Mouse models of myeloproliferative neoplasms for pre-clinical testing of novel therapeutic agents

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Myeloproliferative neoplasms (MPN), are clonal hematopoietic stem cell (HSC) disorders driven by gain-of-function mutations in *JAK2* (*JAK2*-V617F), *CALR* or *MPL* genes. MPN treatment options currently mainly consist of cytoreductive therapy with hydroxyurea and JAK2 inhibitors such as ruxolitinib and fedratinib. Pegylated interferon-alpha can induce complete molecular remission (CMR) in some MPN patients when applied at early stages of disease. The ultimate goal of modern MPN treatment is to develop novel therapies that specifically target mutant HSCs in MPN and consistently induce CMR. Basic research has identified a growing number of candidate drugs with promising effects *in vitro*. A first step on the way to developing these compounds into drugs approved for treatment of MPN patients often consists of examining the effects *in vivo* using pre-clinical mouse models of MPN. Here we review the current state of MPN mouse models and the experimental setup for their optimal use in drug testing. In addition to novel compounds, combinatorial therapeutic approaches are often considered for the treatment of MPN. Optimized and validated mouse models can provide an efficient way to rapidly assess and select the most promising combinations and thereby contribute to accelerating the development of novel therapies of MPN.

Key words: MPN, myeloproliferative neoplasms, *JAK2*, mouse model, pre-clinical models, novel therapies

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INTRODUCTION

Myeloproliferative neoplasms (MPNs) were proposed in 1951 by William Dameshek to represent "somewhat variable manifestations of proliferative activity of bone marrow cells" and to constitute a group of closely interrelated diseases[1]. Today, three disease entities are summarized under the term "BCR/ABL-negative MPN" (ref.[2,3]). Polycythemia vera (PV) is characterized by increased erythropoiesis and is frequently accompanied by thrombocytosis and leukocytosis, while essential thrombocythemia (ET) manifests as augmented megakaryopoiesis with thrombocytosis, but with normal red cell parameters. Primary myelofibrosis (PMF) requires histological proof of reticulin fibrosis and is frequently accompanied by anemia and extramedullary hematopoiesis. The currently used diagnostic criteria for MPN are defined by the World Health Organization (WHO) (ref.[2]). As proposed by Dameshek, MPN subentities are interrelated, as ET can sometimes convert to PV and both ET and PV can progress to myelofibrosis (post ET-MF or post-PV-MF). MPNs have been demonstrated to be clonal diseases of the hematopoietic stem cell (HSCs) driven by acquired gain-of-function mutations in *Janus kinase 2* (*JAK2*), *Calreticulin* (*CALR*) and *Thrombopoietin receptor* (*MPL*) genes[4–10]. These driver gene mutations in most patients are mutually exclusive. Only a few patients with more than one driver gene mutation have been studied in detail and in these cases each driver gene mutation represented an independent clone[11,12].

JAK2-V617F, the most frequent MPN driver mutation, is found in >95% of PV patients, and also in approximately one-half of patients with ET or PMF (ref.[4-7]). MPN progenitors and stem cells are hypersensitive to cytokines due to augmented intracellular signaling by the mutant *JAK2*-V617F (ref.[13]). Mutations in *CALR* and *MPL* also increase the JAK2/STAT signaling by activating cytokine receptors independently of their cognate ligands[14]. In some MPN patients, driver gene mutations are complemented by additional somatic mutations, most frequently affecting genes involved in epigenetic regulation inflammation, DNA damage response and Ras signaling, that can modify the disease course and contribute to disease initiation and/or progression[15].

A large number of mouse models have been developed to study MPN pathogenesis and to advance our current understanding of MPN disease initiation and progression[16-20]. These mouse models have been also used to examine the effects of therapeutic agents on MPN disease manifestations. Identification and validation of novel therapeutic agents in pre-clinical models of MPN is an extremely important and valuable step in the development of novel drugs for MPN patients. In this review we discuss the use of different types of mouse models and experimental setups to study candidate drugs for MPN treatment. We also provide examples of modern therapies that have been first tested in MPN mouse models.

Table 1. MPN mouse models with *JAK2*-V617F.

JAK2-origin	Type of model	Promoter	Phenotype	Background	Ref.
Mouse	retroviral	*viral LTR*	PV	C57BL/6	6
Mouse	retroviral	*viral LTR*	PV	BALB/c and C57BL/6	21
Mouse	retroviral	*viral LTR*	PV	BALB/c and C57BL/6	22
Mouse	retroviral	*viral LTR*	PV	C57BL/6	23
Mouse	retroviral	*viral LTR*	PV	BALB/c	24
Human	oocyte injection	*Vav*	PV	C57BL/6/DBA/2	25
Mouse	oocyte injection	*H-2Kb*	ET/PV	C57BL/6/DBA/2	26
Human	BAC oocyte injection	*hJAK2*	ET/PV	C57BL/6	27
Human	oocyte injection	*TetOCMV*	♀ET/ ♂PV	C57BL/6	29
Mouse	knock-in	*mJak2*	PV	129Sv/C57BL/6	30
Human	knock-in	*mJak2*	ET	129Sv/C57BL/6	33
Mouse	knock-in	*mJak2*	PV	129Sv/C57BL/6	31
Mouse	knock-in	*mJak2*	PV	129Sv/C57BL/6	32

1. Mouse models of MPN

Retroviral transduction models were first used to characterize the *JAK2*-V617F signaling and to confirm its effects on MPN phenotype *in vivo* (summarized in Table 1) (ref.[6,21-24]). Bone marrow cells were transduced with retroviral vectors carrying cDNA encoding *JAK2*-V617F and transplanted into syngeneic recipient mice, resulting in PV phenotype that in most cases rapidly progressed to myelofibrosis. Since retroviral transduction required HSCs to divide in culture, some of the retroviral models exhausted prematurely after a few months and were unable to engraft in secondary transplantations[24].

Transgenic models of MPN (summarized in Table 1) provide a more robust and reproducible system for preclinical drug testing *in vivo*. The first transgenic models used a cDNA encoding mouse or human *JAK2*-V617F that was placed under the control of a ubiquitous or tissue specific promoter and injected into mouse oocyte[25,26]. A more advanced transgenic model used a bacterial artificial chromosome (BAC) containing a large part of the human *JAK2* gene (exons 1-12). The V617F mutation was introduced as a cDNA covering *JAK2* exons 13-25 that were placed in reverse orientation and flanked by antiparallel loxP sites to make expression of *JAK2*-V617F conditional[27]. When activated by Cre-recombinase, these BAC transgenic mice express the human *JAK2*-V617F under the control of the human *JAK2* promoter and manifested with ET or PV phenotypes, with late transformation to post-MPN myelofibrosis recapitulating the full MPN pathophysiology observed in patients. When constitutive *VavCre* was used activate the *JAK2*-V617F transgene, ET phenotype was observed and was associated with lower *JAK2*-V617F expression levels in bone marrow, whereas PV phenotype resulted when conditional *MxCre* or *SclCre* was used activate the transgene, leading to higher *JAK2*-V617F expression[27,28]. Recently, transgenic mice expressing *Jak2*-V617F under the tetracyclin dependent SCL-tTA-2S tet-off system were generated[29]. Using this system, the authors showed that turning on *Jak2*-V617F expression resulted in MPN phenotype and turning off *Jak2*-V617F reversed the phenotypic features, indicating that mutant selective JAK2 inhibitors should profoundly disable the *JAK2*-V617F MPN clone.

Later several transgenic models were generated that introduced the V617F mutation into the endogenous mouse *Jak2* locus as a conditional knock-in[30-32]. These mice express the mouse *Jak2*-V617F and display a strong PV phenotype with moderate thrombocytosis and leukocytosis. In contrast, a knock-in model where exon 2 of mouse *Jak2* was replaced with a cDNA for the human *JAK2*-V617F presented with a very mild ET-like phenotype, but when the knock-in allele was intercrossed to homozygosity, the mice displayed a strong PV phenotype[33,34].

Recently, also mouse models for MPN driven by *CALR* mutations have been generated[35-39]. Most of the models used the *CALRdel52* mutation, but recently also mice expressing the *CALRins5* mutation have been described[40]. Both *CALRdel52* and *CALRins5* mice developed ET phenotype with thrombocytosis, leukocytosis and variable degree of fibrosis similar to MPN patients with *CALR* mutations. So far only a retroviral mouse model has been established for the *MPL*-W515L mutation found in patients with ET or PMF (ref.[41-43]). These mice develop a very rapid MPN-like disease with severe myelofibrosis, and early lethality. The phenotype is likely due to the increased and in part ectopic expression of *MPL*-W515L driven by the retroviral LTR.

Overall, these mouse mutants proved that expression of the *JAK2*, *CALR* or *MPL* mutants can alone cause MPN phenotype in mice and established them as driver gene mutations. Since about half of MPN patients carry additional somatic mutations that can modify the course of the disease[44], the MPN mouse models have then been crossed with mice carrying mutations in such modifier genes. The double mutant mice drive expansion of LT-HSC compartment by increasing their self-renewal, stemness and repopulation capacity, leading to clonal dominance. In most cases these models faithfully recapitulated the acceleration and modifications of the phenotypes that were also observed in patients carrying the same mutations. This was observed in particular with double mutant mouse models with mutations in epigenetic regulator genes, such as *Ezh2* (ref.[45-47]), *Tet2* (ref.[48,49]), *Dnmt3a* (ref.[50]), and *Asxl1* (ref.[51]), which are also among the most frequently found additional mutations in patients with MPN. As an alternative to intercrossing genetically

engineered mouse models, recently techniques using clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) were applied to target specific genomic loci for loss of function mutations *in vivo*. CRISPR/Cas9 techniques are especially useful in modeling complex genotype combinations, exemplified by a study, in which mutations in up to five genes (*Dnmt3a, Runx1, Nf1, Tet2, and Ezh2*) were introduced in a single HSC (ref.[52]). CRISPR/Cas9 *in vivo* system was also used to generate MPN mouse models[39,50,53] that may replace complicated breeding's for studies examining the contribution of multiple loci.

Finally, xenotransplantation models using patient-derived xenografts (PDX) into immuno-compromised recipient mice represent an attractive complement to genetic mouse models. PDX models allow studying human primary cancer and leukemia cells *in vivo* and they have been used for pre-clinical drug testing e.g. in leukemia[54] and solid cancers[55]. They provide the advantage that primary human cells can be studied. However, PDX models for MPN so far showed limited multilineage engraftment and self-renewal capacity, with large patient to patient variability making PDX difficult to use for drug testing compared to standard MPN mouse models[56,57]. Injection of patient-derived CD34 $^{+}$ HSCs into mice that were implanted with ossicles derived from human mesenchymal stem cells showed only transient improvement in engraftment[58]. More recently, humanized Rag2$^{-/-}$Il2rγ$^{-/-}$ immunodeficient mice that are engineered to express a number of non-cross-reactive human cytokines and growth factors became available[59]. One of these strains, the "MISTRG" mice, express human macrophage colony-stimulating factor (M-CSF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), signal regulatory protein a (SIRPα). The interaction between human CD47 on the PDX graft and human SIRPα on mouse macrophages provides a "don't eat me" signal that favors the expansion of human hematopoiesis in mice[60]. MISTRG mice were recently shown to improve engraftment of MPN PDX samples[61].

2. MPN mouse models for pre-clinical testing of new therapeutic agents

Original studies to test the compounds imatinib, dasatinib, AG490, and TG101348 used retroviral models expressing *JAK2*-V617F (ref.[22,62]). These studies provided first *in vivo* proof-of-principle that JAK2 inhibitors have therapeutic effects on MPN (Fig. 1). However, the retroviral models are not optimal for drug studies. The main disadvantages include the unphysiologically high expression levels of the mutant *JAK2*-V617F driven by the retroviral LTR promoter (>10-fold higher that endogenous mouse *Jak2*), variable severity of the phenotype dependent on the retroviral titer and absence of thrombocytosis. However, the *MPL*-W515L retroviral model is still frequently used for drug studies because these mice rapidly develop severe myelofibrosis[63,64].

Early studies in primary transgenic mice monitored normalization of blood counts and splenomegaly, but did not follow changes in the mutant allele burden[26,32]. To expand the size of the cohort that allowed testing of multiple drugs, bone marrow transplantations into irradiated recipients mice were introduced[32]. At the same time competitive transplantations of mixtures of *JAK2*-V617F and wildtype bone marrow cells into recipient mice were performed to mimic the situation in MPN patients, where *JAK2*-mutant and wildtype hematopoietic cells co-exist. Furthermore, to follow the *JAK2*-mutant cells, a polymorphism in the *Ptprc* gene that encodes CD45, also called leukocyte antigen 5 (Ly5) was used[65]. C57BL/6 mice, including most *JAK2*-V617F transgenic models express the CD45.2 variant. Congenic C57BL/6 mice that express CD45.1 were generated by backcrossing and used as donors of the wildtype bone marrow competitor cells and also as recipients (Fig. 2). The first such competitive transplantations were used to determine the effects of

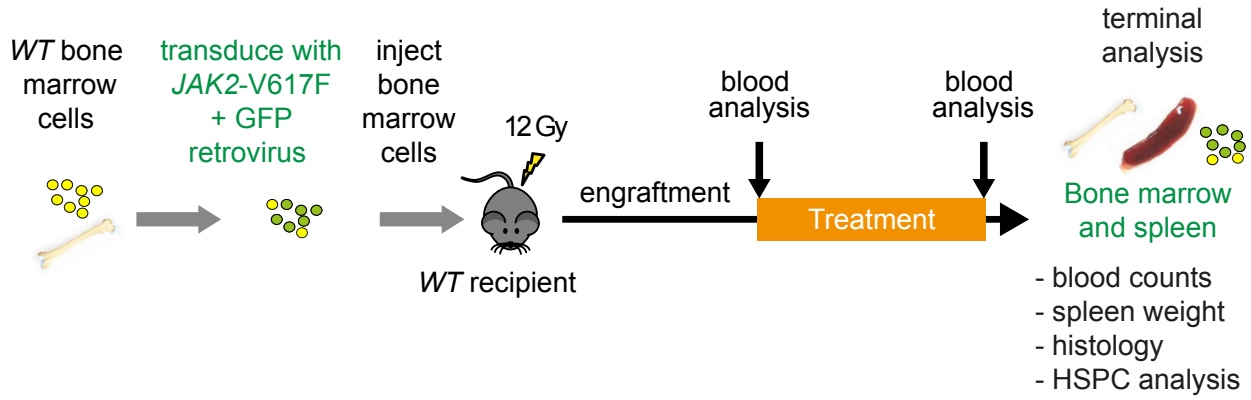

Fig. 1. Experimental setup for candidate drug testing in retroviral MPN mouse models. Wildtype (*WT*) bone marrow cells are transduced with murine retrovirus harboring the mutant *JAK2*-V617F allele. Transduced bone marrow cells are selected based on expression of GFP reporter and transplanted via tail vein injection into the lethally irradiated syngeneic recipient mice. After engraftment, the recipient mice are allowed to develop MPN phenotype and are randomized into treatment or vehicle control groups. Complete blood counts and GFP chimerism are determined in peripheral blood at defined timepoints. At the end of the treatment the mice are sacrificed and terminal analysis of cells and organs is performed.

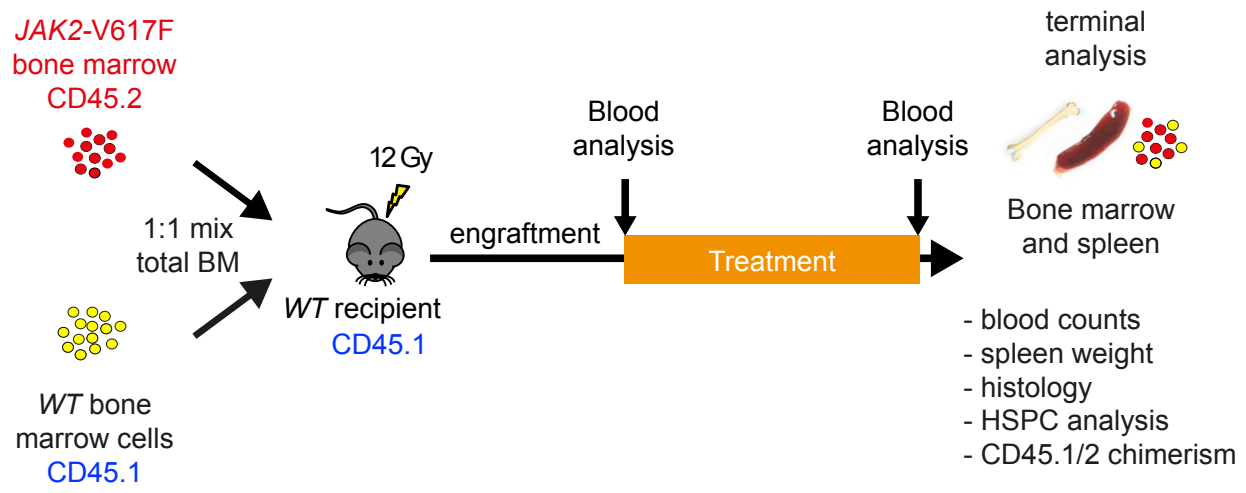

Fig. 2. Experimental setup for candidate drug testing in pre-clinical MPN mouse models with CD45.1/2 reporter gene. Bone marrow (BM) cells expressing *JAK2*-V617F$^+$ and CD45.2 (red) are mixed with wildtype (*WT)* competitor cells expressing CD45.1 (yellow) and injected into lethally irradiated CD45.1 recipient mice (blue). After engraftment, the recipient mice are allowed to develop MPN phenotype and are randomized into treatment or vehicle control groups. Complete blood counts and CD45.1/2 chimerism are determined in peripheral blood at defined timepoints. At the end of the treatment the mice are sacrificed, and terminal analysis of cells and organs is performed.

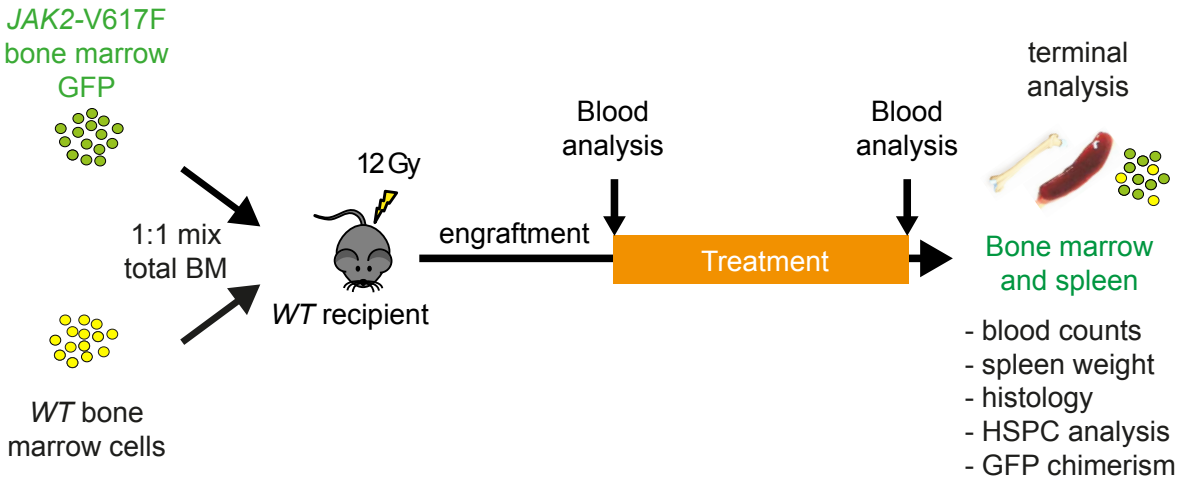

Fig. 3. Experimental setup for candidate drug testing in pre-clinical MPN mouse models with GFP reporter gene. Bone marrow (BM) cells co-expressing *JAK2*-V617F$^+$ and GFP in all hematopoietic cells (green) are mixed with wildtype (*WT)* competitor cells (yellow) and injected into lethally irradiated syngeneic recipient mice. After engraftment, the recipient mice are allowed to develop MPN phenotype and are randomized into treatment or vehicle control groups. Complete blood counts and GFP chimerism are determined in peripheral blood at defined timepoints. At the end of the treatment the mice are sacrificed, and terminal analysis of cells and organs is performed.

vorinostat, a small-molecule inhibitor of histone deacetylation, on *Jak2*-V617F allele burden[66]. For this purpose, bone marrow from inducible *Jak2*-V617F knock-in mice (CD45.2) were mixed at a ratio of 3:1 with the BM from wildtype (CD45.1) mice and injected into lethally irradiated CD45.1$^+$ recipient animals. Similar approach was used in studies of combined JAK2 and Bcl-2/Bcl-xL inhibition by TG101209 and ABT-737 inhibitors[67], interferon-α treatment of *Jak2*-V617F knock-in mice[68-70] and a novel type II JAK2 inhibitor[63]. A disadvantage of the CD45.1/2 reporter lies in the absence of CD45 antigen on a surface of erythrocytes and platelets, two major lineages that define MPN disease phenotype. Therefore, CD45.1/2 does not allow following the *JAK2*-mutant chimerism in peripheral blood Ter119$^+$ red cells and CD61$^+$ platelets. In addition, recent studies show that despite backcrossing into C57BL/6, a small amount of the original SJL mouse background, from which CD45.1 was derived, is retained in C57BL/6 CD45.1 mice from some vendor sources[71]. This includes regions encompassing genes of immunological importance, explaining previously observed problems of hematopoietic stem cell reconstitution being less efficient from C57BL/6 CD45.1, compared to C57BL/6 CD45.2 mice[72-74]. A new congenic CD45.1^{STEM} knock-in

line that carries a single point mutation in *Ptprc* was generated directly in C57BL/6 background[75]. These CD45.1^{STEM} knock-in mice should now overcome this problem.

A more complete labeling of all *JAK2*-mutant cells was achieved using mice that express the green fluorescent protein (GFP) reporter driven by a ubiquitous *UBC*-promoter in all hematopoietic cells[76]. Crossing *UBC*-GFP mice with *JAK2*-V617F mice resulted in double transgenic mice that co-express *JAK2*-V617F and GFP in all hematopoietic cells, including peripheral blood leukocytes, erythrocytes and platelets and represent an improved system for drug studies (Fig. 3). Competitive transplantations with bone marrow cells from such double transgenic mice were first used to compare the efficacy of hydroxyurea and ruxolitinib[28]. The GFP reporter system combined with transgenic models of *JAK2*-V617F is reliable and can be applied also for testing combinations of drug treatment. Recently, this approach was used to show synergism between interferon-α and arsenic[77].

Since $1x10^6$ bone marrow cells from *JAK2*-mutant mice contain about 60-100 HSCs, competitive transplantations at 1:1 ratio with wildtype competitor cells results in MPN that is essentially polyclonal. Drugs that decrease *JAK2*-mutant allele burden may have to overcome potential escape mechanisms though selection of *JAK2*-mutant HSCs that are resistant to the treatment. Therefore, competitive transplantations at higher dilutions of the *JAK2*-mutant cells, e.g. 1:20, could be a better system to test the ability of drugs to selectively target the mutant JAK2 and decrease the mutant allele burden. In principle, recipient mice reconstituted from a single *JAK2*-V617F mutant HSC can be generated by transplantation of single HSCs or by limiting dilution of transplanted bone marrow cells[20]. However, such an approach would require transplanting a large number of recipients, since only about 30% of recipients at limiting dilution engraft and display MPN phenotype and would therefore not be practical for routine use.

CONCLUSIONS

Transgenic MPN mouse models have been developed for *JAK2*-V617F, *JAK2* exon 12 mutation and *CALR* mutations. The optimal experimental setup for candidate drug testing in pre-clinical MPN mouse models should include reporters expressed in all blood cells, e.g. *UBC*-GFP, that allows tracking the MPN cells in all organs including peripheral blood. Competitive bone marrow transplantations allow expanding the cohort for testing multiple compounds and combinations of drugs. Presence of wildtype HSCs, derived from the wildtype competitor bone marrow, can maintain hematopoiesis when MPN HSCs are effectively targeted by new therapeutic approaches. Transgenic mouse models with reversible on-off *JAK2*-V617F expression[29], could serve as a the ideal positive controls for drugs that are selective for the mutant JAK2 proteins.

Mice with more complex mutation genotypes, e.g. combinations of *JAK2*-V617F with mutations in *Tet2*, *Ezh2*, *Dnmt3a* or *Asxl1* that are found in patients with MPN can also be used as bone marrow donors, which will allow testing drugs in these more complex MPN models. The CRISPR/Cas9 technology could further facilitate and speed up the generation of complex genotypes for such studies. Retroviral models, such as *MPL*-W515L can also remain useful in specific setting, e.g. to study rapid progression to myelofibrosis without the need to wait several months for myelofibrosis to develop, as is the case with the transgenic models for *JAK2*-V617F.

The PDX models using improved recipient mice such as MISTERG (ref.[61]) could become the next step in preclinical MPN mouse models for drug testing, if some of the main hurdles can be overcome. These include the difficulties obtaining a large cohort of recipient mice reconstituted from the same MPN patient donor, variability of phenotypes between mice engrafted with cells from the same MPN donor and inter-individual differences in MPN phenotypes between different MPN donors. Overall, the MPN field already now has excellent pre-clinical *in vivo* models for drug testing, which can contribute to accelerating the development of novel therapies of MPN.

Search strategy and selection criteria

Our research strategy was aimed at evaluating studies on the mouse models of myeloproliferative neoplasms used for pre-clinical testing of novel therapeutic agents. Data for this article were identified by searches of PubMed and Google Scholar databases and references from relevant articles using the terms “MPN”, “myeloproliferative neoplasms”, “JAK2”, “mouse models”, “transplantation”, “novel therapies”. All searches were up to date as of December 2020. Only articles published in English were included, abstracts and reports from meetings were not included. We gave preference to publications from the past 10 years.

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