



## Insights into myelodysplastic syndromes from current preclinical models

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### Abstract

In recent years, there has been significant progress made in our understanding of the molecular genetics of myelodysplastic syndromes (MDS). Using massively parallel sequencing techniques, recurring mutations are identified in up to 80% of MDS cases, including many with a normal karyotype. The differential role of some of these mutations in the initiation and progression of MDS is starting to be elucidated. Engineering candidate genes in mice to model MDS has contributed to recent insights into this complex disease. In this review, we examine currently available mouse models, with detailed discussion of selected models. Finally, we highlight some advances made in our understanding of MDS biology, and conclude with discussions of questions that remain unanswered.

**Key words:** Myelodysplastic syndromes; Mouse models; Genetic mutations

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**Core tip:** Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders. In recent years, we have witnessed a rapidly expanding

catalog of MDS candidate genes. Mirroring this, there has been an increased number of candidate genes employed to model MDS. Here, we aim to review currently available mouse models of MDS, highlighting models that are robust and well-characterized phenotypically with a particular focus on models that demonstrate close resemblance to the human disease.

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## INTRODUCTION

Myelodysplastic syndromes (MDS) are neoplastic clonal disorders of ineffective hematopoiesis with an inherent risk of transformation to acute myeloid leukemia (AML)<sup>[1]</sup>. MDS typically manifests as increased intramedullary apoptosis of maturing clonal cells in a hyperproliferative and pro-inflammatory bone marrow<sup>[2-4]</sup>. Clinically, this is seen as peripheral blood cytopenia(s) with accompanying dysplasia in a hyper- or normocellular bone marrow.

It should be noted that apoptosis in the bone marrow is more prominent in low risk MDS, driven by an excess of pro-inflammatory cytokines and altered T cell response<sup>[5-7]</sup>. In advanced MDS, increased expression of BCL2 leads to resistance to apoptosis. Additionally, the acquisition of further molecular defects results in increased proliferation and blocked differentiation in myeloid progenitors, culminating in evolution to AML<sup>[8-11]</sup>.

## CLONAL HEMATOPOIESIS FROM A MDS STEM CELL

MDS is thought to arise from mutations in the hematopoietic stem cell/progenitor (HSPC) CD34<sup>+</sup> cell<sup>[12]</sup>. The founder mutation occurs in a MDS stem cell (or MDS initiating cell) that gives rise to clonal hematopoiesis. Support for this model of clonal architecture of MDS has been illustrated in several studies<sup>[13,14]</sup>. Delhommeau *et al.*<sup>[13]</sup> isolated CD34<sup>+</sup> cells from MDS patients and identified *TET2* mutations only in a small fraction of immature CD34<sup>+</sup>CD38<sup>-</sup> population with higher proportions detected in the CD34<sup>+</sup>CD38<sup>+</sup> mature progenitors. The findings are in keeping with a model in which a *TET2* mutation arose in an immature progenitor cell and was passed on to its more mature progeny. In another study using whole genome sequencing, Walter *et al.*<sup>[14]</sup> reported that about 85%-90% of unfractionated bone marrow cells were clonally derived from the MDS stage and persisted through to leukaemic transformation.

Of interest, whilst the MDS stem cell can establish clonal hematopoiesis, overt hematological manifestations of disease may be absent. It is likely that additional

cooperating genetic and epigenetic events are required to drive disease progression and bring about a clinically apparent phenotype<sup>[15]</sup>. Indeed, age-related clonal hematopoiesis was first described in a group of healthy women over the age of 65. In approximately 23% of these women, a skewed pattern of X-chromosome inactivation was observed in cells taken from the peripheral blood, with some associated with *TET2* mutations<sup>[15,16]</sup>. More recently, whole exome sequencing identified the presence of clonal somatic mutations in genes that are recurrently mutated in hematological malignancy in the peripheral blood of ostensibly healthy elderly individuals<sup>[17-19]</sup>. Jaiswal *et al.*<sup>[17]</sup> reported that the presence of somatic mutations was rare in individuals younger than the age of 40. However, the incidence of clonal mutations increases considerably with successive decades of life thereafter, with the frequency of mutations in individuals 60 years and older exceeding the incidence of hematological malignancy diagnosed in the general population. The most commonly mutated gene was *DNMT3A*, followed by *TET2*, *ASXL1*, *TP53*, *JAK2*, and *SF3B1*. These mutations persisted over time, and were associated with an increased risk, approximately 0.5%-1% per annum, of developing a hematological malignancy. In a second, independent cohort of subjects unselected with respect to hematological phenotypes, Genovese *et al.*<sup>[18]</sup> found that more than 10% subjects aged 65 years or more had evidence of clonal hematopoiesis. In this population the most frequently mutated candidate driver genes were *DNMT3A*, *ASXL1*, *TET2*, *JAK2* and *PPMID* and the presence of a mutant clone was a risk factor for subsequent hematological malignancy or death. Finally, McKerrell *et al.*<sup>[19]</sup> reported that the prevalence of clonal hematopoiesis doubled with each decade of life after the age of 50, rising from 1.5% in those aged 50-59 to nearly 20% in those 90 years and older. The most common mutations were *DNMT3A*, *JAK2*, *SRSF2* and *SF3B1*. Notably, spliceosome mutations at *SRSF2* P95, *SF3B1* K666 and K700 were exclusively observed in individuals older than 70 years. The striking degree of overlap between results of these studies with regards to the driver genes identified and the significantly heightened risk of hematological disease in individuals with clonal hematopoiesis serves to underline the generalizability of these findings.

## CURRENT MOLECULAR INSIGHTS

MDS is a very heterogeneous disease, underscored by significant genomic instability and a complex genetic landscape. The catalog of MDS candidate genes is rapidly expanding with the application of modern techniques in detecting molecular lesions. However, the pathogenesis of MDS remains elusive. The hierarchical significance and functional interplay of the different mutations in the development and progression of the disease are areas of active investigation. Moreover, there is emerging evidence that MDS is not solely an intrinsic hematopoietic disease, with the niche, *i.e.*, bone marrow

microenvironment, also playing a role<sup>[5,6,20,21]</sup>.

Recently, interrogation of MDS samples by massive parallel sequencing technology has allowed the identification of genetic mutations at single nucleotide resolution. Using this technique, mutations are apparent in up to 80% of cases, including many with a normal karyotype<sup>[22]</sup>. Recognition that recurrently mutated genes can be grouped according to the function of the proteins that they encode (epigenetic regulators, transcription factors, spliceosome components, etc.) has greatly improved our understanding of MDS pathogenesis<sup>[14,22,23]</sup>.

## CYTOGENETIC ABNORMALITIES

Cytogenetic analysis of MDS has been instrumental in the diagnosis and prognostication of MDS. Using conventional metaphase cytogenetics, abnormalities are found in up to 50% of patients with MDS<sup>[24]</sup>, with a higher frequency of abnormal and complex cytogenetics (defined as the identification of three or more abnormalities in the karyotype) seen in therapy-related MDS.

As is the case for the recurrent mutations, many cytogenetic lesions seen in MDS are not exclusive to this disorder and also occur in other myeloid malignancies. However, copy number losses or gains are more frequent in MDS than balanced translocations, which tend to predominate in AML. The most common single cytogenetic aberrations include trisomy 8, del(5q), del(20q), and monosomy 7 or del(7q)<sup>[24-27]</sup>. It is thought that, in most cases, abnormalities detected by conventional cytogenetics are secondary genetic events resulting from genomic instability caused by earlier sub-microscopic initiating or founder mutations<sup>[28]</sup>. Furthermore, cytogenetics only detects large-scale genomic changes, *i.e.*, loss or gain of a whole chromosome or most of a chromosome arm and as such, it is difficult to pinpoint candidate driver genes that are involved in a region of copy number change.

Although the abnormality rate for conventional cytogenetics is limited by the low-resolution inherent in using a microscope-based technology for the detection of genomic lesions, the information it provides remains clinically relevant. Conventional cytogenetics has a key role in identifying clonality. This can be central to MDS diagnosis which otherwise relies on subjective morphologic criteria. Importantly, possibly by virtue of the fact that cytogenetic abnormalities are seldom initiating events, cytogenetics provides powerful prognostic information in addition to diagnostic information, as demonstrated by the revised International Prognostic Scoring System (IPSS-R)<sup>[29]</sup>.

## MUTATIONS IN THE SPLICING MACHINERY

Pre-mRNA splicing is catalyzed by the spliceosome, a macromolecule composed of five small nuclear RNAs

associated with numerous proteins to form small ribonucleoproteins. It is a highly dynamic structure, conferring accuracy in constitutively spliced exons and at the same time, allowing the flexibility for alternative splicing to generate genetic diversity and complexity.

Recently, whole genome and exome sequencing of human MDS samples has identified frequent somatic mutations in genes that encode components of the RNA splicing machinery, including *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*<sup>[22,30-32]</sup>. Indeed, RNA splicing is one the most common pathways targeted by mutations in MDS, with up to 50% of patients carrying a mutation in a gene encoding a spliceosome component<sup>[22]</sup>.

Furthermore, mutations in the RNA splicing genes are mutually exclusive and are most often founding events. In fact, the mutant allele burden is typically between 40%-50%, indicating a dominant bone marrow clone that is heterozygous for the mutation<sup>[33,34]</sup>. Given the essential requirement for RNA splicing in generating protein diversity, biallelic mutations would be predicted to be lethal and evidence from mouse models largely supports this. Mutation hotspots in the three most frequently mutated genes, *SF3B1*, *SRSF2*, and *U2AF1*, have been identified. Almost all described mutations are missense, with no evidence of nonsense or frameshift changes, suggesting that they result in altered function rather than loss of function<sup>[30,32,35]</sup>.

The mutations in individual spliceosome components are associated with different phenotypes and distinct clinical outcomes<sup>[22,34]</sup>. *SF3B1* mutations are found almost exclusively in patients with refractory anemia with ringed sideroblasts without or with thrombocytosis [refractory anemia with ringed sideroblasts (RARS) and RARS-T respectively], therefore proposing a causal link between mutation and ringed sideroblasts formation. Most patients with *SF3B1* mutations have good risk disease with a protracted clinical course and a low propensity to AML transformation<sup>[36]</sup>. On the other hand, *SRSF2* mutations are found mainly in patients with multilineage dysplasia and/or excess blasts and predict a high risk of leukemic evolution and poor survival<sup>[37-39]</sup>. *U2AF1* mutations have been reported in various MDS subtypes and found to predict high risk of progression to AML and hence, shorter survival<sup>[30,38]</sup>.

The observation that spliceosome mutations are mainly founding mutations associated with different clinical outcomes led Papaemmanuil *et al.*<sup>[22]</sup> to hypothesise that they give rise to initiating clones with different genetic predestination. Through specific cooperating genetic lesions, the initial driver mutations likely shape the trajectory of clonal evolution leading to more or less aggressive MDS phenotypes.

Spliceosome mutations are rarely found in childhood myeloid neoplasms. Moreover, they are rarely detected in the blood of young healthy individuals but increase in prevalence in an age-dependent manner in people aged 70 years and over, and confer an increased risk of myeloid malignancy<sup>[19]</sup>. These findings suggest RNA

splicing mutations are typically acquired with age and support the hypothesis that they occur early in disease pathogenesis<sup>[17-19]</sup>.

## MUTATIONS IN GENES INVOLVED IN EPIGENETIC REGULATION

Alterations in epigenetic processes, including DNA methylation, histone modifications and miRNA are now well-described and are pivotal in the pathogenesis of MDS.

Promoter-associated CpG island hypermethylation is seen in about 3%-5% of MDS, may occur early in the course of the disease and is associated with a more rapid progression to AML<sup>[40]</sup>. Recurrently mutated genes in MDS known to be involved in the regulation of DNA methylation include *TET2*, *DNMT3A* and *IDH1/2*<sup>[41-45]</sup>.

Post-translational modification of histones plays an important part in epigenetic regulation. These proteins can be acetylated, methylated, and ubiquitinated by a group of histone-modifying enzymes. Loss-of-function mutations occur in histone modifiers, such as *ASXL1* and *EZH2*, and they are associated with a poor prognosis and reduced survival<sup>[46-49]</sup>.

## MUTATIONS IN OTHER PATHWAYS

Mutations in signalling molecules, transcription factors, and *TP53* are often subclonal, driving disease progression and are associated with adverse clinical outcomes<sup>[22,50-54]</sup>. They tend to occur in advanced disease, with the exception of *TP53*, which may occur at an early stage in del(5q) MDS and therapy-related disease<sup>[55]</sup>. In the context of del(5q) MDS, mutated *TP53* is associated with lower response rates to lenalidomide treatment and an increased risk of leukaemic transformation<sup>[56]</sup>.

## MODELING MDS IN MICE

Animal models are valuable pre-clinical tools to advance our understanding of human diseases, as well as facilitating the development and evaluation of novel therapeutic agents. The laboratory mouse (*Mus musculus*) is the model of choice to phenocopy and to investigate the biology of human cancer for a variety of reasons including its small size, well-characterized physiology and rapid breeding cycle. Moreover, the frequently used C57BL/6J mouse strain has a fully sequenced genome, with 75% orthology to human<sup>[57]</sup>.

In the case of MDS, mouse models are particularly useful to study the biology of this disease. By expressing MDS candidate genes in mice, the function of the various genes and their role in the pathogenesis and progression of the disease can be evaluated in detail. These models can also serve as a platform to identify and test novel therapeutic candidates. Additionally, they can also be used to evaluate the mechanism of action of therapies currently used in clinic, for example lenalidomide which

is used in 5q- syndrome, and hypomethylating agents such as azacitidine and decitabine.

## DIAGNOSING MDS IN HUMANS AND MICE

The diagnosis of MDS in humans is predominantly based on morphology. Based on the 2008 World Health Organisation (WHO) MDS classification, the minimum requirement for diagnosis include the presence of > 10% dysplasia morphologically, significant cytopenia in at least one lineage, and < 20% blasts<sup>[1]</sup>. The thresholds for significant cytopenias as recommended by the IPSS are hemoglobin < 10 g/dL, absolute neutrophil count <  $1.8 \times 10^9/L$ , and platelet count <  $100 \times 10^9/L$ . The only exception to meeting the minimum diagnostic prerequisites is evidence of clonality, *i.e.*, an abnormality from a pre-defined list of characteristic cytogenetic abnormalities is present. This is sufficient for a diagnosis of MDS, provided cytopenia is present and that AML has been excluded in the basis of the blast count<sup>[1]</sup>.

Using the morphological criteria outlined above, MDS can be sub-classified depending on the number of lineages affected by cytopenias and dysplasia, and the enumeration of blasts in the bone marrow. Groups classified according to WHO criteria have prognostic significance, which can be refined further with cytogenetic information.

The presence of excess blasts, immediately places patients in a high-risk group. There are two groups, stratified according to blast count: Refractory anemia of excess blasts-1 (RAEB-1, 5%-9% marrow blasts) and RAEB-2 (10%-19% marrow blasts). The low-risk MDS comprises subtypes with only single-lineage cytopenia and dysplasia, and includes refractory anemia, and RARS. It should be noted that ringed sideroblasts can also be seen in other subtypes of MDS, and carry no independent prognostic significance. The intermediate-risk MDS, refractory anemia with multi-lineage dysplasia is associated with bi- or tri-lineage dysplasia.

Taking into consideration the diagnostic criteria and other salient characteristics described, the key features of human MDS that are desirable to recapitulate in mouse models include peripheral blood cytopenias, bone marrow dysplasia, ineffective hematopoiesis, a propensity to transform to secondary acute leukemia after a long latency, transplantable disease into secondary mice, and the ability to mimic therapeutic responses to treatments with established efficacy in human MDS.

It should be noted that mouse hematopoiesis differs from human hematopoiesis. In humans hematopoiesis is largely confined to the epiphyses from adulthood and compensatory hematopoiesis can occur in the bone marrow. In steady-state murine hematopoiesis, the bone marrow is 95% occupied leaving the spleen as the major site for compensatory blood cell production, and the spleen in mouse remains an important hematopoietic organ throughout life. As such, compensatory spleno-





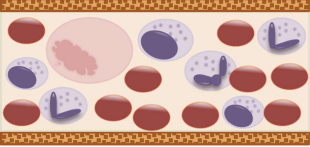
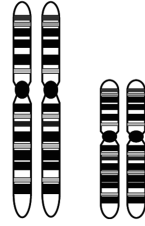
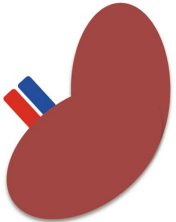
		Human	Mouse
Cytopenia		Yes	Yes
Dysplasia		Yes	Yes
Bone marrow cellularity		Usually increased	Usually increased
Cytogenetic abnormality		Yes	No recurrent abnormalities identified to date
Mutations	ACCGTGAAAGCCTA TGGCACU TTCGGAT	Yes	Yes
Splenomegaly		Usually absent	Possible

Figure 1 Key features of myelodysplastic syndromes in humans and mouse.

**Table 1** Bethesda diagnostic criteria for myeloid dysplasia in mice

(1) At least one of the following
A: Neutropenia
B: Thrombocytopenia (without leucocytosis or erythrocytosis)
C: Anemia (without leucocytosis or thrombocytosis)
(2) Maturation defect in myeloid cells manifest as at least one of
A: Dysgranulopoiesis, dyserythropoiesis, or dysplastic megakaryocytes with or without increased myeloid immature forms or blasts
B: At least 20% myeloid immature forms or blasts
(3) Disorder is not AML
Subclassification
Myelodysplastic syndrome if meeting criteria (2)A
Cytopenia with increased blasts if meeting (2)B

Summarised from Kogan *et al*<sup>[60]</sup>. AML: Acute myeloid leukemia.

megaly in a cytopenic mouse may be considered the equivalent of bone marrow hypercellularity in a human and should not necessarily be regarded as evidence of a myeloproliferative neoplasm<sup>[58,59]</sup>. Thus, compensated or uncompensated anemia in a mouse with an MDS phenotype may be accompanied by splenomegaly. In addition, AML in mice will often involve both the bone marrow and the spleen, unlike in humans where leukemia tends to be confined to the bone marrow. Figure 1 illustrates the key features of myelodysplasia

expected in both humans and mice, and also points out the differences between them.

## DIAGNOSIS OF MDS IN MICE - THE BETHESDA CLASSIFICATION

Recognizing the need for consensus in the classification of murine hematopoietic lesions within the scientific community, the hematopathology subcommittee of the Mouse Models of Human Cancer Consortium (MMHCC) proposed diagnostic criteria for the classification of nonlymphoid hematopoietic neoplasms in mice (Table 1). There is a myeloid dysplasia category and within this, disease can be subclassified as either myelodysplastic syndrome or cytopenia with increased blasts<sup>[60]</sup>. To qualify as a myeloid dysplasia, acute nonlymphoid leukemia (*i.e.*, AML) must first be excluded. A diagnosis of AML should be applied if there are greater than 20% blasts, disseminated tissue infiltration and biologically aggressive disease that is rapidly fatal<sup>[60]</sup>.

The defining criteria for myeloid dysplasia require the presence of cytopenia. Evidence of myeloproliferation in the form of erythrocytosis, leucocytosis and thrombocytosis must be absent. If there is morphologic evidence of dysplasia in at least one hematopoietic lineage, a myelodysplastic syndrome is said to be present

**Table 2** Candidate genes used to model myelodysplastic syndromes in mice and their correlation to human myelodysplastic syndromes

Gene(s) studied	Chromosomal location	Frequency in human MDS	Equivalent human MDS subtype according to WHO 2008 classification	Ref.
Transcription factors				
<i>NUP98-HOXD13</i>	t(2;11)(q31;p15)	Rare	RCMD	[69]
<i>RUNX1</i>	21q22.3	15%-40%	RAEB	[105]
<i>EVI1</i>	3q26.2	Rare	RCMD	[136]
<i>SALL4</i>	20q13.2	15%-40%	RCMD	[139]
<i>NPM1</i>	5q35.1	About 4%	RCMD	[138]
Signalling molecules				
<i>NRAS</i>	1p13.2	About 20%	RAEB	[142]
<i>BCL2</i>	18q21.33	Unknown		
<i>PTEN</i>	10q23.3	Unknown	RCMD	[140]
<i>SHIP</i>	2q37.1	Unknown		
Epigenetic regulators				
<i>TET2</i>	4q24	20%-30%	RCMD/CMML	[115]
<i>ASXL1</i>	20q11.21	15%-20%	RCMD	[122,123]
<i>EZH2</i>	7q36.1	2%-6%	RCMD/MPN	[141]
<i>MLL5</i>	7q22.1	Unknown	No definitive MDS	[145]
RNA spliceosome				
<i>SRSF2</i>	17q25.2	15%-30%	RCMD	[98]
<i>U2AF1</i>	21q22.3	11%	No definitive MDS	[100]
<i>SF3B1</i>	2q33.1	10%-20%	RARS, RARS-T	[101]
Telomere function				
<i>TERT</i>	5p15.33	Unknown	RCMD	[124]
5q-				
<i>RPS14</i>	5q33.1	About 10%	5q-like	[91]
<i>CD74-SMIM3 (NID67)</i>	5q32-q33.1			[92]
<i>SPARC</i>	5q33.1			[97]
<i>MIR145/146A</i>	5q32-34			[143]
<i>APC</i>	5q22.2			[99]
<i>CSNK1A1</i>	5q32			[94]

WHO: World Health Organization; RARS: Refractory anemia with ringed sideroblasts; RARS-T: Refractory anemia with ringed sideroblasts and thrombocytosis; RCMD: Refractory anemia with multilineage dysplasia; RAEB: Refractory anemia with excess blasts; MPN: Myeloproliferative neoplasm; MDS: Myelodysplastic syndromes; CMML: Chronic myelomonocytic leukemia.

(Table 1). Morphological features of dysplasia are subtler in mice than in humans and can be difficult to identify. The MMHCC subcommittee lists features considered speculative evidence of dyspoiesis. In the erythroid lineage, dyserythropoiesis includes megaloblastic maturation, increased mitotic figures, multinucleation, and nuclear irregularity. Ringed sideroblasts are also a feature of dysplasia, but are rare in mice. Dysgranulopoiesis may manifest as hypogranular neutrophils, and lobated neutrophils as opposed to ring-shaped nuclei. For megakaryocytes, micromegakaryocytes, large megakaryocytes with unlobated nuclei or bizarre hypersegmented nuclei, and megakaryocytes with separated nuclei are all regarded as signs of dysplasia. Where AML has been excluded and morphologic dysplasia is lacking but there is cytopenia with more than 20% blasts in the bone marrow or spleen, the diagnosis cytopenia with increased blasts can be made. Provision is also made within the MMHCC criteria for disease in mice that very closely approximates a defined human MDS subtype. In this situation the disease may be labelled "myelodysplastic syndrome with features of a named human MDS"<sup>[60]</sup>.

Little is known about the acquisition of cytogenetic abnormalities in mouse models of MDS. Thus, recurrent cytogenetic abnormalities cannot be used to aid the

diagnosis and classification of disease in mice, in the way that they can be used in humans.

## AVAILABLE MOUSE MODELS OF MDS

There are more than 20 published mouse models of MDS in the literature, and they are summarised in Tables 2 and 3. Several strategies have been employed to create these models. They include genetic engineering of mouse hematopoietic cells using knock-in or knock-out strategies, and xenotransplantation of human MDS cells, the latter of which has proven to be technically difficult.

Mirroring the rapidly expanding catalog of MDS candidate genes, there have been an increasing number of genetically modified mice being reported as MDS mouse models (Figure 2). These models mostly examine the effects of a single gene modification in the pathogenesis of MDS, and many of them displayed features reminiscent of MDS. Considering the genetic complexity inherent to MDS, it is not surprising that a single unifying model that faithfully mimics the MDS phenotype in its entirety is still lacking. More recently, there have been efforts to incorporate more than one mutation in modeling MDS, which has certainly provided insights into the functional interactions of different genes in the

**Table 3** Published mouse models of myelodysplastic syndromes

Gene(s) studied	Model/technique	Tractability to human MDS								Ref.
		Anemia	Multi-lineage cytopenias	Dysplasia	Bone marrow cellularity	HSPC skewing	Secondary leukemia	Latency to leukemia	Survival	
<i>NUP98-HOXD13</i>	Transgenic	Yes	Yes	Yes	Hyper-	Yes	Yes <sup>2</sup>	14 mo	-	[69]
<i>Cd74-Nid67</i>	Large scale chromosomal deletion, RPS14 haploinsufficient	Yes	No	Yes	Hypo-	Yes	No	-	<sup>1</sup>	[92]
<i>SPARC</i>	Knock-out	No	No	Yes	<sup>1</sup>	<sup>1</sup>	No	-	<sup>1</sup>	[97]
<i>MIR-145, MIR146a</i>	Retroviral transduction	No	No	Yes	<sup>1</sup>	<sup>1</sup>	Yes	4-14 mo	-	[143]
<i>APC</i>	Transgenic, haploinsufficient	Yes	No	Yes	<sup>1</sup>	Yes	No	-	3-8 mo	[99]
<i>Csnk1a1</i>	Transgenic, inducible	Yes	Yes	Yes	Yes	Yes	No	-	<sup>1</sup>	[94]
<i>Srsf2</i>	Transgenic, inducible	Yes	Yes	Yes	Normal	Yes	No	-	<sup>1</sup>	[98]
<i>U2af1</i>	Transgenic, inducible	No	No	No	Normal	Yes	No	-	-	[100]
<i>Sf3b1</i>	Transgenic, haploinsufficient	Yes	No	Yes	<sup>1</sup>	<sup>1</sup>	No	-	About 12 mo	[101]
<i>RUNX1-D171N</i>	Retroviral transduction	Yes	Yes	Yes	Normal to hyper-	<sup>1</sup>	Yes	4-13 mo	-	[105]
<i>RUNX1-S291fsX300</i>	Retroviral transduction	Yes	Yes	Yes	Normal to hyper-	<sup>1</sup>	Yes	4-13 mo	-	[105]
<i>RUNX1S291fs/Ezh2</i>	Retroviral transduction	Yes	Yes	Yes	Variable	<sup>1</sup>	No	-	262 d	[125]
<i>Tet2</i>	Transgenic, hypomorphic	Yes	Yes	Yes	Hyper-	Yes	No	-	11 mo	[115]
<i>Tet2/Ezh2</i>	Transgenic, inducible	Yes	Yes	Yes	Hyper-	Yes	No	-	10 mo	[141]
<i>ASXL1</i>	Transgenic, inducible	Yes	Yes	Yes	Hypo-	Yes	No	-	Median 50 wk	[122]
<i>Asx1</i>	Transgenic, constitutive	Yes	Yes	Yes	Normal to hyper-	Yes <sup>3</sup>	16 mo	8-42 d	-	[123]
<i>TERT</i>	Transgenic, inducible	Yes	Yes	Yes	Hyper	Yes	Yes	<sup>1</sup>	12 mo	[124]
<i>Evi1</i>	Retroviral transduction	Yes	Yes	Yes	Hyper-	<sup>1</sup>	No	-	10-12 mo	[136]
<i>SALL4</i>	Transgenic	Yes	Yes	Yes	Hyper-	Yes	Yes	6 wk to 12 mo	-	[139]
<i>Npm1</i>	Transgenic, haploinsufficient	No	No	Yes	Hyper	No	Yes	24 mo	-	[138]
<i>NRASD12-BCL2</i>	Transgenic, inducible/ constitutive	<sup>1</sup>	No	Yes	<sup>1</sup>	Yes	Yes	3-6 mo	-	[142]
<i>Pten/Ship</i>	Transgenic, Pten +/- Ship -/-	Yes	Yes	<sup>1</sup>	Hypo	Yes	No	-	5 wk	[140]
<i>Dido</i>	Knock-out	Yes	No	Yes	<sup>1</sup>	<sup>1</sup>	No	-	1	[137]
<i>Arid4a</i>	Knock-out	Yes	Yes	Yes	Hyper-	<sup>1</sup>	Yes	5 mo	-	[144]
<i>Mll5</i>	Knock-out	Yes	No	No	<sup>1</sup>	<sup>1</sup>	No	-	-	[145]

<sup>1</sup>Denotes not reported, denotes not applicable; <sup>2</sup>Some mice developed pre-T cell acute lymphoblastic leukemia; <sup>3</sup>Only 1 of 18 mice developed AML. HSPC: Hematopoietic stem and progenitor cells; MDS: Myelodysplastic syndromes; AML: Acute myeloid leukemia.

## biology of MDS.

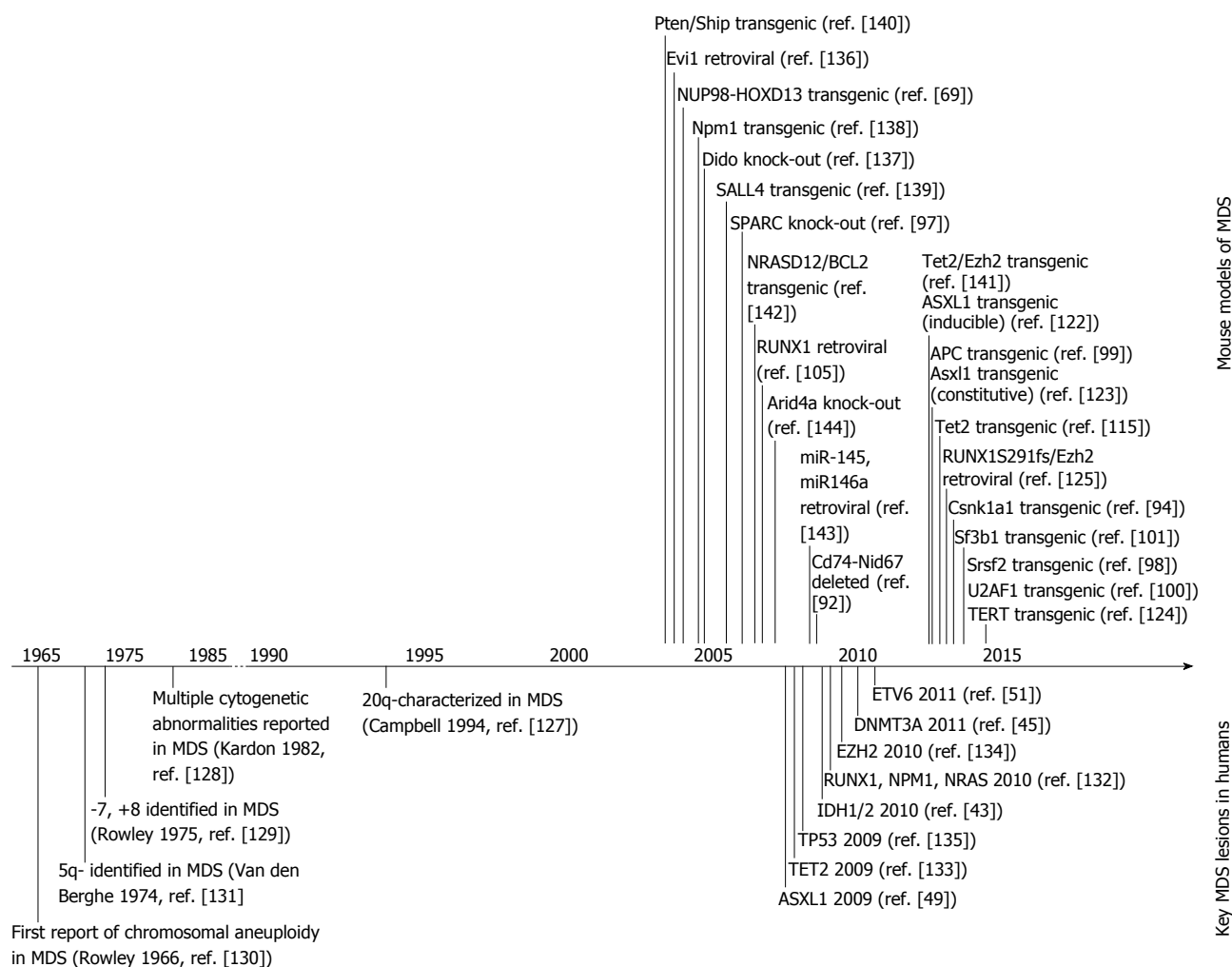
Amongst the published mouse models, anaemia was the most common cytopenia reported with a significant proportion showing multilineage cytopenia including pancytopenia in some models. Accompanying dysplastic changes were often noted in more than lineage. It should be noted that unlike the number of cytopenias which is indicative of MDS severity, the extent of dysplasia carries no prognostic implications. Several of these models also showed alterations in the hematopoietic stem cell (HSC) and a propensity to transform to secondary AML, further improving their tractability to the human disease.

## XENOGRAFT MOUSE MODELS OF MDS

The establishment of immunodeficient mice harbouring

malignant human xenografts is an attractive approach to model and study malignancy of the hematopoietic system. Establishing human malignant cells in the mouse host has been technically challenging, but has proven feasible in hematological disorders such as AML and acute lymphoblastic leukemia (ALL). In contrast, the propagation of MDS clones has been met with limited success. This has been attributed to a number of factors including host anti-tumor/human immune response, inadequate microenvironment for tumor growth or survival, and toxicity from *ex vivo* manipulation of malignant cells.

Techniques have improved considerably over the last ten years, and the generation of transgenic severe combined immunodeficient (SCID) mice expressing human granulocyte-macrophage colony-stimulating



**Figure 2** Time-line showing key genetic aberrations identified in myelodysplastic syndromes and published mouse models. MDS: Myelodysplastic syndromes.

factor and interleukin-3 (IL-3) has improved the engraftment of an immortalized cell line derived from a patient with MDS (F-36P). Engraftment was further improved with the pre-administration of IL-2 receptor antibodies, which suppressed natural killer (NK) cell function<sup>[61]</sup>.

This led to the development of non-obese diabetic (NOD)-SCID mice that have reduced natural killer cell function, as well as deficiencies in T- and B-cells. However, injection of progenitor cells from MDS patients with del(5q) and trisomy 8 into these mice showed poor engraftment<sup>[62,63]</sup>. Although one of seven mice with del(5q) showed low-level of engraftment, no clinical phenotype of MDS was observed. In another study, bone marrow cells from MDS patients and healthy controls were injected into sublethally irradiated NOD-SCID mice, with or without human cytokines<sup>[64]</sup>. Cells from patients with MDS demonstrated reduced marrow repopulating ability compared to healthy controls. Moreover, previously observed karyotypic abnormalities could not be identified in recipient mice, suggesting that most of the engrafted human cells were derived from normal bone marrow. Taken together, these studies showed

that the NOD-SCID environment could not reliably and reproducibly support the expansion of human MDS cells.

The generation of NOD-SCID beta2-microglobulin-null mice (NOD-SCID-B2m<sup>-/-</sup>) that have suppressed NK cell function, but express human cytokines and steel factor (c-kit ligand) has allowed the repopulation of MDS clones<sup>[65]</sup>. However, the level of engraftment was less than 1% of nucleated cells, and the mice did not develop clinical disease.

More recently, intravenous co-administration of the human marrow stromal cell line HS27a with CD34<sup>+</sup> MDS cells in NOD-SCID gamma (NSG) mice was explored and showed considerable promise with engraftment documented in 44 of 46 (95%) mice<sup>[66]</sup>. Co-localisation of the stroma and CD34<sup>+</sup> cells were seen in the spleen of the recipient mice, and furthermore, these cells also engrafted successfully in secondary NSG recipients. This study suggested that HS27a stromal cells in direct contact with the hematopoietic precursors supported their propagation. In another study, overproduction of niche factors such as CDH2 (N-Cadherin), IGFBP2, VEGFA, and LIF enhanced the expansion of MDS mesenchymal stromal cells, highlighting the complexity of the



disease and that it requires the engagement of both the hematopoietic and stromal elements to propagate<sup>[67]</sup>.

Collectively, these studies demonstrate ongoing progress in the development of xenograft models of MDS. Overall, more robust and more consistent engraftment of MDS cells that can result in clinical disease is needed to improve the utility of this approach. While poor engraftment of MDS cells remains the main drawback, the requirement of an immunocompromised host with this technique makes it unsuitable for the bone marrow niche to be examined.

## GENETICALLY ENGINEERED MOUSE MODELS OF MDS

Generally, genetic engineering of hematopoietic cells of mice has been accomplished using two approaches. The first approach involves *in vitro* transduction of bone marrow with viral overexpression/shRNA vectors and subsequent transplantation into a histocompatible, irradiated host. The second approach involves modification of the mouse germline to generate mice with altered expression of a particular gene of interest. These approaches can be further refined with the employment of the Cre-Lox recombination system which allows gene expression to be controlled in a temporal, cell type and spatial manner<sup>[68]</sup>.

Indeed, conditional knock-in mice are currently the most favored technique in generating mouse models of MDS. The gene in question can be manipulated easily and importantly, is expressed at more physiological levels. The host is often immunocompetent, and the bone marrow niche can also be examined. In comparison, retroviral models require transplanting transduced cells into lethally irradiated recipients and hence, results in supraphysiological levels of gene expression. Moreover, the bone marrow niche is altered through the process of irradiation and transplantation. As a result, the observed effects of altered gene expression in this context is not entirely representative.

In the subsequent sections, we will focus our discussions on selected mouse models of MDS (Table 3). We will highlight models that are robust and well-characterized phenotypically, as well as models that illustrated different genetic lesions that are clinically relevant. We have particularly focused on the mouse models that demonstrate synergy to human disease.

## MODELLING CYTOGENETIC ABNORMALITIES - CHROMOSOMAL TRANSLOCATIONS

### *NUP98-HOXD13*

Of all the approaches that have been explored to model MDS to date, the *NUP98-HOXD13* mouse model is the best established, and perhaps the only published model that has been able to recapitulate many of the key

features of MDS<sup>[69]</sup>.

The *NUP98-HOXD13* involves the fusion of two genes: Nucleoporin protein, *NUP98*, with homeobox D13, *HOXD13*. The *NUP98-HOXD13* fusion gene, which is generated by the chromosomal translocation t(2;11)(q31;p15), was initially identified in a patient with therapy-related MDS (t-MDS)<sup>[70]</sup>. Although numerous partner genes of *NUP98* have been reported in various hematopoietic malignancies, balanced translocations are rare in MDS<sup>[71,72]</sup>, and there are very few cases of MDS bearing the t(2;11) reported in the literature<sup>[73]</sup>.

The first reported *NUP98-HOXD13* mouse model was established by a retroviral system. Pineault *et al.*<sup>[74]</sup> constructed *NUP98-HOXD13* (*ND13*) cDNA using *ND13* cDNA fragment isolated from a patient with t-MDS, and transplanted transduced murine bone marrow cells into irradiated recipient mice. *ND13* expressing mice showed a preferential increase in myelopoiesis at the expense of B and T-cell lymphopoiesis, and developed overt features of myeloproliferative disease five months post-transplant. The mice did not progress to AML, however accelerated leukemic transformation was observed when the *HOX* cofactor, *Meis1*, was co-transduced with *ND13*.

Subsequently, conditional *NUP98-HOXD13* (*NHD13*) transgenic mice were developed, using a *vav* promoter to drive *NHD13* expression in hematopoietic tissues<sup>[69]</sup>. At 4 to 7 mo, these mice developed anemia and neutropenia, with variable degree of macrocytosis and thrombocytopenia. This was accompanied by normal or hypercellular bone marrow with dysplasia observed in multiple lineages. In line with human MDS, about half of *NHD13* mice with MDS developed acute leukemia, typically at 10 to 14 mo of age. Although AML was the most common type of leukemia reported, several mice also developed precursor T-cell lymphoblastic lymphoma/leukemia (T-ALL), which is rarely reported in human MDS. The T-ALL predisposition may be related to increased levels of *Hoxa* cluster genes, such as *Hoxa7* and *Hoxa9*, which have an association with T-ALL<sup>[75]</sup>.

In addition to the key features of MDS described above, it is noteworthy that the *NHD13* mice showed marked reductions in undifferentiated lineage negative (*lin*<sup>neg</sup>) hematopoietic precursors *in vitro* and *in vivo*, which are comparable to results from studies performed on MDS patients<sup>[76-79]</sup>. This was further accompanied by impaired differentiation with the majority of the *NHD13* *lin*<sup>neg</sup> cells undergoing apoptosis, which is a salient feature in human MDS. Gene expression profiling of Lineage<sup>-</sup>, c-kit<sup>+</sup>, Sca-1<sup>-</sup> (LKS<sup>-</sup>) myeloid progenitor cells from 3-mo-old *NHD13* mice that displayed macrocytic anemia showed 3.6-fold reduction in *BCL2*<sup>[79]</sup>. Enforced expression of *BCL2* inhibited apoptosis at the HSPC level, rescued the macrocytic anemia and interestingly, also abrogated leukaemic transformation<sup>[79]</sup>.

The *NHD13* model has also been used to identify secondary mutations that lead to acute leukemia in the mouse. An increased frequency of *Nras* and *Kras* mutations has been noted in *NHD13* mice that pro-

gressed to leukemia<sup>[80]</sup>. In contrast, *Npm1*, *Trp53*, *Runx1*, *Kit*, and *Flt3* mutations were not increased, and *Meis1*, which induces leukaemic transformation in the retroviral model, was not altered in the transgenic model<sup>[69,80]</sup>.

The initial transgenic *NHD13* study was performed using FVB/N background mice. The entire study was subsequently repeated with C57Bl/6 mice with similar findings, demonstrating that effects of the transgene were reproducible and not compounded by the genetic background of the mice<sup>[69]</sup>. In comparison, there are considerable differences between the transgenic and retroviral transduction models, which may be explained by *ex vivo* manipulation of cells, differences in mouse strain, amount of overexpression of *NHD13* achieved and/or the differential effects of *ND13* retrovirus on the bone marrow hematopoietic stem/progenitor cells and their subsequent bone marrow reconstitution.

## MODELLING CYTOGENETIC ABNORMALITIES - CONTIGUOUS GENE DELETION SYNDROMES

### 5q-

Deletion of the long arm of chromosome 5, del(5q), is the most common cytogenetic abnormality found in MDS, accounting for approximately 10%-15% of cases<sup>[24,81]</sup>. The 5q- syndrome is recognized as a distinct clinical entity in the 2008 WHO classification, and is defined by del(5q) being the sole karyotypic abnormality<sup>[1]</sup>. It has a female preponderance, and a distinct phenotype characterized by refractory anemia with normal or increased platelet count, erythroid hypoplasia, hypolobated megakaryocytes, < 5% blasts, and lenalidomide responsiveness. MDS associated with isolated del(5q) carries a good prognosis with a low risk of transformation to AML<sup>[82-84]</sup>.

There are two distinct commonly deleted regions (CDR) in 5q- syndrome. The more distal CDR is mapped to a 1.5-megabase region between bands 5q31 and 5q33<sup>[85,86]</sup>.

The CDR contains 24 known genes, 16 predicted genes, and four known microRNAs (*MIR584*, *MIR143*, *MIR145* and *MIR378A*)<sup>[85]</sup>. The more proximal CDR contains 18 genes, and has been associated with more advanced MDS and AML<sup>[87,88]</sup>. Point mutations in *CSNK1A1* occur in approximately 5% cases of 5q- syndrome<sup>[89]</sup>, however point mutations have not been identified in the remaining coding genes in the distal CDR. The absence of point mutations in the majority of cases suggests that haploinsufficiency of one or more genes, or the epigenetic inactivation of a retained tumor suppressor allele are responsible for the disease phenotype<sup>[90]</sup>. The study of the haploinsufficient effect of the coding genes in the distal CDR led to the identification of *RPS14*, which encodes a component of the 40S ribosomal subunit. Reduced *RPS14* expression leads to defects in ribosome biogenesis and protein translation, resulting in apoptosis of erythroid cells

and macrocytic anemia<sup>[91]</sup>. Furthermore, this phenotype was rescued *in vitro* by enforced expression of *RPS14* in CD34<sup>+</sup> bone marrow cells derived from 5q- syndrome patients indicating that haploinsufficiency of *RPS14* is responsible for the erythroid phenotype in the 5q- syndromes.

Subsequently, Barlow *et al*<sup>[92]</sup> generated a mouse model using Cre-loxP recombination to delete a large region on chromosome 18 flanked by the *Cd74* gene and small integral membrane protein 3 (*Smim3*, also known as *Nid67*) in the mouse. The haploinsufficient region in this model is syntenic to a region within the 5q- CDR in humans that contains *RPS14*. The *Lmo2Cre<sup>+</sup> Cd74-Nid67* deleted mice developed severe macrocytic anemia, prominent dyserythropoiesis and monolobated megakaryocytes, in keeping with the characteristics of 5q- syndrome. On the other hand, these mice developed thrombocytopenia, which is generally not seen in 5q- syndrome unless in the context of disease progression or leukaemic transformation.

In addition, the *Cd74-Nid67* deleted mice had hypocellular bone marrow with 50%-60% reductions in cell numbers, accompanied by defective production of progenitor cells with proportionally reduced trilineage colony-forming potential *in vitro* compared to controls. Deletion of various segments of mouse chromosomes 11 and 18 syntenic to other regions of the 5q- CDR and exclusive of *Rps14* did not give rise to red cell phenotype. The deleted region of this *Cd74-Nid67* mouse contains five candidate genes (*Synpo*, *Myoz3*, *Rbm22*, *Dctn4* and *Nid67*) in addition to *Rps14*. Nonetheless, the fact that it was only mice in which the region containing *Rps14* was deleted that had a macrocytic anaemia phenotype was consistent with the findings of Ebert *et al*<sup>[91]</sup> that *RPS14* is the key contributor to the erythroid phenotype seen in 5q- syndrome.

Of interest, *Cd74-Nid67* deleted mice showed increased intracellular Trp53 (p53) in their immature progenitor cells. Although not statistically significant, there was a trend to an increase in annexin-V<sup>+</sup> (early apoptotic) cells in the *Cd74-Nid67* deleted marrow compared to control mice, suggesting that cell cycle arrest and apoptosis was probably enhanced by the stabilization of Trp53 in these cells. Following this, Barlow *et al*<sup>[92]</sup> elegantly showed that homozygous *Trp53* deletion rescued the progenitor deficits and normalized the peripheral blood phenotype observed in *CD74-Nid67* deleted mice. Taken together, these findings suggest that the loss of *RPS14* results in impaired ribosomal biogenesis and consequently TP53 activation, leading to increased apoptosis and erythroid hypoplasia.

More recently, there have been two studies that explored the role of casein kinase 1A1 (*CSNK1A1*) in the pathophysiology and treatment of 5q- syndrome<sup>[93,94]</sup>. *CSNK1A1* is located in the distal CDR, and encodes a serine/threonine kinase. Gene expression analysis of CD34<sup>+</sup> cells from MDS patients with del(5q) demonstrated the haploinsufficiency of *CSNK1A1* with approximately 50% of normal expression<sup>[90]</sup>. Furthermore, studies in

solid organ malignancies showed that *CSNK1A1* acts as a tumour suppressor gene through regulation of the  $\beta$ -catenin pathway, and also regulates TP53 activity<sup>[95,96]</sup>.

Schneider *et al.*<sup>[94]</sup> generated an *Mx1Cre*-inducible *Csnk1a1* exon 3 knock-out mouse model, and demonstrated that activation of  $\beta$ -catenin activity was proportional to the allelic loss of *Csnk1a1*. Accumulation of  $\beta$ -catenin was noted in both hematopoietic and stromal cells consistent with the expression of *Mx1Cre* in bone marrow stroma<sup>[97]</sup>, with more pronounced expression in homozygous knock-out mice. As such, the function of mesenchymal stem cells in supporting hematopoiesis is significantly impaired in the knock-out mice, with inactivation of  $\beta$ -catenin rescuing the effect.

Homozygous knock-out mice (*Csnk1a1*<sup>-/-</sup> *Mx1Cre*<sup>+</sup>) rapidly developed profound pancytopenia, fulminant bone marrow failure, multi-organ ischemia and death in 5-17 d, demonstrating the critical role of *Csnk1a1* in hematopoiesis<sup>[94]</sup>. Moreover, there was accumulation of Trp53 and its target, Cdkn1a (p21), leading to induction of early and late apoptosis with a marked decrease in cells in G0 and a significant increase in cells in S/G2/M phases in keeping with stem cell exhaustion.

Transplantation of bone marrow cells from heterozygous knock-out mice (*Csnk1a1*<sup>+/-</sup>) into lethally irradiated mice showed that transplant recipients developed normal to hypercellular bone marrow, accompanied by increased and mildly dysplastic hypolobated megakaryocytes, as well as thrombocytosis over time<sup>[94]</sup>. Additionally, non-competitive transplantation of *Csnk1a1*<sup>+/-</sup> bone marrow showed increased proportions of HSC-enriched Lineage<sup>-</sup> ckit<sup>+</sup>Sca-1<sup>+</sup> (LKS<sup>+</sup>) cells in contrast to reduced Lineage<sup>-</sup> ckit<sup>+</sup>Sca-1<sup>-</sup> (LKS<sup>-</sup>) myeloid progenitor cell populations. This was further demonstrated to be related to exit of *Csnk1a1*<sup>+/-</sup> HSCs from quiescence, with reduced cells in G0 and a significantly increased proportion of cells in the cycling G1 fraction and S phase, which was due to increases in  $\beta$ -catenin activity and expression of cyclin D1. A competitive advantage was demonstrated in *Csnk1a1* haploinsufficient bone marrow using long-term repopulating assays, where haploinsufficient cells were significantly more abundant than controls at 16 wk following primary and secondary transplant, specifically with increased LKS<sup>+</sup> cells and increased myeloid progenitor cells and CD3<sup>+</sup> T cells. Collectively, the hypolobated megakaryocytes and self-renewal cells in *Csnk1a1*<sup>+/-</sup> cells highlight the role of *Csnk1a1* haploinsufficiency and  $\beta$ -catenin in the megakaryocyte phenotype and clonal expansion that occur in 5q-syndrome.

Correlating this clinically, the group performed whole-exome sequencing on MDS samples, and identified a small proportion of patients (3 of 43) with somatic mutations in *CSNK1A1*. All three patients had mutations that resulted in the same amino acid change (E98K or E98V), and all had wildtype *TP53*. Analysis by SNP array showed a high variant allelic frequency of the del(5q) MDS clone, indicating that the deletion of chromosome 5q preceded *CSNK1A1* mutation in remaining allele.

The functional consequence of *CSNK1A1* E98V mutation was then examined by retroviral transduction of mutant cDNA in *Csnk1a1*<sup>-/-</sup> *Mx1Cre*<sup>+</sup> haematopoietic cells and transplantation into lethally irradiated recipients. Firstly, it was noted that cDNA overexpression of *CSNK1A1* E98V mutation rescued the HSC ablation in *Csnk1a1*<sup>-/-</sup> *Mx1Cre*<sup>+</sup> cells. Secondly, in comparison to cells transduced with the WT cDNA, *CSNK1A1* E98V transduced cells have increased nuclear  $\beta$ -catenin activity but do not cause increased p53 activation. Collectively, these findings provided evidence that that *CSNK1A1* E98V mutation do not cause of loss of function, but conversely confer selective advantage and drives clonal dominance of del(5q) MDS cells.

Finally, the group showed that *Csnk1a1* haploinsufficiency sensitizes cells to casein kinase 1 inhibition with D4476. Using purified myeloid progenitors, *Csnk1a1* haploinsufficient cells demonstrated reduced viability and increased apoptosis compared to control cells at a range of D4476 drug concentrations.

In another study by the same group, Krönke *et al.*<sup>[93]</sup> showed that reduced *CSNK1A1* levels sensitize hematopoietic cells to lenalidomide. Inhibition of cell growth and proliferation was observed in the presence of lenalidomide using transduced primary human CD34<sup>+</sup> hematopoietic stem and progenitor cells with shRNA knockdown of *CSNK1A1*. Overexpression of *CSNK1A1* in bone marrow samples of MDS patients with del(5q) led to reduce *in vitro* sensitivity to lenalidomide in 3 of 5 patients, which correlated with the clinically observed cytogenetic response. In contrast, overexpression of *CSNK1A1* had no effect in normal donors and in MDS with a normal karyotype, highlighting the therapeutic window for selectively targeting MDS cells by lenalidomide in del(5q) MDS.

Lenalidomide induces the ubiquitination of casein kinase 1A1 (CK1a) *via* the E3 ubiquitin ligase CUL4-RBX1-DDB1-CRBN (known as CRL4<sup>CRBN</sup>) in a species-specific manner. Mice are insensitive to the teratogenic effects of thalidomide. Similarly, *Csnk1a1*<sup>+/-</sup> murine cells are insensitive to lenalidomide because degradation of CK1a does not occur after binding of lenalidomide to mouse Crbn. This is due to a single amino-acid difference between cereblon in mice and humans. Substitution of isoleucine for the human valine at position 391 of mouse Crbn (CrbnI391V) is sufficient to rescue lenalidomide sensitivity. This data illustrates the importance of taking into account and leveraging differences between mice and humans when using mice to model human diseases.

## MODELLING SINGLE GENE MUTATIONS AND OTHER SUBMICROSCOPIC CHANGES

### Modelling mutations in the RNA splicing genes

Somatic mutations in components of the 3' pre-mRNA splicing machinery are common, and are frequently early pathogenetic events in MDS. However, the functional

contribution of these mutations in the evolution of MDS remains to be delineated. It is unclear whether mutation in a splicing factor affects the splicing of a single gene or large number of genes, or even whether the downstream impact of these mutations is mRNA splicing-dependent.

### SRSF2

*SRSF2* is mutated in 20%-30% cases of MDS, and about 50% cases of chronic myelomonocytic leukemia (CMML). Importantly, it is associated with an inferior prognosis<sup>[32,35]</sup>. *SRSF2* is a member of the serine/arginine-rich protein family, and binds to exonic splicing enhancer sequences (ESEs) within pre-mRNA through the RNA recognition motif domain.

To study the functional impact of *SRSF2* mutations on hematopoiesis or splicing, Kim *et al.*<sup>[98]</sup> generated a hematopoietic-specific conditional *Srsf2* knock-in mouse model with the commonly occurring *SRSF2*<sup>P95H</sup> mutation. Heterozygous transgenic mice were generated and crossed to *Mx1-cre* mice. Bone marrow mononuclear cells from *Srsf2* wildtype (WT), *Srsf2*<sup>fl</sup>/WT (heterozygous deletion of one copy of *Srsf2*), *Srsf2*<sup>fl</sup>/fl (homozygous floxed mice for both copies of *SRSF2*), and *Srsf2*<sup>P95H</sup>/WT were transplanted into lethally irradiated recipients, followed by polyinosine-polycytosine treatment four weeks later.

Mice transplanted with BM cells harbouring the homozygous *Srsf2* deletion or the *Srsf2*<sup>P95H</sup>/+ mutation developed significant anemia and leucopenia at 18 wk post-transplant<sup>[98]</sup>. In addition to the observed bicytopenia, *Srsf2*<sup>P95H</sup>/+ mice also displayed macrocytic erythrocytes, accompanied by normocellular bone marrow with multilineage dysplasia in the erythroid and myeloid lineages, mimicking features of human MDS. In contrast, and consistent with the original published full knock-out model, homozygous *Srsf2* deletion led to profound bone marrow aplasia without evidence of dysplasia morphologically.

Moreover, *Srsf2*<sup>P95H</sup>/+ mice showed increased LKS<sup>+</sup> cells, increased early apoptosis and increased proportion of cells in the S-phase of the cell cycle<sup>[98]</sup>. The increase in HSC and progenitor cells in conjunction with peripheral cytopenia is suggestive of impaired differentiation. Flow cytometry showed that the observed peripheral leucopenia was predominantly due to reduced B-lymphopoiesis. *Srsf2*<sup>P95H</sup> mice also had reduced early erythroid progenitors with reduced pre-MegE and pre-colony-forming units, erythroid. Of note, none of the non-transplanted *Srsf2* P95H mice developed overt MDS phenotypes or acute leukemia even well past a year of monitoring and the primary phenotypes reported were all present only in the context of transplant studies.

Subsequently, the authors showed that *Srsf2* P95H mutation altered the normal function of SRSF2, instead of resulting in haploinsufficiency or a dominant negative form. Using RNA sequencing, they found that *Srsf2* mutation led to genome-wide alteration, rather than loss, of its normal ESE recognition activity. Wild-type

SRSF2 recognizes the consensus binding sequences CCNG and GGNG with similar affinity<sup>[99]</sup>. In contrast, *SRSF2* mutation resulted in preferential recognition of cassette exons containing C- vs G-rich ESEs. This was further supported by biochemical analysis which demonstrated that *SRSF2* mutation was associated with a conformational change in its RNA recognition motif domain, consequently altering the interaction specificity between *SRSF2* and pre-mRNA.

At a functional level, the authors proposed that this drives recurrent missplicing of key hematopoietic regulators, including *SRSF2* mutation-dependent splicing of *Ezh2*. Missplicing of *Ezh2* leads to nonsense-mediated decay, reduced *Ezh2* protein expression and in turn, contributes to impaired hematopoietic differentiation. *SRSF2* and *EZH2* mutations are known to be mutually exclusive in MDS, and findings in this study have provided a potential mechanistic explanation for this observation<sup>[22]</sup>. Finally, the authors showed that overexpression of normally spliced *Ezh2* cDNA in progenitor cells from *Srsf2* P95H/+ mice partially rescues the hematopoietic defect induced by *SRSF2* mutation in methylcellulose colony forming cell assays.

### U2 small nuclear RNA auxiliary factor 1

U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) is one of the most commonly mutated genes in MDS, and can be found in approximately 11% of patients<sup>[30,32]</sup>. It is typically a founder mutation, and is associated with a less favorable prognosis with a high risk of transformation to AML<sup>[14,22]</sup>. Previous studies using a retroviral overexpression model of mutant *U2AF1* have demonstrated that transduced murine bone marrow cells have reduced repopulating potential *in vivo*<sup>[32]</sup>.

More recently, Shirai *et al.*<sup>[100]</sup> generated a doxycycline-inducible transgenic mouse model with the most commonly identified *U2AF1* (S34F) mutation. Human cDNA encoding for *U2AF1* (S34F) or *U2AF1* (WT) were inserted into the *Col1a1* locus of KH2 mouse embryonic stem cells, which contain the M2rtTA tetracycline-responsive transactivator protein (rtTA) ubiquitously expressed from the *Rosa26* locus to allow for induction of the transgene. Bone marrow cells from transgenic mice were transplanted into lethally-irradiated wild-type congenic mice, and allowed to engraft prior to the induction of transgene expression with doxycycline treatment for 12 mo.

Peripheral blood leucopenia was observed in the *U2AF1* (S34F) mice after one month of doxycycline treatment, and appears to be related to B-lymphopenia and monocytopenia based on flow cytometry. Leucopenia persisted up to 12 mo, with white cell counts recovering to levels similar to that of controls following withdrawal of doxycycline treatment, suggesting a relationship between expression of mutant *U2AF1* and the phenotype seen.

Strikingly, *U2AF1* (S34F) mice showed increased proportions of HSPC in the bone marrow, particularly in the multipotent progenitors and common myeloid



progenitor (CMP) compartments. Moreover, there was increased cell cycling in the LKS<sup>+</sup> population as evidenced by increased Ki67 staining. Overall, the bone marrow cellularity of *U2AF1* (S34F) mice was not significantly different to controls. In mature cell lineage analysis, B-lymphopenia and monocytopenia were also noted in bone marrow. This appeared to be due to neutrophilia and increased in apoptosis of the monocytes.

Interestingly, there was no morphological evidence of dysplasia despite 12 mo of doxycycline treatment. Whilst there were convincing features of perturbed hematopoiesis, and bone marrow characteristics reminiscent of MDS in *U2AF1* (S34F) mice, they do not meet the Bethesda criteria for a myelodysplastic syndrome. They also failed to develop AML. It would be interesting to establish whether other cooperating mutations such as *ASXL1* give rise to MDS and AML in these mice. However, this work remains important as it has shed light on the effects of *U2AF1* mutation on hematopoiesis. In addition, the authors used RNA sequencing data to identify a splice junction sequence-specific pattern of altered splicing induced by *U2AF1* mutation. Exons skipped more frequently and alternative splice sites used more often than canonical splice sites by *U2AF1* (S34F) were enriched for a uracil in the minus 3 position relative to the AG dinucleotide, consistent with published reports of mutant *U2AF1*-associated splicing abnormalities seen in other malignancies<sup>[101]</sup>. Moreover, integration with human RNA sequencing datasets determined that common mutant *U2AF1*-induced splicing alterations are enriched in RNA processing genes, ribosomal genes, and recurrently mutated MDS and acute myeloid leukemia-associated genes. Taken together, this supports the hypothesis that *U2AF1* mutation alters downstream gene isoform expression, thereby contributing to abnormal hematopoiesis in MDS.

Collectively, studies modelling RNA splicing mutations in mice have provided insights into genetic lesions affecting spliceosome function and mRNA splicing. These findings have already improved our mechanistic understanding of the role of spliceosome mutations in altering the transcriptome, and its effect on normal hematopoiesis and MDS pathogenesis.

## MODELLING MUTATIONS IN TRANSCRIPTION FACTORS AND EPIGENETIC MODIFIERS

### *RUNX1*

The *RUNX1* gene, also known as *AML1* or *CBFA*, plays a key role in hematopoiesis, and is frequently mutated in MDS, *de novo* AML and secondary AML<sup>[102,103]</sup>. The vast majority of *RUNX1* mutations are located in the Runt homology domain (RHD) which mediates binding to DNA and core binding factor beta, although mutations in the C-terminus outside the RHD have also been reported<sup>[104]</sup>.

Watanabe-Okochi *et al.*<sup>[105]</sup> developed a *RUNX1* mouse model using retroviral constructs based on two types of

*RUNX1* mutations identified in patients. AML1-D171N (hereafter D171N) has a point mutation in the RHD resulting in the loss of its DNA binding site, while the AML1-S291fsX300 (hereafter S291fs) has a frameshift mutation outside the RHD that results in C-terminal truncation, leading to the loss of transactivation potential but increased DNA-binding ability. Both are dominant negative forms, with the latter being more potent than the former<sup>[106,107]</sup>.

Both D171N and S291fs mice developed macrocytosis, multi-lineage dysplasia, progressive cytopenias in a normal or hypercellular bone marrow, and transformation to leukemia in 4 to 13 mo. However, they displayed quite distinct phenotype and disease kinetics. D171N mice had a more proliferative phenotype with leukocytosis due to increased myelopoiesis, more prominent granulocytic dysplasia, accompanied by marked hepatosplenomegaly, and a higher percentage of blasts. In contrast, S291fs developed pancytopenia with a more marked erythroid dysplasia. This study showed that different mutations within the same gene could induce heterogeneous disease with different biological outcomes. This may be explained in part by the structural and functional differences between the mutants.

Of note, a fraction of the D171N mice had the *Evi1* locus as the retroviral integration site. This reduced the latency of leukaemic transformation to 3 to 5 mo, hence, providing evidence that *Evi1* co-operates with mutant *RUNX1* to facilitate disease progression.

### Ten-eleven-translocation-2

Ten-eleven-translocation-2 (*TET2*) belongs to a 3-member family of genes (*TET1-TET3*). It encodes a  $\alpha$ -ketoglutarate-dependent enzyme that catalyzes the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethyl cytosine (5-hmC), which is the first step of active demethylation. It is frequently mutated in myeloid malignancies (up to 30% of MDS)<sup>[108,109]</sup>.

Loss of *TET2* leads to a reduction in the amount of 5-hmC, and this has been demonstrated in samples of patients with myeloid malignancies, suggesting that *TET2* acts as a tumour suppressor gene<sup>[110]</sup>. Indeed, several groups have reported that the loss of *TET2* resulted in deregulated self-renewal of hematopoietic stem cells and the development of CMML-like disease<sup>[111-114]</sup>. It should also be noted that mice that were hypomorphic or heterozygous for the *TET2* allele showed similar phenotypes, suggesting a haploinsufficiency effect of *TET2* in the development of hematopoietic malignancies.

Recently, Muto *et al.*<sup>[115]</sup> described mice hypomorphic for *TET2* as a mouse model of CMML and MDS. In that study, *TET2* gene trap mice (*TET2*<sup>KD/KD</sup>) were engineered to express approximately 20% of the *TET2* mRNA of WT mice. *TET2*<sup>KD/KD</sup> mice developed overt features of myeloid malignancy after about 11 mo. Whilst the majority had features of CMML, 3 out of 13 mice developed MDS with pancytopenia, granulocytic dysplasia, and increased erythroid apoptosis.

Comparable to the *NHD13* model, *TET2*<sup>KD/KD</sup> mice



that developed CMML or MDS showed skewing in their HSPC compartment. These mice had a greater proportion of Lineage<sup>-</sup>, Sca-1<sup>+</sup>, c-kit<sup>+</sup> (LKS<sup>+</sup>) cells in their bone marrow. Interestingly, mice with CMML had a greater proportion of CMP and granulocyte-macrophage progenitors (GMP) in comparison to mice with MDS who had a greater proportion of megakaryocyte/erythroid progenitors (MEP).

Of note, both mice with CMML and MDS developed splenomegaly, with further analysis showing marked increases in LKS<sup>+</sup> cells and disruption of the splenic architecture due to extramedullary hematopoiesis. It should be noted that although splenomegaly in association with CMML is well documented clinically, splenomegaly does not tend to feature in MDS patients. This can be explained by the hematopoietically active role of the spleen in the lifespan of the mice, but not in humans. As such, splenomegaly can be seen in MDS mice in the context of a compensatory response to underlying erythroid changes.

This study showed that alterations in TET2 expression resulted in two distinct phenotypes, CMML and MDS, after a considerable latency. Recurrent somatic *TET2* mutations have been identified in normal, elderly individuals with acquired clonal hematopoiesis without overt clinical manifestations<sup>[16]</sup>. In line with this, findings from this mouse study support a role for *TET2* mutations as early, founder or initiating mutations in myeloid malignancies with later, acquisition of additional, co-operating mutations required to bring about overt disease. The distinct phenotype observed, whether predominantly myelodysplastic, myeloproliferative or myelodysplastic/myeloproliferative may be determined by the nature of the secondary mutations.

### Additional sex combs-like 1

Additional sex combs-like 1 (*ASXL1*) plays an important role in regulating *Hox* genes through its interaction with the polycomb group of proteins<sup>[116,117]</sup>. *ASXL1* mutations are reported in approximately 15%-20% of MDS patients<sup>[47,49,118]</sup>. They are usually heterozygous with most mutations located in the 5' region of the last exon (exon 12), resulting in the expression of a truncated *ASXL1* protein<sup>[119]</sup>. *ASXL1* mutations are generally subclonal, indicating that they are acquired later in the course of the disease. They have been reported to promote leukaemic transformation, and their presence is an independent predictor of adverse prognosis in MDS<sup>[120,121]</sup>.

There are two mutated *ASXL1* mouse models of MDS in the literature<sup>[122,123]</sup>. In the first study, Inoue *et al.*<sup>[122]</sup> developed retroviral constructs with a C-terminal-truncating *ASXL1* mutation, FLAG-*ASXL1*-MT1 and FLAG-*ASXL1*-MT2 (collectively termed *ASXL1*-MTs) derived from patients with MDS harbouring mutated genes of 1934dupG;G646WfsX12 and 1900-1922del;E635RfsX15 respectively. GFP-positive cells of *ASXL1*-MTs mice showed preferential myelopoiesis in the marrow at the expense of reduced B-lymphopoiesis at 6 mo post

transplantation. At approximately 12 mo, mutant mice developed features of MDS with display of pancytopenia, multi-lineage dysplasia and impaired myeloid differentiation. Additionally, some of the secondary transplant recipients progressed to secondary leukemia. Gene expression profiles of hematopoietic cells from mice that developed MDS showed de-repression of homeobox a9 (*Hoxa9*) through inhibition of polycomb repressive complex 2 - mediated methylation of histone H3K27. Moreover, the *ASXL1* mutation led to upregulation of *Mir125a* and subsequent suppression of C-type lectin domain family 5, member a (*Clec5a*), which is involved in myeloid differentiation. Thus, this study identified an *ASXL1*-MT-*Hoxa9*-*Mir-125a*-*Clec5a* axis that is critical for *ASXL1*-mediated MDS pathogenesis.

In another study, Wang *et al.*<sup>[123]</sup> constitutively deleted *Asx1* (*Asx1*<sup>-/-</sup>), which resulted in significant embryonic lethality. Surviving *Asx1*<sup>-/-</sup> mice showed profound developmental abnormalities that included dwarfism and anophthalmia. Perinatal mortality was high; 78% of mice died within 24 h of birth and no mice lived longer than 42 d. Hematologically, the surviving mice displayed features of MDS with multilineage cytopenia and dysplastic neutrophils in the peripheral blood. In the bone marrow, *Asx1*<sup>-/-</sup> mice showed normal to increased bone marrow cellularity, with accompanying myeloid hyperplasia and reduced erythroid precursors. Moreover, *Asx1*<sup>-/-</sup> mice had reduced LKS<sup>+</sup> cells and altered myeloid progenitors with increased GMP and reduced MEP, accompanied by increased apoptosis in the bone marrow.

Subsequently, the generation of heterozygous *Asx1* mutation (*Asx1*<sup>+/-</sup>) mice showed that haploinsufficiency was sufficient for the development of MDS. These mice generally had a milder MDS phenotype, with some developing a phenotype that was more reminiscent of CMML.

Taken together, these two studies demonstrated that *ASXL1* mutation or deletion gives rise to MDS phenotypes, and suggest a tumour suppressor function for *ASXL1* in hematopoiesis.

### TERT

Telomere shortening or dysfunction has been linked to advancing age, however, its direct role in causing MDS is unclear. Colla *et al.*<sup>[124]</sup> recently engineered an inducible telomerase mouse model to study the chronic physiological DNA damage in the hematopoietic system. *TERT*<sup>ER</sup>, a telomerase reverse transcriptase-estrogen receptor fusion protein was used, and inter-generational crosses of *TERT*<sup>ER/ER</sup> mice was carried out to elicit progressive telomere erosion. By the fourth and fifth generations (G4/G5), telomere dysfunction with attendant DNA damage signalling and severe tissue degeneration was evident<sup>[125]</sup>.

The G4/G5 *TERT*<sup>ER/ER</sup> mice displayed characteristics of MDS as early as 3 mo. They demonstrated significant cytopenias including anemia, reduced lymphopoiesis with accompanying hypercellular bone marrow, increa-

sed myeloid-to-erythroid ratio, increased apoptosis and multi-lineage dysplasia<sup>[124]</sup>. Moreover, approximately 5% of the aged G4/G5 *TERT*<sup>ER/ER</sup> mice progressed to AML.

In the hematopoietic progenitor compartment, G4/G5 *TERT*<sup>ER/ER</sup> mice showed a significant increase in GMP, accompanied by loss or markedly reduced MEP and CMP that were not attributed to an increase in apoptosis. Further analysis showed a preferential accumulation of  $\gamma$ -H2AX and 53BP1 DNA damage foci in the CMP subpopulation, but not in GMP or MEP. Of note, tamoxifen induction of *TERT* at G4/G5 stage was able to restore telomeres, ceased DNA damage signalling and reversed the degenerative tissue phenotype seen.

Subsequently, long-term HSC isolated from 3-mo-old G0 or G4/G5 mice were transplantable into wild-type congenic recipients<sup>[124]</sup>. Transplant recipients developed a more severe phenotype, with skewed myeloid differentiation, trilineage dysplasia and an excess of blasts. Notably, one of the six mice transplanted with G5 HSC progressed to AML. Defective CMP differentiation suggested from *in vivo* studies was confirmed using *in vitro* methylcellulose clonogenic assays, which showed a profound impairment of myeloid differentiation with preferential granulo-monocytic commitment at the expense of the erythroid lineage.

At a molecular level, the defect in CMP differentiation was found to be related to decreased expression of genes involved in the 3'mRNA splicing or processing genes, resulting in abnormal RNA splicing<sup>[124]</sup>. It was noted that 40% of the aberrant splicing events in *TERT*<sup>ER/ER</sup> cells resulted in exon skipping, and a higher proportion of in exon retention. Moreover, RNA-sequencing analyses of *TERT*<sup>ER/ER</sup> CMP cells identified aberrantly spliced genes to be associated with various pathways linked to MDS pathogenesis including DNA repair, chromatin remodeling and histone modification. These gene sets were also enriched in CMP of mice and patients with *SRSF2* mutations.

This report provides the first evidence linking telomere dysfunction to reduced expression of splicing factors, which consequently drives abnormal myeloid differentiation.

## CONCLUSION

At present, we have an expanding list of MDS mouse models, most of which displayed a range of typical features of MDS including cytopenias, dysplasia, ineffective hematopoiesis, and some of which also have the ability to transform to leukemia. The bone marrow environment has a distinct role in MDS, as highlighted by the ongoing challenges faced in xenografting MDS cells into the murine system.

Several candidate genes used to model MDS have demonstrated their role in the pathogenesis of MDS, and in some cases, also revealed other collaborating mutations leading to leukaemic transformation. Additionally, the impact of specific recurrent mutations within a single gene has also begun to be elucidated. More

recently, the therapeutic potential and the mechanism of action of lenalidomide has also been explored using a del(5q) mouse model, and has certainly enhanced our understanding of this disease with further implications in the clinical context.

In this era of flourishing genetic medicine, there is no doubt that existing and emerging mouse models will continue to be valuable tools in improving our insights into this disease. The availability of the novel CRISPR-Cas9 genomic editing system is likely to hasten the generation of more models, and importantly, the engineering of more complex models that better reflects the disease heterogeneity.

It should be noted that the utility of mouse models in the study of MDS can only be optimized by a careful and systematic approach to their characterization, including the distinction between features that are common to a variety of myeloid malignancies and those that are unique to MDS. Drawing similarities to humans, a diagnosis of MDS is difficult to conclude without the triad of cytopenia, dysplasia and absence of AML (< 20% blasts). Secondly, evidence of increased apoptosis in the marrow, which is characteristic of MDS, will make the diagnosis more convincing.

To make a diagnosis of MDS, close examination of various haematopoietic samples is essential. The normal ranges of different cellular compositions within the different haematopoietic compartments in a normal mouse should first be appreciated, and this was very well-illustrated and described by Yang *et al.*<sup>[126]</sup>. Ideally, morphological assessments should be carried out by an experienced pathologist.

When mice are monitored for features of MDS, peripheral blood sampling should be examined over various time points. The presence of peripheral blood cytopenia that is persistent over time may point to the development of MDS, particularly if this is accompanied by dysplastic changes morphologically. Anaemia may be normocytic or macrocytic, the latter clearly more convincing of MDS. However, peripheral blood assessments alone are insufficient to confirm MDS, and a thorough cytological and histological examination of the bone marrow and spleen is required<sup>[59,60]</sup>. Additional examination and histological assessment of other tissues may also be required especially if AML is suspected.

Cytological assessments for MDS should include the enumeration of marrow and spleen cellularity, the review of cytopsin preparations for dysplastic features and calculation of myeloid to erythroid ratio. This can be further complemented by immunophenotypic analysis of the various mature and immature cellular populations within bone marrow, including the haematopoietic stem and progenitor compartments. Perls Prussian blue staining should also be carried out on cytopsin preparation to assess for the presence of ringed sideroblasts. Ringed sideroblasts are rare in mice, but would be pathognomonic of MDS if present. Histological examination of the bone marrow and spleen is useful to confirm tissue cellularity, and importantly, it provides

better morphological evaluation of megakaryocytes, which can be underrepresented in cytopins preparations.

Finally, the understanding of the MDS initiating cell, and mechanisms responsible for leukemic transformation are some of the major questions that remained to be answered in MDS. It is hopeful that new mouse models created will shed more light on the functional interplay amongst the various genetic mutations present. An ultimate goal of this area of research is to use animal models to facilitate the development of new therapeutics in MDS and improve clinical outcomes.

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