Engineering mouse models with myelodysplastic syndrome human candidate genes; how relevant are they?

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ABSTRACT

Myelodysplastic syndromes represent particularly challenging hematologic malignancies that arise from a large spectrum of genetic events resulting in a disease characterized by a range of different presentations and outcomes. Despite efforts to classify and identify the key genetic events, little improvement has been made in therapies that will increase patient survival. Animal models represent powerful tools to model and study human diseases and are useful pre-clinical platforms. In addition to enforced expression of candidate oncogenes, gene inactivation has allowed the consequences of the genetic effects of human myelodysplastic syndrome to be studied in mice. This review aims to examine the animal models expressing myelodysplastic syndrome-associated genes that are currently available and to highlight the most appropriate model to phenocopy myelodysplastic syndrome disease and its risk of transformation to acute myelogenous leukemia.

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Introduction

Myelodysplastic syndromes (MDS) are thought to arise from a malignant hematopoietic stem cell (HSC) that has sustained irreversible genetic or epigenetic events resulting in dominance of the abnormal clone. Approximately 30% of MDS cases progress to acute myelogenous leukemia (AML). Dysmyelopoiesis and increased apoptosis are the main features of MDS. Bone marrow (BM) isolated from MDS patients exhibits dysplasia of at least one lineage (according to the World Health Organization (WHO) classification),[1,2] hypercellularity, and increased apoptosis correlating with peripheral blood cytopenia.[3,4] Because MDS patients present with a range of different characteristics, classification has become important, and prognostic scoring techniques have been developed to address patient management and treatment.[2,5,6] Recently, these classification and scoring techniques have also taken into account the presence of cytogenetic abnormalities. Cytogenetic abnormalities are relatively frequent in MDS; deletions and rare chromosomal reciprocal translocations have been identified, while DNA sequencing and micro-arrays (RNA, CGH) have confirmed additional mutations and deletions.[7,8] Thus, genetic and epigenetic abnormalities are linked to MDS pathogenesis.[9] Efforts have been made to develop accurate animal models of MDS that would help us understand the pathogenesis of the disease and the mechanisms of its transformation to AML. Various strategies have been used to express MDS-associated candidate genes in animal models, either by transduction of candidate genes under the control of retroviral promoters and subsequent transplantation of transduced mouse BM cells into syngenic mice, or by the establishment of transgenic mice expressing the candidate genes under the control of myeloid-specific promoters (Figure 1). Indications of function have also been obtained by analysis of knock-in (KI) and knock-out (KO) models of various genes. Recently, analyses of xenografts of human MDS cells have yielded promising results. Hematopoietic analysis of these animal models has revealed a great variety of hematologic abnormalities, some of which are not necessarily reminiscent of either the human MDS or MDS/myeloproliferative neoplasms (MDS/MPN). This review focuses on the animal models expressing the most frequent genetic abnormalities associated with MDS and MDS/MPN, including chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML), as well as those models that resemble the human MDS disease regardless of the expressed or deleted genes (Table 1). The WHO classification of human MDS and MDS/MPN is shown in the *Online Supplementary Tables S1 and S2*.[1,2] Our working model of disease progression is illustrated in Figure 2. Starting with an initiation event targeting the stem cell, an abnormal clone arises which has a selective growth advantage and expands. This is the phase that we recognize as pre-leukemia or MDS. Several events then follow until a leukemic clone arises and accelerates the disease to give rise to acute leukemia. The genes that drive this progression are those that code for differentiation, proliferation and apoptosis. A block in differentiation can occur in any compartment; although in MDS it is thought to occur at a very immature stage, giving rise to leukemic stem cells (LSC), so-called because they are cells that have acquired self-renewal capacity. Early MDS is often characterized as pro-apoptotic, and as disease progresses, the cells become more anti-apoptotic. There is abnormal proliferation throughout the disease process.

Novel signaling pathways and cells involved in MDS/MPN

Recent insights into the pathogenesis of MDS have highlighted the importance of genomic instability (GI), epigenetic

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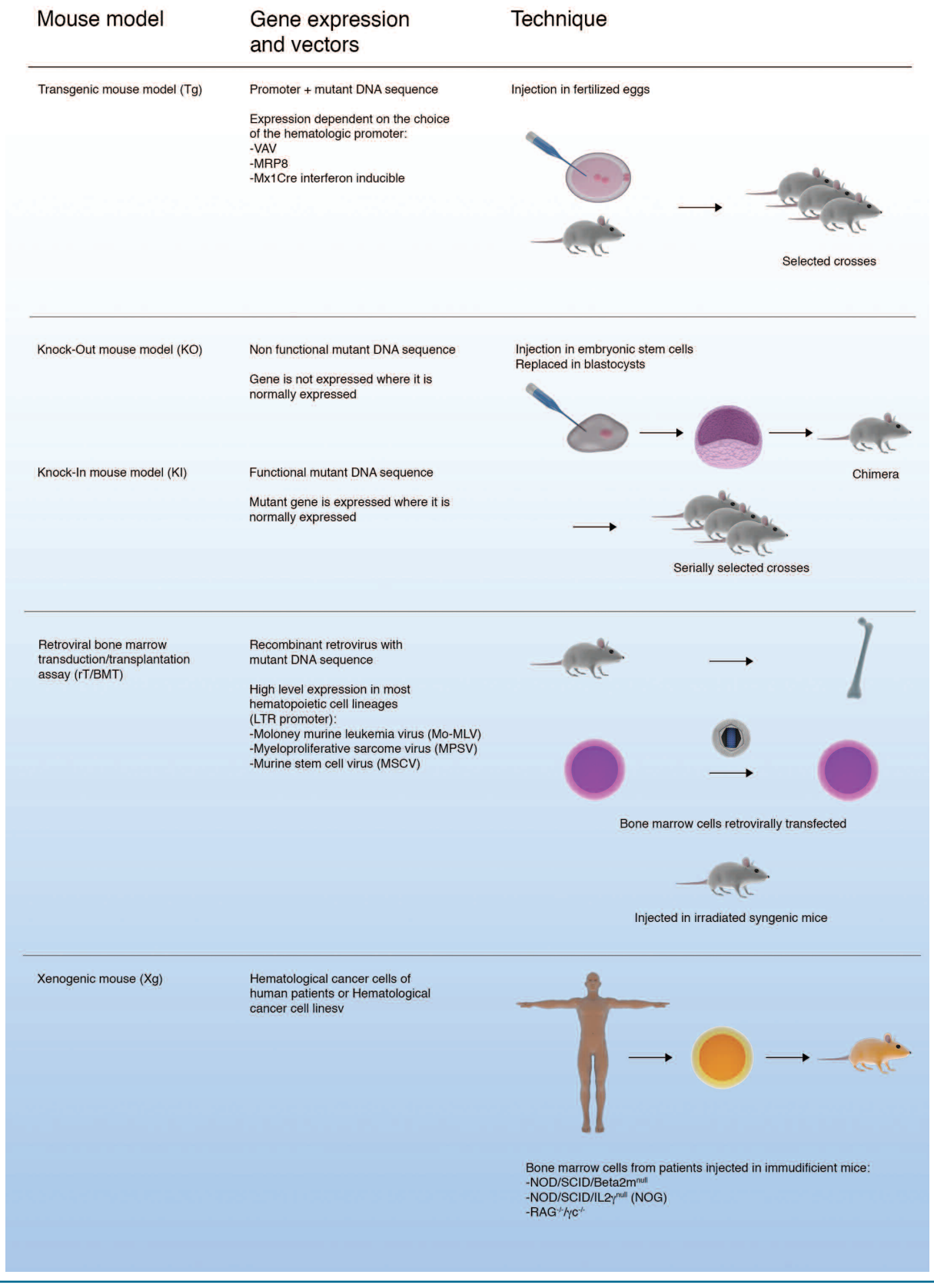

Figure 1. Mouse models to study malignant hematologic disease. Several strategies can be employed to create mouse models of disease. Candidate genes can be introduced into the germline under the control of appropriate promoters to drive expression in certain cell compartments. Knock-out strategies create gene deficient mice, whilst knock-in strategies use the endogenous promoters; but again these tend to be conditional systems requiring specific promoters to determine in which cell the genes are introduced. Transduction of bone marrow and reinfusion is a powerful tool. Xenograft models are theoretically the best if the techniques involved can be improved.

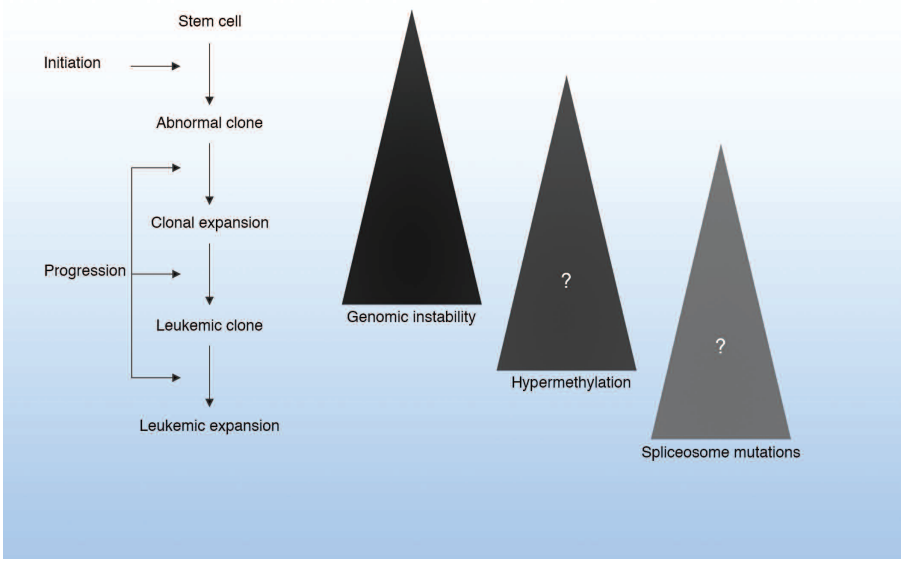

Figure 2. Multi-step model of leukemogenesis. Starting with a stem cell, an initiating event occurs which gives rise to an abnormal clone with a growth advantage, which expands. Further genetic events drive the progression of the disease towards overt leukemia. Genomic instability and misrepair increase as disease progresses. Some of the events that may drive progression include increased methylation to down-regulate genes and mutations of the ribosome machinery to further deregulate genes.

alterations and abnormalities of the spliceosome machinery. The MDS stem cell and its niche have also proved to be of significance (Figure 2).

Genomic instability (GI)

Karyotypic changes are indicative of increased GI. Increased GI can result in increased misrepair. Non-homologous end joining (NHEJ), the main pathway for double-strand break repair, gives rise to chromosomal deletions and translocations. It has been hypothesized that increased GI can drive initiation and progression of malignant disease.[68] Aging mice display increased chromosome instability mediated by MDM2, a regulator of the p53 tumor suppressor and DNA repair.[69] The expression of many oncogenes, such as mutant RAS, results in increased DNA damage-inducing reactive oxygen species (ROS). As MDS is essentially a disease associated with aging, it is not surprising that increased chromosome instability has been observed in a subgroup of MDS that is associated with a poor prognosis (20%), thereby suggesting that GI may be a driver of disease progression.[70] GI in mouse models of MDS has yet to be investigated; although a mouse model of AML with MDS-related changes mediated by RAS and BCL-2[33] displayed increased ROS, DNA damage and misrepair which can be rescued by treatment with either RAC1 inhibitors or antioxidants.[71] Furthermore, BM cells isolated from *NPM1*+/- MPN-like mice display multiple centrosomes, thus indicating that centrosome amplification is associated with *NPM1* loss and may participate in leukemia pathogenesis in this mouse model.[14] DNA damage and chromosomal instability is observed in Fanconi anemia (FA) patients, which is an important cause of childhood MDS.[72] Unfortunately, mouse models harboring a disrupted mouse homolog of FANCC fail to develop MDS.[73] However, the double mutant *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ mice develop spontaneous hematologic abnormalities including BM failure, AML, MDS and complex random chromosomal abnormalities that the single mutant mice do not display.[74]

Altered gene expression through epigenetic modifications and gene silencing

Dysregulation of the epigenetic machinery can lead to oncogenic transformation. Approximately 30% of MDS patients show inactivation, by hypermethylation, of the gene encoding *p15INK4b*.[75] This strongly suggests that the INK4 family member plays a role in the regulation of cell cycle arrest at the early G1 phase as a myeloid tumor suppressor.[76] *Ink4b* KO mice (Ink4b-/-) show defects in the differentiation of common myeloid progenitors, resulting in an imbalance between erythroid and myeloid potential, although follow-up analysis of these mice did not reveal any signs of MDS.[77] Other genes modified by epigenetic mechanisms have been identified, such as *DAPK1*, *CDH1* (E-Cadherins), and *TSP1* (thrombospondin).[78,79] Hypermethylation of DNA is known to contribute to progression of MDS to AML, thereby inhibiting different tumor suppressor genes. Mouse models designed to address this epigenetic progression have yet to be characterized. An exception is the EVI1 mouse model, in which EVI1 expression in the BM gives rise to an MDS phenotype and causes silencing of the miRNA124 by methylation. This epigenetic event results in perturbation of cell division and self-renewal in this mouse model.[80]

The *TET2* gene (Ten-Eleven Translocation-2) was originally identified from an MDS patient with a rearrangement of 4q24[24] and numerous groups have identified somatic inactivating mutations (frameshifts or misense) in MDS, MPD and CMML patients.[23-25] As one of the most frequent mutations found in myeloid malignancies, *TET2* has been proposed to be a tumor suppressor as loss of function tends to lead to disruption of progenitor myeloid differentiation leading to disease progression.[81] Its function is to convert 5-methlycytosine to 5-hdroxymethyl cytosine (5-hmc) and it is, therefore, involved in the epigenetic

control of gene regulation; 5-hmc has been found to be decreased in granulocyte DNA from MPD patients with *TET2* mutations.[81] Animal models of *TET2* using conditional knock-out systems give rise to CMML-like diseases: $Tet2^{LacZ}$ mice, $Tet2^{Lox}$ mice[26] and $Tet2^{-/-}$VavCre+ mice[27] develop a CMML-like phenotype with splenomegaly, leukocytosis with abnormal myelomonocytic differentiation, and expansion of the hematopoietic stem cell compartment.[26,27]

Altered stem cell and niche in MDS mouse models

Blood and BM samples from most MDS patients are technically difficult to engraft into NOD/SCID-β2mnull mice. When xenografts are obtained, they regenerate progeny that display phenotypic and genotypic abnormalities of the original neoplastic clone, such as trisomy 8 and del(5q). Interestingly, injecting BM cells isolated from MDS patients with human stroma-derived cells, via intramedullary injection, into NOD/SCID-β2mnull mice[82] showed the engraftment of early precursors containing del(5q) with a loss of more mature cells that had additionally acquired trisomy 8. Therefore, the 5q deletion may be an earlier event than trisomy 8.[82] In another study, the results of MDS sample engraftment suggested the presence of a relatively late type of 'multilineage but myeloid-restricted' neoplastic 'stem' cell with repopulating activity, limited self-renewal ability, and skewed differentiation potential.[83] These findings also show that the repopulating cells present in MDS patients include residual normal or pre-neoplastic repopulating cells with normal features that outcompete the neoplastic clone if adequately stimulated. Recently, a transgenic approach has provided evidence that disturbance of the endosteal niche can result in MDS.[22] Transgenic mice lacking Dicer1 expression in mesenchymal cells of the osteolineage were generated, *Osx*Cre+/*Dicer*fl/fl.[22] The *Osx*Cre+/*Dicer*fl/fl mutant mice develop fatal neutropenia with hyperplastic BM and dysmyelopoiesis which is highly suggestive of MDS. Increased apoptosis of Lin-/c-KIT+ progenitor cells has also been observed in these mice. However, the MDS phenotype was not transplantable. Interestingly, when *Osx*Cre+/Dicerfl/fl mice were transplanted with BM cells from wild-type mice they developed the same MDS phenotype. Although mutations of Dicer have yet to be discovered in MDS patients, gene expression analysis of *Dicer-/-* osteoprogenitors showed a downregulation of SDB, the gene found mutated in Schwachman-Bodian-Diamond syndrome, which induces BM failure and MDS in children. Recently, downregulation of Dicer has been found associated with poor prognosis in chronic lymphocytic leukemia (CLL) patients.[84] Recent studies have highlighted the role of innate immune signaling molecules in the physiopathology of BM failure: in 5q- patients, deletion of chromosome 5 correlates with a loss of two miRs, miR145 and miR146a,[85] which are abundant in hematopoietic progenitors and target two molecules implicated in innate immune signaling,[86] Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) and tumor necrosis factor receptor-associated factor-6 (TRAF6), respectively. In KO mouse models of miR145 and miR146a targeted specifically in hematopoietic stem and progenitor cells, animals develop thrombocytosis, mild neutropenia, and megakaryocytic dysplasia, which represent several clinical features of 5q-MDS.[87]

MDS mouse models of chromosomal deletions: candidate genes for 5q- syndrome

Deletion of chromosome 5 (5q-) is the most common cytogenetic abnormality found in MDS (10-15% of patients). It is associated with refractory anemia, thrombocytosis, hypolobulated megakaryocytes and low risk of evolving into AML.[16] Because deletion of 5q represents the major cytogenetic abnormality associated with MDS, it has been hypothesized that this region may contain an important MDS suppressor gene.[16,18] One such candidate is the *NPM* (Nucleophosmin) gene, located in the 5q region. Mutations of the NPM1 gene have also been found in 4.4% of MDS patients.[12] NPM is a nuclear phosphoprotein, which has a putative function in ribosome assembly and DNA repair. While *NPM1-/-* mice lack definitive hematopoiesis, *NPM1+/-* mice develop features similar to the human 5q-syndrome, such as dyserythropoiesis and dysmegakaryopoiesis that is sometimes associated with thrombocytosis,[13] albeit after a long latency period of 10-24 months.[14] The commonly deleted region (CDR) associated with the 5q-syndrome has been narrowed down to a region of approximately 1.5 Mb located between 5q31 and 5q32.

The CDR is divided into two regions of synteny on mouse chromosomes 11 and 18. Partial deletion of this region in mice results in macrocytic anemia, prominent erythroid dysplasia, and monolobulated megakaryocytes, and is associated with moderate thrombocytopenia and neutropenia.[17] The candidate genes included in this region are: *Cd74*, *RPS14, NDST1, SYNPO, MYOZ3, RBM22*, *DCTN4* and *NID67*. In humans, the candidate genes of the CDR include *FAT2* (FAT tumor suppressor homolog 2), SPARC (secreted protein, acidic and rich in cysteine), and *RPS14* (40S ribosomal protein S14). The *FAT2* candidate gene was evaluated *in vivo* but deficient mice failed to develop hematologic disease.[17] *RPS14* is a strong candidate as demonstrated *in vitro*, whereby silencing RPS14 expression by RNA interference in human BM cells reproduced a 5q-cell phenotype.[15] Interestingly, Diamond-Blackfan anemia is also associated with expression of the ribosomal protein RPS19[88] and transgenic mice expressing an inducible transgene of a mutant form of RPS19 (loss of function) develop anemia with dyserythropoiesis, reduced erythroid progenitors and a block in terminal erythroid maturation.[89] RPS14 is thought to be a tumor suppressor.[16]

SPARC is a component protein of the extracellular matrix. SPARC-null mice develop mild thrombocytopenia and exhibit an impaired ability to form BFU-E colonies.[18] SPARC is also a candidate tumor suppressor as methylation of the *SPARC* promoter has been observed in both lung and pancreatic cancers[90,91] and is often associated with breast cancer cell invasion.[92]

The human *APC* (adenomatous polyposis coli) gene is located on chromosome 5q22, in close proximity to the region that is deleted in 5q-syndrome. APC is a well-characterized tumor suppressor associated with colon cancer. *APC/min* mice (carrying a truncated, non-functional allele of *APC*) develop an MDS/MPN hematologic disease with macrocytic anemia, leukocytosis, monocytosis and splenomegaly.[19] The mice display altered stem cell function demonstrated by an impaired repopulating potential in secondary recipients.[19] However, *APC* haploinsufficiency is observed in 95% of MDS patients with 5q-syndrome and *APC*+/- mice develop a disease that recapitulates several characteristic features of human MDS.[20] Thus, hap-

Table 1. Gene alterations found in MDS patients and their corresponding mouse models with corresponding hematologic disease entities according to WHO classification 5.

Gene	Alterations found in MDS patients MDS disease entities (Frequency)	Alterations studied in mouse model	Type of mouse model	Mouse models disease entities according to WHO Classification 2008	Hematologic features described
Gene silencing and altered tumor suppressor genes					
NPM1	t (3,5) translocation NPM/MLF1 MDS, AML with MDS <1%[10,11]	NPM1	NPM1+/-	Some features of MDS[13] MDS/MPN like and Myeloid leukemia[14]	Dyserythropoiesis , Dysmegakariopoiesis[13] Leukocytosis with organs infiltration[14]
	NPM1 Mutations MDS/AML[12] (4.4%)				
RPS14	Haplo insufficiency 5q- syndrome[15,16]	LSCE** Cd74-Nid67region	KO	MDS with isolated del(5q) 5q- syndrome like[17]	Macrocytic Anemia , Mild thrombocytopenia and mild neutropenia, Dyserythropoiesis Dysmegakariopoiesis, Pro-apoptotic profile
SPARC	Haplo insufficiency 5q- syndrom[16]	SPARC	KO	MDS with isolated del(5q) Some features of 5q- syndrome[18]	Thrombocytopenia, No Dysplasia Impaired ability to form BFU-E
APC	Haplo insufficiency 5q- syndrome[16] (95%)	APC	Heterozygote KO	MDS/MPN CMML like[19,20]	Macrocytic anemia, Leukocytosis, Monocytosis, Splenomegaly
BAP1	Heterozygous frameshift mutation RMCD[21]	BAP-1	Conditional KO CreERT2	MDS/MPN CMML like[21]	Anemia, thrombocytopenia, leukocytosis with monocytosis, splenomegaly, dyserythropoiesis, dysmyelopoiesis
Altered stem cell and niche					
Dicer 1	No	Dicer	Tg Inducible Osterix	MDS RCMD like[22]	Neutropenia +/-Anemia +/- Thromcytopenia Dysgranulopoiesis Dysmegariopoiesis Pro-apoptotic profile, 2% AML transformation
Tet2	Mutations (loss of function) CMML 20%,[23] 22%[24] MDS 6%,[23] 19%[24] MPN 23%[25] MPD 12%[24]	Tet2	KO Tet2$^{LacZ/LacZ}$	MDS/MPN CMML like[26] (Incomplete penetrance)	Leukocytosis Monocytosis Anemia Thrombocytopenia Splenomegaly
			Conditional KO Mix1Cre		
			Conditional KO VavCre	MDS/MPN CMML like[27]	Leukocytosis Monocytosis Myeloid dysplasia, Splenomegaly
RAS signaling molecules					
NRAS	Point mutation MDS, CMML 20 to 40%[28-30]	NRASG12D	rT/BMT LTR- LK *	AML with maturation or MDS/MPN Atypical CML like[31]	Leukocytosis Monocytosis +/- high count of myeloblasts Pro-apoptotic profile of lymphocytes and myelomonocytic cells
		NRASG12D	rT/BMT LTR- MCSV*	AML (monocytic/monoblastic)[32] Or MDS/MPN CMML like	High BM blasts with monocytic/monoblastic differentiation Leukocytosis with Monocytosis Hepatosplenomegaly Thrombocytopenia Dysmyelopoeisis, dysplasia, propapoptotic profile BM blasts about 15% Thrombocytopenia Neutropenia, some dysplasia, anti-apoptotic profile, BM blasts >60%
		NRASG12D	Tg MRP8 promoter Cooperation with BCL-2	MDS[33] RAEB-1 like or AML post-MDS[33]	
		LSL-NRASG12D	Conditional KI Mix1Cre	MPD[34]	Leukocytosis, splenomegaly
		LSL-NRASG12D	Conditional KI Mix1Cre +MOL4070LTR	AML-like[34]	Myeloblasts M4/M5 subtype

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KRAS	Point mutation MDS,CMML, JMML 20 to 25%(62;70;71)	LSL-KRASG12D	Conditional KI Mix1Cre	MDS/MPN JMML or CMML like[35,36]	Anemia Dyserythropoiesis Leukocytosis Monocytosis
		KRASG12D	rT/BMT LTR-MCSV*	MDS/MPN CMML like[37]	Leukocytosis with Monocytosis Hepatosplenomegaly
FLT3	FLT3-ITD MDS, CMML 5%(40)	FLT3-ITD	KI Endogenous	MDS/MPN CMML like[38]	Leukocytosis Monocytosis Splenomegaly
PDGFRβ	Translocation t(5;12) CMML with eosinophilia Rare(95)	TEL-PDGFRβ	BMT LTR-MCSV*	MDS/MPN CMML like[40]	Leukocytosis with Neutrophilia and Monocytosis Hepatosplenomegaly
NF1	Loss and mutation Pediatric MDS/MPN 14%(84)	NF1	Inducible KO Mx1CreXNF1flox	MDS/MPN JMML like[42]	Leukocytosis Monocytosis Splenomegaly
PTPN11	Mutations JMML 34%(86)	Ptpn11D61Y	KI Endogenous	MDS/MPN JMML like[44]	Anemia, Leukocytosis Monocytosis, Splenomegaly Hypersensivity to GM-CSF
Nuclear and Transcription Factors					
EVI1 Evi1-MDS1	Inv(3), t(3,12), t(3;21) MDS, MDS/AML, t-MDS/AML (Rare)[45-47] Overexpression (Common)[48]	Evi1	rT/BMT LTR-MSCV* rT/BMT	MDS RCMD like[49]	Pancytopenia, Dyserythropoiesis Dysmegakariopoiesis, Pro-apoptotic profile
RUNX1/AML1	Mutations MDS subgroup MDS/AML (5 to 20%) (7) t-MDS/AML (40%)[50]	Mutant AML1 D171N	LTR-Retrovirus* (unspecified)	AML with MDS related changes[51]	Multilineage, Dysplasia, Leukocytosis, Hepatosplenomegaly, High blasts count in BM
		Mutant AML1 S971fs		MDS RAEB-2[51]	Multilineage, Dysplasia, Pancytopenia BM blasts<20%
NUP98	Translocations MDS, MDS/AML, t-MDS/AML 1 - 2 %[52]	NUP98-HOX	Tg VAV promoter	MDS RCMD like sometimes AML[53,54]	Thrombocytopenia, Anemia, Neutropenia, Multilineage dysplasia Pro-apoptotic profile
CBFβ	Translocation Inv 16 t-MDS/AML 1,5%[55]	CBFβ-MYH11	Conditional KI Mx1Cre promoter	AML[56]	AML with monocytic differentiation Multilineage differentiation impairment
MLL	Translocations t-MDS, t-MDS/AML 12%[57-59]	MLL-AF9	KI Endogenous promoter	AML[61]	No MDS features AML with Mac-1+/c-KIT+ phenotype
	Amplification MDS Mostly RAEB, t-MDS, AML with MDSrc <1%[60]	MLL-AF9	rT/BMT LTR-MSCV*	AML[62]	No MDS features AML with Mac-1+/c-KIT+ phenotype
		MLL-ENL	Conditional KI endogenous promoter	MPD[63]	Accelerated to AML with caffeine, an inhibitor of DNA damage response kinases ATM and ATR with increase in Mac-1/c-KIT/GR-1 phenotype
C/EBPα	Mutations MDS, CMML Rare[64,65] AML 5 to 14%[64,65]	C/EBPα mutant p30	KI Endogenous Promoter	AML[66]	No MDS features AML with Mac-1+/c-KIT+ phenotype
SALL4	Overexpression AML and MDS 60% (4B isoform)[67]	SALL4B isoform	Tg CMV promoter	MDS RCMD like[67]	Neutropenia, Multilineage dysplasia 50% AML transformation Pro-apoptotic profile in young mice

loinsufficiency is a mechanism of activating the oncogenic potential of a gene. However, other candidate genes located in the 5q-region either fail to confer hematopoietic disease or have not yet been studied.

Spliceosomal mutations

Recurrent mutations in a number of genes encoding core spliceosomal proteins have been identified in all subgroups of MDS. Heterozygous missense mutations in the *U2AF1* and *SF3B1* genes that encode spliceosome subunits have been described. *SF3B1* mutation is prevalent in both low-risk MDS with ringed sideroblasts and chronic lymphocytic leukemia (CLL) and is associated with a good prognosis. *U2AF1* and *SRSF2* are frequently mutated in myeloid hematopoietic malignancies, especially in CMML and advanced forms of MDS, and are associated with shorter survival.[93-96] *SF3B1* null homozygous mice are not viable and heterozygous mutant mice are healthy with

reduced levels of transcripts and protein exhibiting skeletal alterations with associated changes in ectopic homeobox transcription factor *HOX* gene expressions in the vertebrae of embryos, leading to the conclusion that SF3B1 mediates repression of *HOX* genes.[97] *HOX* gene abnormalities have been implicated in fusions giving rise to myeloid malignancies, as described below. GEP of MDS patients show *HOX* deregulation with high risk of transformation to AML[98] and that HOX upregulation is associated with poor prognosis AML,[99] although no study has yet correlated the deregulation of *HOX* genes with *SF3B1* mutations. However, the study in mouse[97] shows that SF3B1 spliceosomal protein is required to repress *HOX* genes, so there may well be a link between these two genes in the context of human disease, as predicted by the mouse model.

An epigenetic regulator belonging to the polycomb group of proteins, ASXL1, is involved in the activation and silencing of *HOX* genes and *ASXL1* mutations do occur in a proportion of MDS patients and are associated with poor prognosis.[100-102] However, the ASXL1-deficient mice have a mild phenotype with defects in differentiation of progenitors and do not develop MDS, possibly due to redundancy of pathways.[103]

These abnormalities and deregulation of target genes identify a ubiquitous pathway that undoubtedly contributes to malignancy.

Tumor suppressors

The p53 protein, a tumor suppressor involved in many types of cancer, has been reported to be mutated in up to 10% of MDS patients with high-risk subtypes.[28] Although *p53* KO mice are highly susceptible to develop AML after benzene exposure,[104] and mutant p53 KI mice are susceptible to develop cancers such as lymphoma, thymoma and sarcoma, no signs of MDS have been reported in association with p53 mutations in these animals. Many pathways converge to the p53 pathway leading to deregulation of apoptosis. Examples are the RPS14 and SPARC described above as potential candidates of the 5q- deletion have increased p53 expression.[17,91,105] Reports describing the increased expression of the tumor suppressor gene *p53* in 5q- syndrome patients, and the partial rescue of the phenotype observed when *Cd74-Nid67* deficient mice are crossed with *p53*-/- mice, provide further insight into the development of the disease. Upregulation of p53 has been observed in a subset of patients. RPS19 deficiency also activates p53 and, like RPS14 deficiency, is associated with dyserythropoiesis.[105]

Likewise, although lack of the *IRF-1* gene expression has been observed in 100% of MDS patients assessed in one study,[106,107] *IRF-1*-/- mice show no sign of MDS development despite impaired granulocytic differentiation and decreased expression of C/EBPα and C/EBPε in $CD11b^+$ cells.[108] This tends to suggest that these abnormalities are not sufficient by themselves to confer disease.

Mutations of the deubiquitinating enzyme BAP1 is found in hereditary cancer syndrome and predisposes to various malignancies, particularly mesothelioma and uveal melanoma. In mice, the *BAP1* knockout is lethal. However, when a conditional knockout is employed and deletion as an adult is induced, the mice develop features of human MDS.[21] KI mice expressing BAP1 interact with the polycomb proteins ASXL1. These are frequently mutated in MDS[101] and ASXL2. The authors postulate that a BAP1/ASXL1 complex may suppress the development of MDS. A heterozygous frameshift mutation of *BAP1* was identified in an MDS patient with refractory cytopenias and multi-lineage dysplasia (RCMD, WHO classification),[2] similar to the features observed in the mouse model. In GEP studies, *BAP1* expression was found to be reduced in $CD34^+$ cells from MDS patients compared to healthy controls,[96] consistent with *BAP1* being a tumor suppressor. In the mouse model, loss of only 1 copy of *BAP1* ($Bap1^{fl/+}$) was shown to have a mild phenotype but had progressive hematologic defects, as predicted by the heterozygous mutation found in the MDS patient. The identification of additional chromosomal deletions associated with MDS such as 7q- and 20q, suggest that a tumor suppressor gene may map to these regions. Mice with a 7q22 deletion have been created but no phenotype was found, and no cooperation was found with oncogenes. Interestingly, the death inducer-obliterator (*Dido*) gene, which maps to 20q, has been targeted in mice and when deleted gives rise to a transplantable hematologic disease with features consistent with MDS/MPN.[109]

Mouse models of activated signaling pathways in MDS, MDS/MPN and MDS/AML

Mutations of genes coding for proteins of the RAS signaling pathway have been reported to participate in the pathogenesis of MDS. The RAS pathway is complex and integrated within a network of pathways known to be implicated in cell proliferation, differentiation and apoptosis. Deregulation of the RAS pathway may result from genetic alterations of the genes coding for tyrosine kinase receptors or RAS/GTPase activating protein (GAP)-related proteins.

RAS mutations have been described in MDS and MDS/MPN patients correlating with prognosis in earlier studies;[28-30] mutations have been identified in up to 20% of patients with MDS. Point mutations interfere with the intrinsic activity of RAS and result in constitutive signaling and subsequent activation of downstream components. *NRAS* mutations are more frequent in myeloid malignancies than KRAS mutations, while *HRAS* mutations are rare. The most common *NRAS* mutations are found at residue G12 in the P-loop and at the catalytic residue Q61. The glycine to valine mutation at residue 12 renders the RAS GTPase domain insensitive to inactivation by GAP, which causes the protein to remain in an 'on' state, whereas the glutamine to lysine mutation at residue 61 reduces the rate of RAS-GTP hydrolysis. NRAS mutations are associated with a poor prognosis in MDS patients and an increased rate of transformation to AML.[28,30] Over the past ten years, mouse models have been established to study the effects of increased signaling through the RAS pathway. The results of a comparative analysis of the different models emphasize that the promoter determines the temporal and the spatial expression of the mutant gene as well as the strength of its expression. Activation of the RAS pathway may also be able to block differentiation[110] and induce AML, either when expressed at high levels, by activation of the pathways downstream of RAS or with co-expression of other co-operating genes.

RAS mutation mouse models of MDS/MPN

Several transplantation assays using retrovirally-infected BM cells from donor mice to express mutant NRASD12 in murine hematopoietic cells give rise to an MDS/MPN (CMML)-like,[32,37] and AML-like[32] diseases. Transgenic mice

expressing KRASG12D from its endogenous locus using an Mx1Cre system also develop a rapid and lethal MDS/MPN with leukocytosis, monocytosis, severe anemia and BM features of dyserythropoiesis resembling JMML and CMML.[35,36,111,112] Furthermore, mice expressing NRASG12D from its endogenous locus (the Mx1Cre Lox-STP-Lox (LSL)-NRASG12D mice) develop an indolent MPD with a long latency, and eventually die of MPD, MDS-like lymphoproliferation found concomitant with myeloid disease and histiocytic sarcoma. Together with the MOL4070LTR retrovirus, these mice develop AML,[34] stressing once again the requirement of genetic events working together for the onset of disease. Overexpression of EVI1 as a co-operating event for AML transformation was identified. A transgenic model expressing mutant NRASG12V via the VAV promoter[113] and rt/BMT models expressing either MRASQ71L (a recently identified RAS isoform, also known as RRAS) or HRASG12V develop mast cell MPN-like diseases including mastocytosis, mast cell leukemia and malignant histiocytosis.[114]

Mutated RAS mouse models of MDS and MDS/AML

Kogan *et al.* have described a transgenic mouse model expressing mutant NRASD12 under the control of the myeloid promoter MRP8 with a phenotype associated with dysplastic features in the myeloid BM.[115] Crossing NRASD12 and hBCL-2 transgenic mice yields two distinct models of MDS and AML with MDS-related changes depending on the promoter driving BCL-2 expression: when the expression of the MRP8-driven NRASD12 transgene is associated with the expression of the *hBCL-2* gene driven by the MMTV-LTR promoter, mice develop a disease resembling human MDS with excess BM blasts or MDS-like and increased apoptosis. However, when both transgenes, *NRAS* and *hBCL2*, are driven by the same MRP8 promoter, the mice develop a disease with characteristics of human AML, high BM blast counts, and persistence of MDS dysplastic features in myeloid cells, and an apoptosis resistance profile. Expanded LSK (Lin-/Sca-1$^+$/c-Kit$^+$) populations and increased hBCL-2 expression in the RAS-GTP complex within the expanded Sca-1$^+$ compartment are observed in both disease models. The diseases are transplantable, which underscores the view that these genetic alterations are functional at the stem cell level.[33]

In the MDS-like mouse model, most RAS and BCL-2 double-staining localizes to the plasma membrane where active pro-apoptotic RAS is normally located, whereas in the AML post-MDS disease, RAS and BCL-2 co-localize at the mitochondria, where anti-apoptotic BCL-2 is normally found. The co-localization of NRAS and BCL2 was also found in MDS patients.[33] The apoptotic profile of these NRASD12/hBCL-2 mice has been characterized by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) of liver sections showing increased TUNEL in the single mutant NRASD12 mice and the MMTVtTA/TBCL-2/NRASD12 mice.[33] Analysis of the AML post-MDS model shows a stepwise increase in double-strand breaks, increased misrepair and increased ROS production.[71]

Other RAS pathway mouse models of MPN/MDS

Approximately 30% of MDS/MPN JMML patients harbor an *NF1* gene mutation, and approximately 14% of JMML patients are also diagnosed with neurofibromatosis 1.[41] NF1 encodes neurofibromin, a tumor suppressor gene product that has a GAP function. Transgenic mice created using the inducible Mix1-cre system to express an inactive, mutant NF1 protein develop a progressive and fatal MDS/MPN with granulocytosis, monocytosis and splenomegaly, thus representing an appropriate model for JMML.[42] Likewise, *PTPN11* encodes the tyrosine phosphatase SHP2, which is frequently mutated in JMML patients (34%).[43] Chan *et al.* have developed an inducible KI mouse model that expresses the inactive PTPN11D61Y mutation, these mice die of a fatal myeloproliferative disease (MPD). The selective hypersensitivity of BM cells to GM-CSF, the hallmark of JMML, confirms the JMML model.[44]

Abnormalities of the RAS signaling pathways tend to suggest that these mouse models are master regulators and in some cases, depending on the context, can alone give rise to rapid disease.

Tyrosine kinases

Amongst the genes coding for tyrosine kinase receptors, the *FLT-3* Internal Tandem Duplication (ITD) mutation is found in 5% of MDS patients including CMML,[116] while mutation incidence is 33% for patients who progress to AML.[117] *RAS* mutations and *FLT3* ITD have rarely been found together, suggesting that their pathways are convergent or that only one of these pathways needs to be altered to give rise to disease. Various methods have been used to express *FLT3*-ITD in hematopoietic cells in animal models, which mainly develop an MPN phenotype.[118-120] In particular, two KI models closely resemble human CMML, exhibiting leukocytosis, monocytosis and splenomegaly.[38,121]

Translocations involving *TEL*, a member of the *ETS* transcription factor family, have been found to be associated with MDS and MDS/MPN. The fusion partners of *TEL* identified in association with MDS include *SYK, MDS2, CDX2* and *MN1*, while the expression of the TEL-PDGFR fusion protein has been shown to be associated with CMML. The translocation t(5;12) (TEL-PGFRβ) rarely occurs in MPN and MDS.[39] Murine transplantation of BM cells transfected with TEL-PDGFRβ results in the development of an MDS/MPN disease resembling CMML.[40]

MDS mouse models of altered transcription factor and nuclear factor expression in MDS/MPN

Unlike in AML, alterations in transcriptional factor activity resulting from chromosomal translocations are less frequent in MDS (13-16%).[122,123] Nevertheless, several transcription factor genes have been reported to be mutated or truncated in MDS, including *RUNX1 (AML1), CBFβ, C/EBPα, TEL, MLL, EVI1, RARα, P53, IRF-1*, and *SALL4*.[7] Both point mutations in the RUNT domain and truncation of the C-terminus domain of the *RUNX1* (*AML1*) gene are found in 15-40% of MDS patients with excess blasts.[7] Mice expressing the *RUNX1* (*AML1*) S291fs mutation develop MDS with excess blasts and erythroid dysplasia.[51] TEL has been discussed above.

Abnormalities of EVI1 (ectopic viral integration 1), such as Inv3, t(3;3), t(3;21), are found in up to 2% of MDS patients.[45-47] EVI1 is a nuclear transcription factor that is required for normal hematopoiesis; EVI1 deficient mice die as embryos and it has been shown to be essential for the maintenance of HSCs.[124-126] GATA-2, essential for proliferation of definitive HSCs, is one of the targets of EVI1 and is reduced in HSCs of EVI1 deficient mouse embryos. Expression of EVI1 is frequently increased in MDS[48] and

associated with poor prognosis in AML patients.[127] Transplantation of lineage-negative BM cells infected with the MSCV retrovirus containing the *EVI1* gene induces fatal MDS with pancytopenia, hypercellular BM, dyserythropoiesis and dysmegakaryopoiesis through molecular inhibition of GATA-1 by EVI1.[49,128] Transgenic mice with *EVI1* driven by the Sca1 promoter had impaired erythropoiesis[129] and increased susceptibility to development of myeloid leukemia upon retroviral infection of newborn mice with the Cas-Br-M MuLV.

Translocations involving NUP98 have been identified in both MDS and therapy-related MDS (t-MDS) patients.[52,130,131] Nucleoporins are molecules involved in the nuclear import and export of both proteins and RNA. NUP98 has been identified as a fusion partner of 19 various proteins classified into two groups: the homeobox genes (*HOXA9, HOXD13* and *PMX1*) and non-homeobox genes (*DDX10*, RNA helicase; and *TOP1*, human DNA topoisomerase 1).[52,132] *NUP98-HOXD13* fusions under the control of the VAV promoter develop fatal MDS within 14 months.[52-54,130,131] CBFβ-MYH11-mediated translocation (inversion 16) is not frequently found in MDS but is associated with t-MDS with eosinophilia.[55] *CBFβ-MYH11* transgenic mice expressing the fusion protein under the control of the MRP8 promoter have been reported to develop dysgranulopoiesis[115] whereas the CBF-MYH11 KI mouse model develops AML.[56] Chromosomal translocations involving MLL, such as t(11;16)(MLL-CBP)[58,59] and t(11;19),[57] as well as *MLL* gene tandem duplications (MLL-PTD),[7] have been described in MDS and t-MDS, although frequencies are low. However, none of the *MLL-AF9* mouse models, whether it be KI[61] or retroviral BM transduction/transplantation assay (rt/BMT) models, appear to develop MDS, and overt AML rapidly develops.[62,133] The conditional MLL-ENL mice develop MPD early (within 7 months) and later develop AML. This can be accelerated by treating with caffeine, an inhibitor of ATM and ATR kinases which are part of the fail-safe DNA damage response machinery,[63] thus, demonstrating the importance of GI in the development of disease. Likewise, KI mice with *CEBPα* mutations develop AML.[66]

SALL4 overexpression has also been detected in MDS.[67,134,135] Interestingly, transgenic mice expressing the SALL4B isoform under the control of the CMV promoter develop MDS-like features within 6-8 months, with dysplastic neutrophils, erythrocytes and megakaryocytes, as well as increased blast counts leading to AML transformation in 50% of mice.[67]

Growth factors

Myeloid differentiation-associated cytokine pathways have been implicated in MDS. Approximately 40% of patients with severe congenital neutropenia express truncated granulocyte colony-stimulating factor receptor (G-CSFR) protein, and those who develop secondary MDS typically harbor an acquired *G-CSFR* mutation.[136] Alterations in erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF signaling have been recapitulated in mice. Alone they do not induce MDS but instead confer maturation disturbances, proliferative advantage of an abnormal clone, and consequent effects on apoptosis.[137-139] The macrophage colony-stimulating factor receptor (M-CSFR) encoded by *FMS* is mutated in approximately 12% of MDS patients and 20% of CMML patients and is associated in some cases with a poor outcome.[140] CSF-1 deficient osteopetrotic Op/Op mice display a macrophage deficiency and monocytopenia as well as defective bone formation, but do not develop MDS.[141-143]

Conclusions

To engineer relevant animal models of MDS, we must ask what genetic alterations are important, how many genetic events are required, and in which stem cells should they be expressed for the development of a given MDS disease in patients. However, this review emphasizes that the expression or inactivation of one such candidate gene in mouse models can generate various disease profiles, MDS, MDS/MPN and AML, depending on the vector, the promoter or the mouse strain with a predominant description of the MDS/MPN or AML phenotypes. This suggests that, very often, the driver of the malignant clone is proliferation and/or that induction of dysmyelopoiesis with increased apoptosis is either rare or difficult to analyze. The Knudson 2-hit hypothesis of loss of one allele and mutation of the other in the case of p53 may not be appropriate for MDS as time is needed for disease to develop; the age of onset in the majority of cases is 65-75 years. This suggests that multiple steps may be required. It is also feasible that mutations in a gene that controls chromatin structure/methylation may hit several genes at once, and again such a mutation could initiate the disease but will still need other events for the disease to develop if it is to resemble the human condition. This has hampered the identification and establishment of true MDS mouse models. Nevertheless, this review shows that there are now a number of mouse models that may qualify as bona fide MDS models based on their pro-apoptotic features, and possibly more may qualify once their apoptotic status has been investigated. It is clear from the use of the emerging technologies of gene expression profiling, sequencing and epigenomics that the expression of one gene or transgene in human or mouse stem cells results from and in the deregulation of a number of networks and pathways. Increased genomic instability as a result of increased inflammation, irradiation, genotoxic stress, and mutagens, is one way of generating mutations, deletions, and translocations, and these alterations in turn enhance genomic instability. Thus mouse models may serve as tools that can be used to investigate the integrity of the genome and dissect the various altered pathways. The identification of the NRAS:BCL2 complex in MDS and MDS-AML patients from data observed in MDS and AML-post MDS mouse models is one such example.

Of the animal models for which apoptosis has been identified, the genes used include NRASD12 in co-operation with BCL-2,[33] transcription factors of the Class II type *EVI1*,[49] *Sall4B*,[67] genes important in ribosome biogenesis *NPM*,[13] *RPS14*,[17] NUP98-HOXD[13,53,54] and the microenvironment Dicer 1,[22] which appear to confer MDS-like diseases.

However, despite the power of mouse model-based biology, there is no getting away from the criticism that mice are not men. The ongoing development of immunodeficient mice that can be xenografted with human MDS BM hematopoietic and/or stromal cells offers a further level of understanding of MDS-genesis and a patient-tailored study that is not limited to one or few transgenes.

Finally, the potential of these mice as pre-clinical models is unique as they allow us to test the drugs required to tar-

get a specific pathway (apoptosis, proliferation, differentiation), a disease status (MDS, MDS/MPD or AML), and hopefully in the near future a diseased stem cell (MDS, AML or mesenchymal). Furthermore, mouse models that resemble MDS transformation to AML further highlight the possibility of discovering drugs that may either prevent transformation or allow us to reverse an AML to an MDS phenotype. Recent clinical data have stressed the survival advantage obtained with such therapeutic approaches.

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