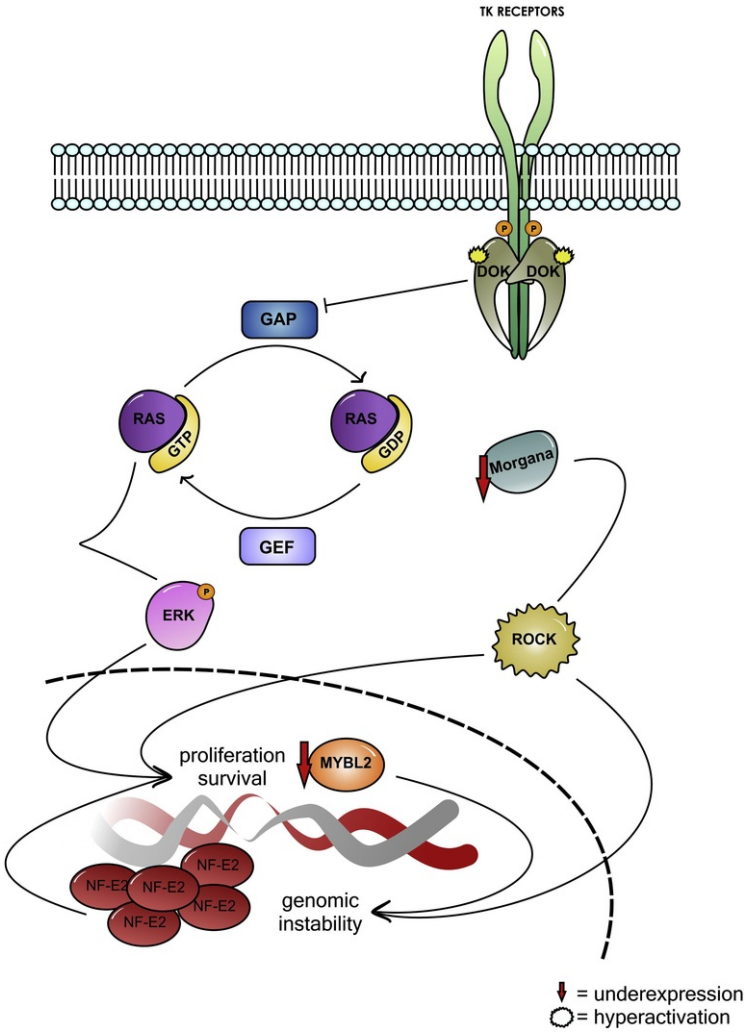
Various mouse models have been generated to assess the contribution of TET2 [83–87,88,89], DNMT3A [90–93], ASXL1 [94,95,96], EZH2 [97,98] and IDH1/2 [99–101] genes in hematopoiesis and leukemogenesis. All these models demonstrated that these genes are involved in the regulation of the HSC self renewal and, to some extent, in the development of AML, MDS and MPN. However, only one DNMT3A null mouse model was strongly associated with the development of a rapid and fatal MPN disorder [93]. Conversely, the IDH1 R132H knock-in mouse had normal lifespan, even if IDH1 mutation significantly affected the murine hemopoiesis [99]. Similarly, TET2 mutations/deletions showed a low penetrance in promoting a MPN phenotype [102] and some mutant strains did not develop the disease even during a 2 years follow up [88]. These observations were also reported by a recent work, where one-third of *Tet2* null and 8% of *Tet2* heterozygous mice were shown to die for leukemia or MPN in one year follow up [87]. Similarly, the phenotype of ASXL1 mutant mouse models was highly variable being in some cases associated with MDS development [96], and in others with the perturbation of the hematopoiesis but not with the onset of MDS/AML/MPN [95]. These data indicate that TET2, DNMT3A, ASXL1, EZH2 and IDH1 play a key role in hematopoiesis. The ability of epigenetic regulators to shape chromatin accessibility, besides sustaining cancer development and cooperating with other mutations, may induce new genetic lesions and fuel genomic instability [26,103,104]. In this view, a high degree of variability is conceivable in mouse models carrying mutant epigenetic regulators. Similarly, recent essential observations with human samples have further expanded the landscape and complexity of epigenetic regulator mutations [105]. The definition of the role of these genes, still highly challenging, will not be unfolded in detail in this review.

~~3.~~3 From mouse models to novel human MPN-associated genes

~~3.1.~~3.1 Morgana

Morgana is a ubiquitous chaperone protein [106] that can bind to the Rho kinases ROCKI and ROCKII and can inhibit their kinase activity [107,108]. Rho kinases, despite being highly homologous and sharing common substrates, show defined functions [109], and their deregulation produces specific pathologic effects. While ROCKII hyperactivation induces centrosome amplification, causing aneuploidy and genomic instability [107,110], ROCKI has been recently described as a potent proliferation signal in myeloid cells [111,112]. While morgana null mice die very early during embryogenesis at a peri-implantation stage [107], with age heterozygous mice develop a lethal and transplantable MPN resembling aCML [113]. The disease onset is preceded by ROCK hyperactivation, centrosome amplification, and aneuploidy in the BM. Diseased mice that are morgana heterozygous present myeloproliferation, leukocytosis, anemia, anisocytosis, circulating reticulocytes, and presence of immature myeloid cells in the peripheral blood, with no expansion of monocytes or eosinophils. These mice also show splenomegaly, hepatomegaly and, in 50% of cases, myeloid infiltration in the liver [113].

Morgana deficiency, by activating both ROCKI and ROCKII, can confer a particularly aggressive signal combination to myeloid cells, as ROCKI hyperactivation induces hyperproliferation, while ROCKII fuels genomic instability, thus inducing further oncogenic hits able to deregulate cell cycle check points [114] (Fig. 2).



~~Figure 2~~Fig. 2 Novel human MPN-associated genes.

Schematic representation of DOK, Morgana, MYBL2 and NF-E2 aberrant signaling in MPNs.

alt-text: Fig. 2

Notably, immunohistochemical analysis on the BM of six aCML patients highlighted a very low morgana expression in all samples [113]. However, further analysis on a higher number of aCML patients are needed to confirm the relevance of morgana downregulation in this pathology. It would be interesting to widen the analysis on morgana expression levels and on possible mutations in the morgana coding gene (*CHORDC1*) in different myeloproliferative diseases.

~~3.2.~~3.2 DOK1/DOK2

DOK1 and DOK2 are adaptor proteins that are involved in regulating tyrosine kinase signaling. DOK1, also known as $p62^{dok}$, was originally cloned as a BCR-ABL substrate in CML [115]. DOK proteins are part of a family of seven related proteins that are substrates of relevant tyrosine kinases, such as ABL, EGF-receptor, PDGF-receptor and others. Exogenous expression of DOK1 and DOK2 has been associated with negative regulation of ERK and MYC

signaling. In particular, both DOK1 and DOK2 have been shown to interact with RAS-GAP, which in turn inhibits RAS activation [116] (Fig. 2). Murine knock-out models of *dok1*, *dok2* and *dok3* were shown to develop fatal MPDs. Notably, *dok1* and *dok2* oppose BCR-ABL-mediated leukemogenesis [117–119]. Recently, we also demonstrated that DOK1 and DOK2 behave as tumor suppressors in lung cancer, in addition to MPDs [120]. In particular, DOK1, DOK2, and DOK3 single and compound heterozygous mice developed both lung cancer and MPD. The results obtained with these murine models led to the search for *dok* gene mutations in a cohort of MPDs. Currently, to our knowledge, only CMML resulted positive for mutations in *dok* genes. In particular, mutations in dok1 (L60Q and D263E) and dok2 (R201H, L238P and R215H) were identified in CMML and in a myeloproliferative/myelodysplastic unclassifiable disease [121].

~~3.3.~~3.3 MYBL2

Heterozygous deletion of the long arm of chromosome 20 is a common genetic alteration in MPDs, and has also been described in myelodysplastic syndromes (4%) and in acute myeloid leukemia (1%) [122,123]. MYBL2 has been identified as a tumor suppressor gene, residing in the 20q region, the deletion of which is responsible for aberrant myeloproliferation. In particular, MYBL2 haploinsufficiency confers an advantage to hematopoietic progenitor cells and induces myeloid malignancies [124,125]. The *MYBL2* gene codes for MYB-related protein B (MYB-B), a transcription factor implicated in cell cycle regulation, genome stability and senescence (Fig. 2). Remarkably, expression levels of MYB-B are reduced, even in patients affected by myeloid malignancies carrying a normal pair of chromosomes 20, indicating that MYB-B downregulation is a common feature associated with the pathology, which may occur via different modalities [124,125].

To model MYB-B reduction in vivo, mouse Lin- mononuclear BM cells were infected with a lentivirus coding for shRNAs against MYBL2, followed by transplantation into lethally irradiated syngeneic mice. Transplanted mice showed clonal dominance in a competitive reconstitution assay with cells carrying control shRNAs. Moreover, after 6 months, transplanted mice displayed a clonal and transplantable MPD with dysplastic features associated with anemia and splenomegaly [125].

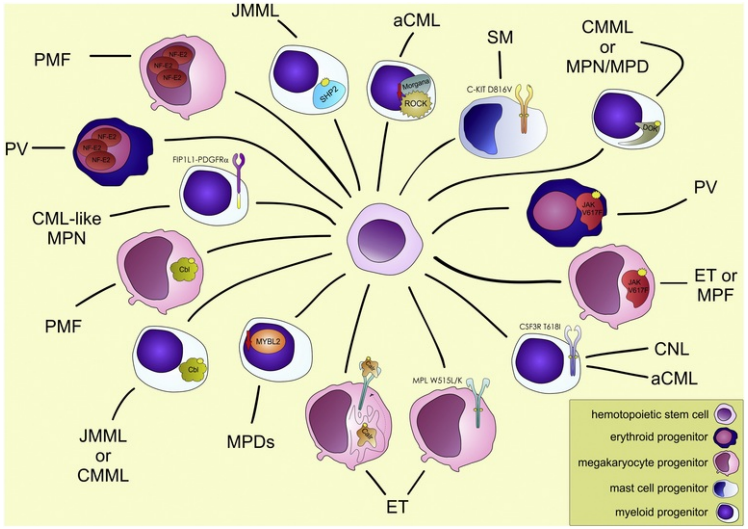
MYBL2 is essential for inner cell mass formation in early mouse development, however heterozygous mice are viable [126]. Clark et al. took advantage of MYBL2 heterozygous mice to study the role of MYBL2 haploinsufficiency in the development of myeloid malignancies. They demonstrated that at 22 months of age, the majority of MYBL2 heterozygous mice showed hematological disorders and splenomegaly. Interestingly, as in human patients, the MYBL2 haploinsufficiency predisposes mice to multiple myeloid disorders. In particular, upon aging, 46% of mice developed MDS, 38% MPN and 7% myeloid leukemia. Moreover, of the five mice affected by MPN, three showed leukocytosis, while two developed severe thrombocytosis [124]. Given that MYBL2 heterozygous mice were maintained in a pure C57/B6 background, the phenotype heterogeneity is likely due to genome instability induced by MYBL2 haploinsufficiency.

~~3.4.~~3.4 NF-E2

Investigating the role of nuclear factor erythroid-2 (NF-E2) in MPN is the paradigm of how murine models can identify novel candidate oncogenes/tumor suppressors. NF-E2 was originally found to be over-expressed in MPN patients and to play an essential role in hemopoiesis [127–130]. Following these observations, a transgenic murine model was designed to up-regulate the expression of NF-E2 in the BM [131]. As a consequence of NF-E2 over-expression, mice developed an MPN disease that evolved into acute myeloid leukemia (Fig. 2). Based on the phenotype of transgenic mice, the genetic status of NF-E2 in MPN patients was investigated and it was found that the *NF-E2* gene is mutated in a subset of patients with MF and PV [132]. The mutations consist of insertions and deletions causing the expression of truncated proteins able to potentiate the wild type NF-E2 transcriptional activity. To clearly demonstrate the causative role of these mutations, BM cells of CD45.2 mice were infected with lentiviruses expressing NF-E2 mutations and transplanted into CD45.1 recipient mice. As a consequence, transplanted animals developed an MPN disease, clearly demonstrating that the NF-E2 mutations are responsible for MPN development.

~~4.~~4 Discussion

This review has highlighted the relevant contribution of mouse models for analyzing the MPN genotype-phenotype relationship (Fig. 3). Mouse models have allowed the assessment of both the causative role of several genetic aberrations found in human MPN (a ‘human-to-mouse’ direction) and the identification of novel MPN-associated genes, serendipitously discovered in genetically modified animals (a ‘mouse-to-human’ direction).



~~Figure 3~~**Fig. 3** Lineage commitments and phenotypic outcomes induced by different mutations.

Schematic representation of the different lineage commitments and phenotypic outcomes induced by the mutations discussed in the review.

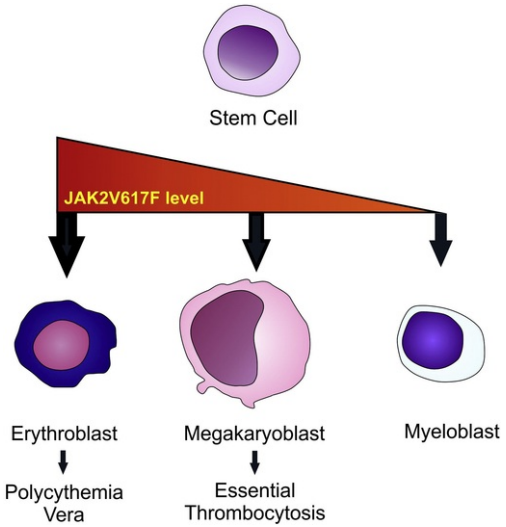
alt-text: Fig. 3

Overall, the significant results from these models can be highlighted as follows:

i) Mouse models have substantially confirmed the causative role of the majority of MPN-associated gene mutations. To our knowledge, no other cancer modeling has been associated with such a stringent genotype-phenotype relationship. Simplistically, it could be speculated that several of these MPN models are generated with aberrant expression of activated tyrosine kinases and therefore the proliferative signal represents an ‘obvious’ consequential phenotype. However, it should also be noted that expression of the tyrosine kinases (i.e. FLT3-ITD) associated with AML in humans did not cause MPN in mouse models but AML itself. Therefore, the genotype/phenotype correlations appear to perfectly match in the myeloid scenario, although the underlying reasons are unclear.

ii) Some human MPN-associated mutations sustain the development of different MPN diseases. For example, JAK2 V617F is associated with either PV, or ET, or PMF; CSF3R mutations with familiar disorders, aCML and CNL; and many others. While several speculations have been made to explain this heterogenic phenotype, the biological mechanisms are almost unknown. Notably, the same phenotypical heterogeneity is also observed in JAK2 V617F mouse models. This stringent biological correlation between human and mouse models is again remarkable.

Analyzing the different mouse models, and the fact that homozygous JAK2 V617F mutations are correlated with PV in human patients, while heterozygosity is more frequent in ET and PMF [29,30,133], suggest that the relative amount of mutated JAK2 is relevant in determining the pathological manifestation of the disease [41]. The impact of JAK2 mutation on the different hematopoietic lineages may depend on differences in signaling requirements during differentiation. It has been demonstrated that JAK2 signaling is essential for erythropoiesis, in particular for the transition from primitive erythrocytes to cKit^{+} Ter119^{+} positive cells [134,135]. Megakaryocytic differentiation also requires JAK2 activation via the thrombopoietin receptor MPL [136], while granulocytic differentiation mainly occurs through JAK1 [137,138]. However, high levels of the JAK2 mutant interfere with megakaryopoiesis, while granulopoiesis and erythropoiesis are less sensitive [41]. It is thus conceivable that subtle variations in JAK2 signaling requirements in the differentiation of erythroid and megakaryocytic lineages may account for the different phenotypic outcomes (Fig. 4) [33,133].



~~Figure 4~~Fig. 4 JAK2 mutation and differentiation of hematopoietic lineages.

Variations in JAK2 signaling requirements in the differentiation of erythroid, megakaryocytic and granulocytic lineages may account for the different phenotypic outcomes in human patients carrying JAK2 mutations.

alt-text: Fig. 4

iii) Several MPN models develop a myeloproliferative phenotype in a rapid timeframe, while many acute myeloid leukemia models require a longer latency. A long latency indicates that additional lesions are required to achieve an oncogenic phenotype, while the rapid onset observed in MPN models suggests that MPN-associated lesions are sufficient for developing the disease in mice.

A growing body of research indicates that murine models are extremely powerful tools for determining the oncogenic potential of human mutations and in order to faithfully recapitulate the most relevant aspects of human MPN. However, it should also be noted that some of these mutations are found in the normal population [139,140], raising the question of which additional events are required to determine the human disease.

Practice points

- Mouse models have confirmed the causative role of the majority of MPN-associated gene mutations.
- Phenotypical heterogeneity induced by certain mutations is remarkably conserved between human patients and mouse models.
- Mouse models allow the evaluation of the latency of the pathology and often recapitulate the evolution of the human pathology.

Research agenda

- Investigation of molecular mechanisms of MPN progression in mouse models.
- Use of mouse models to test innovative treatments.
- Searching, in human patients, for alterations in genes found to be associated with the MPN phenotype in genetically modified mice.

Disclosures

The authors report that there are no conflicts of interest.

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