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SPOTLIGHT REVIEW

Epigenetics of myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) are clonal diseases of the elderly characterized by chronic cytopenias, dysplasia and a variable risk of progression to acute myeloid leukemia (AML). Aberrant methylation of tumor-suppressor gene promoters has been established for many years and recently tracked to the most immature cells of MDS, suggesting that these alterations are drivers of MDS pathogenesis. In recent years, recurrent somatic mutations in genes encoding proteins involved in DNA methylation and demethylation and in covalent histone modifications have been reported in myeloid malignancies, including MDS. Whole-genome epigenetic profiles of MDS are also emerging. In parallel with these advances in the molecular pathogenesis of MDS, clinical trials have established hypomethylating agents (HMAs) as the mainstay of therapy in the advanced forms of the disease. In this review, we summarize the current understanding of the molecular machinery involved in epigenetic regulation, discuss how epigenetic alterations arise in MDS and contribute to its pathogenesis and then discuss the mode of action of HMAs in MDS.

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INTRODUCTION

The notion of 'epigenetics' refers to all the information transmitted through cell division that is not encoded in the DNA sequence. However, it is generally used in a restrictive sense as information governing transcription regulation not coded in the DNA sequence. In this sense, epigenetics corresponds to covalent modifications of DNA and histones that affect chromatin accessibility and gene expression. Of these processes, DNA methylation is the most extensively studied, but epigenetics also include alternative base modifications such as hydroxymethylation, as well as covalent histone modifications (including acetylation, methylation, and ubiquitinylation). Transcriptional regulation is increasingly viewed as an intricate process, coupled to DNA replication and repair, and to pre-messenger RNA splicing. Thus, epigenetic regulation not only involves the basic transcription machinery, transcription factors and covalent histone modifications but also a broad range of multiprotein chromatin-modifying complexes serving as writers, erasers or readers of the chromatin state, as well as microRNAs and long non-coding RNAs (lnRNAs).

Myelodysplastic syndromes (MDS) are a group of diseases of the elderly that initiates in a hematopoietic stem cell (HSC) and is characterized by clonal hematopoiesis with quantitatively and qualitatively abnormal myeloid differentiation, leading to chronic cytopenias and to a variable risk of progression to AML. Epigenetic processes have key roles in the differentiation and aging of normal HSC. Stemming from early studies showing epigenetic silencing of tumor-suppressor genes (TSG) in MDS,[1] the interest for studying the epigenetics of MDS, in particular DNA methylation, has been amplified by the recent availability of whole-genome profiling of epigenetic modifications in MDS,[2–5] the discovery of recurrent somatic mutations in genes encoding epigenetic regulators (summarized in Table 1) and the clinical activity of the 'hypomethylating agents' (HMAs) 5-azacytidine (azacitidine) and 5-azadeoxycytidine (decitabine) in MDS. Because of the limited number of animal models and human cell lines of MDS, most progresses have come from the study of primary patient samples.

In this Spotlight review, we summarize the current understanding of the molecular machinery involved in epigenetic regulation, discuss how epigenetic alterations arise in MDS and contribute to its pathogenesis and then discuss the mode of action of HMA in MDS.

ALTERATIONS IN DNA METHYLATION AND DEMETHYLATION PATHWAYS

DNA methylation is the transfer of a methyl group to a cytosine residue (5-methylcytosine (5mC)) within a CpG dinucleotide, catalyzed by DNA methyltransferases (DNMT). Most CpGs in the genome are methylated, especially in repeated regions, contributing to heterochromatin stability. Regions enriched in CpG ('CpG islands' (CGI)) are also found in the promoters of half of the genes, and the majority of those CGI are unmethylated. Promoter CGI methylation is associated with stable repression of gene expression, 'locking' gene silencing downstream of histone modifications through physical constraints (nucleosome compaction, blockage of transcription factor binding, and prevention of DNA melting) and by recruiting histone deacetylases (HDACs) and repressive histone modifications through methyl-CpG-binding domain proteins. Early studies on the DNA methylation relied on PCR methods assessing repeated elements (for example, LINE-1) as a reflection of 'global' heterochromatin methylation and candidate gene promoters. The 'methylome' of leukemic cells was described as globally hypomethylated, with aberrant hypermethylation of some gene promoters. This aberrant methylome was thought to result in increased genetic instability and silencing of certain TSG. The prototype of epigenetic deregulation in MDS is silencing of the p15/*INK4B* cell cycle regulator by aberrant promoter methylation, which is found in one-third of MDS, and is associated with disease progression.[1] Epigenetic silencing of p15 is likely to be a

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Table 1. Somatic mutations in genes encoding epigenetic regulators in MDS

Gene	*Frequency*	*Type*	*Epigenetic pathway*
IDH1/2	1–12%	Gain of function	DNA hydroxymethylation; histone demethylation
TET2	15–25%	Loss of function	DNA hydroxymethylation
ASXL1	10–20%	Loss of function	Histone modifications
DNMT3A	5–10%	Loss of function, DN?	DNA methylation
BCOR/L1	5–6%	Loss of function, DN?	Transcription coregulation
EZH2	3–7%	Loss of function?	Histone methylation
STAG2	6%	Hypomorphic?	Long-range interactions
RAD21	1%	Hypomorphic?	Long-range interactions
SMC3	1%	Hypomorphic?	Long-range interactions
EED	<1%	Loss of function?	Histone methylation
JARID2	<1%	Loss of function?	Histone methylation
SUZ12	<1%	Loss of function?	Histone methylation
BAP1	<1%	Loss of function	Histone deubiquitination
CTCF	<1%	Loss of function?	Long-range interactions; insulation
UTX	<1%	Loss of function?	Histone demethylation
SETBP1	2%	Gain of function?	Histone acetylation?

Abbreviations: DN, dominant negative; MDS, myelodysplastic syndromes.

driver event in MDS pathogenesis, as inactivation of p15 in mouse results in a MDS/MPN (myeloproliferative neoplasm) phenotype, with frequent progression to AML.[6] Aberrant methylation is found in MDS stem cells, further suggesting that some epigenetic alterations have a driver role in MDS pathogenesis,[7] though a majority may be passenger events. Average methylation levels increase during the progression from low-to-high risk MDS and AML,[3] and most studies on MDS methylation have been carried on high-risk patients or WHO-defined AML.

New methods based on next-generation sequencing or microarrays now allow the assessment of CpG in all gene promoters or even in the whole genome. Similar technologies can be combined to chromatin immunoprecipitation to assess histone modifications. These tools have revealed the extent of non-promoter CGI methylation: gene body methylation stimulates transcription elongation and splicing and thus active transcription. Methylation is also found in enhancers and around transcription factor binding sites.

The same technologies have led to a reappraisal of the cancer methylome, where aberrations predominate in fact in regions adjacent to CGI ('CGI shores'), possibly contributing to alternative promoter usage or to alternative splicing. These studies also revealed the profound inter-cellular heterogeneity of methylation levels between cancer cells, mirroring the clonal (genetic) heterogeneity of neoplasms. Available MDS methylomes focus on promoter methylation and point to global deregulation of DNA methylation in MDS.[2,3,5] Of note, age matching of controls could not always be performed, making age-related methylation changes a potential bias of those studies.

DNA methylation is performed by three different DNMT during replication: *de novo* methylation by DNTM3A and DNMT3B and maintenance of methylation patterns by DNMT1. Loss-of-function or dominant-negative mutations impairing the enzyme's processivity have been reported in *DNMT3A*, but not *DNMT3B*, in 2–8% of MDS.[8]

The mechanisms of DNA demethylation have recently been uncovered, fostered by the discovery of recurrent mutations in the *TET2* gene in various myeloid malignancies, including MDS (reviewed in Solary *et al.*[9]; Figure 1). TET2, together with other TET enzymes not significantly expressed in hematopoietic cells, is a Fe^{++} and 2-oxoglutarate-dependent dioxygenase that catalyzes the conversion of 5mC to 5-hydroxymethylcytosine (5hmC). TET enzymes can also further convert 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 2-Oxoglutarate is generated

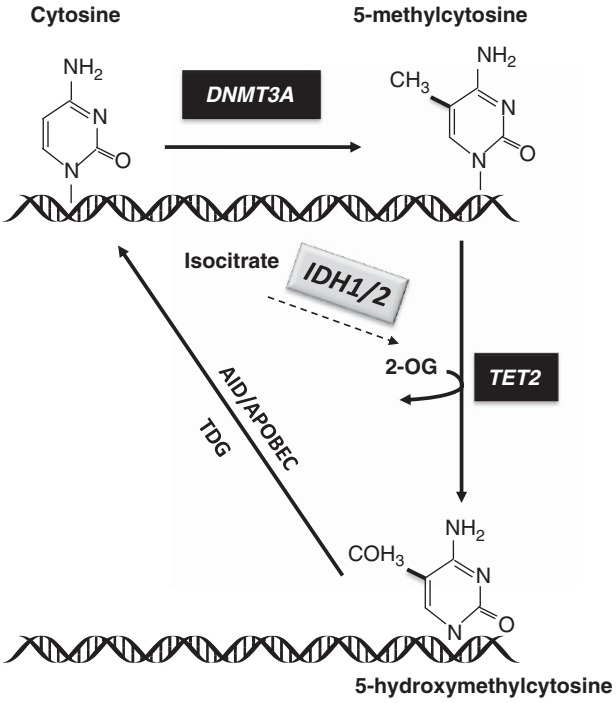

Figure 1. DNA methylation, hydroxymethylation and demethylation in MDS. Arrows depict the normal function of the enzymes and boxes the consequences of mutations found in MDS, with loss-of-function and gain-of-function mutations indicated in black and grey boxes, respectively. AID/APOBEC, activation-induced deaminase/APOlipoprotein B editing complex; 2-OG, 2-oxoglutarate (also called alpha-ketoglutarate); TDG, thymine DNA glycosylase. Details reviewed in Solary *et al.*[9]

by isocitrate dehydrogenases IDH1 and IDH2.[10] 5hmC represents 1% of total 5mC in differentiated cells but 5–10% in stem cells, while 5fC and 5caC are present in much smaller amounts. 5hmC is an intermediate species leading to active and passive DNA demethylation: 5hmC is not recognized by DNMT1, leading to passive demethylation; 5hmC also leads to active demethylation through several molecular pathways involving the base excision repair machinery. Whereas 5fC and 5caC are short-lived demethylation intermediates, the relatively long half-life of 5hmC suggests that, beyond its implication in demethylation, it could also be a distinct epigenetic mark with specific readers.[11] 5hmC is present mostly at promoter CGI, where it is associated with active gene expression, as well as in enhancers, especially around transcription factor binding sites. Though TET2 may be able to bind DNA through its cystein-rich domain, TET2 lacks the CXXC DNA-binding domain of other TET enzymes and is instead recruited by the protein IDAX, which also triggers its degradation by caspases, therefore contributing to its regulation.

Heterozygous or homozygous loss-of-function *TET2* mutations are found in up to 35% of MDS and lead to reduced 5hmC contents.[12] Besides *TET2* mutations, other mechanisms lead to 5hmC depletion.[12] Substitutions in *IDH2* (or very rarely in *IDH1*) have been identified in a subset of these 5hmC-low cases, and *IDH1/2* mutations are found in 5% of total MDS cases.[13] These mutations generate neomorphic enzymes catalyzing the reduction of isocitrate to α-2-hydroxyglutarate, thereby inhibiting the function of 2-OG-dependent genes, including *TET2*.[10] Overexpression of mir-22 has also been shown to downregulate TET2 expression.[14] Most of the studies on TET enzymes and hydroxymethylcytosine have been performed in embryonic tissue. Research on their role in normal and myelodysplastic hematopoiesis is ongoing.

DEREGULATION OF HISTONE MODIFICATIONS

A variety of covalent modifications can be added or removed from histones, resulting in a combinatorial 'histone code' (Figure 2). Acetylation, regulated by histone acetyltransferases and HDACs, changes the charge of the histone tail, resulting in a relaxed (acetylated) or compact (deacetylated) chromatin architecture, respectively, promoting and repressing gene expression. Acetylated histones can also recruit bromodomain-containing proteins. Histone lysine (K) or arginine (R) methylation (mono-methylation, dimethylation or trimethylation) does not affect nucleosomal compaction but serves as binding sites for a broad range of readers. Compared with DNA methylation, histone modifications are much more dynamic. Whether they are autonomously transmitted during cell division and thus represent true 'epigenetic' information remains unclear.

Trimethylation of lysine 4 (H3K4me3) and of lysine 27 (H3K27me3) of histone H3 are prototypical 'active' and 'inactive' histone modifications, respectively. Mapping of these marks in MDS cells is only beginning.[4] Trimethylation of H3K27 is catalyzed by the multiprotein 'Polycomb repressing complex 2' (PRC2). The PRC2 is recruited at gene promoters by transcription factors (for example, RUNX1) or directly bind promoters containing Polycomb Repressive Elements, such as the *HOX* gene cluster, which is crucial to hematopoiesis and leukemogenesis.[15] The PRC2 has a key role in MDS pathogenesis, as loss-of-function mutations in *EZH2* (Enhancer of Zest homolog 2), which encodes its catalytic subunit, are observed in ~5% of MDS.[16] Rare mutations in *EED* (embryonic ectoderm development), *JARID2* (jumonji, AT-rich interactive domain 2) and *SUZ12* (suppressor of zeste 2), which encode other PRC2 subunits, have also been reported in MDS and MPN.[17–19] Inactivating mutations in *UTX*, which encodes KDM6A, a H3K27me3 demethylase, have also been identified in up to 9% of chronic myelomonocytic leukemia patients but not in other subtypes of MDS.[20] Though the finding of inactivating mutations in enzymes with seemingly opposite enzymatic function may seem paradoxical, UTX/KDM6A has been found to convert H3K27me3 to H3K27me1 rather than to unmethylated H3K27. H3K27me1 is also a repressive, stable, histone mark.

PRC2-mediated gene silencing has been attributed to recruitment of other chromatin-modifying complexes, such as the 'Polycomb repressing complex 1' (PRC1), which mediates H2AK119 mono-ubiquitination, and of DNTM3A/B,[21] resulting in higher-order chromatin compaction. Though fine-tuning of PRC1 activity has been involved in the balance between self-renewal and differentiation of HSCs,[22] there is so far limited evidence of PRC1 deregulation in MDS.[23] The evidence supporting a role for the Polycomb Repressive Deubiquitinase (PR-DUB) complex in MDS is more compelling.[24] The PR-DUB complex, including the BAP1 deubiquitinase and the ASXL1 DNA-binding subunit, opposes PRC1 action through H2AK119 deubiquitination. Recurrent inactivating mutations in *ASXL1* (Additional sex combs-like 1) have been reported in 15–25% of MDS and carry a poor prognosis.[25] Though recurrent *BAP1* mutations have yet to be reported, inactivation of *Bap1* generates a MDS/MPN phenotype in mice.[24] In fact, the important tumor-suppressor role of *ASXL1* in MDS has been reported to be independent of the PR-DUB complex: ASXL1 may also recruit PRC2 to inactive expression of the leukemogenic *HOXA* cluster.[26]

CONNECTIONS BETWEEN DNA METHYLATION AND HISTONE MODIFICATIONS

Mutations in DNA methylation regulators (*TET2*, *DNMT3A*) and histone regulators (*ASXL1*, *EZH2*) are not mutually exclusive in MDS, suggesting that DNA methylation and histone modifications must be envisioned as intricate and dynamic processes regulating gene expression and genomic stability in combination with transcription factors, such as *RUNX1* (which is also mutated in 10% of MDS). Recent progress in the molecular biology of normal and myelodysplastic cells has added layers of complexity to this picture (Figure 3).

First, the same chromatin modification can have dual functions: 5hmC is a mark of active transcription in gene bodies and a repressive one, possibly through PRC2 recruitment, in gene promoters. The connection between TET2 and the PRC2 has since been confirmed by several studies.[14,27–31]

Next, epigenetic regulators mutated in MDS can also recruit other epigenetic regulators, independently of their enzymatic activity. TET2,[32] but also BAP1,[24] can recruit the glycosylase O-GlcNAc transferase, which post-translationally regulates several

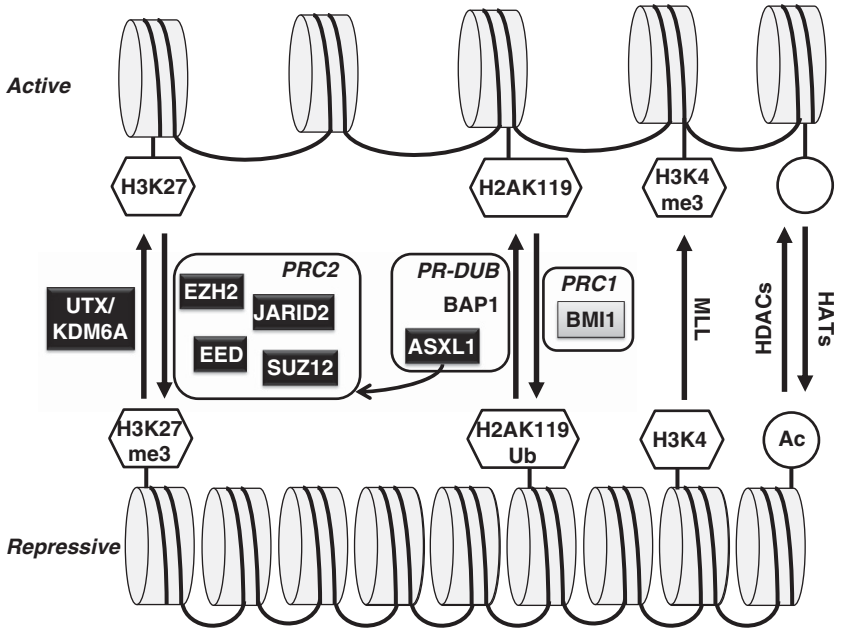

Figure 2. Histone modifications in MDS. Loss-of-function mutations and overexpression are indicated in black and grey boxes, respectively. MLL, mixed-lineage leukemia.

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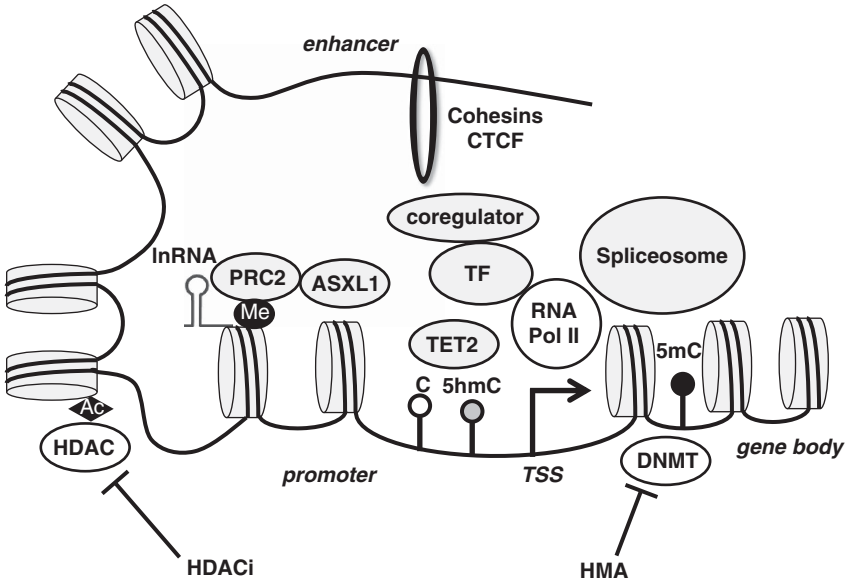

Figure 3. Epigenetic deregulation in MDS. Proteins or complexes targeted by somatic gene mutations are indicated in grey. TSS, transcription start site.

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proteins, including epigenetic regulators. IDH1/2 oncoproteins not only inhibit TET dioxygenases but also jumonji-domain histone demethylases[33] and prolyl hydroxylases involved in response to hypoxia, the latter having a critical role in IDH1/2 leukemogenesis.[34]

NEWLY IDENTIFIED ALTERATIONS IN EPIGENETIC REGULATORS

Cohesins and CTCF

Recently, somatic mutations in genes encoding for components of the cohesin complex (STAG21, SMC1A, SMC3 and RAD21) or its partner CTCF have been reported in myeloid malignancies, including 8% of MDS.[35] These heterozygous mutations are mutually exclusive and presumably lead to a reduced, but not abolished, activity of the cohesin complex. This multimeric ring-shaped complex is involved in sister chromatid cohesion during cell division. However, this function is preserved in cohesin-mutated MDS cases, which generally have a normal diploid karyotype. The cohesin complex and its partner CTCF also contribute to long-range chromatin interactions, such as chromatin looping allowing interaction between promoters and enhancers. It is therefore possible that cohesin mutations induce transcriptional deregulation by perturbing these long-range interactions, for instance at the *RUNX1* locus.[36] CTCF also establishes boundaries defining repressive heterochromatin regions ('insulation').[37] Defective insulation has recently been involved in abnormal methylation patterns in lymphoid malignancies.[38] Very rare somatic mutations in the *ATRX* SWI/SNF chromatin remodeler critical for α-globin expression have also been reported in MDS with acquired alpha thalassemia.[39] Finally, recurrent mutations in the related *BCOR* and *BCORL1* transcriptional co-repressors have recently been reported in 5–6% of MDS cases.[40]

Non-coding RNAs

The key role of miRNAs in the pathogenesis of MDS has been demonstrated by their involvement in the megakaryocytic phenotype of the 5q- syndrome (reviewed in Rhyasen and Starczynowski[41]). The relationship between miRNAs and components of the epigenetic machinery is twofold. First, miRNAs can be deregulated by aberrant epigenetic silencing, independent of recurrent chromosomal deletions. Conversely, miRNAs can regulate the epigenetic machinery: for example, overexpression of miR-29b in AML induces downregulation of DNMT3s and thus hypomethylation.[42] Impaired synthesis of miRNAs by MDS mesenchymal stromal cells could also contribute to MDS pathogenesis.[43,44] LnRNAs have also been recently linked to MDS. Xist, the best studied lnRNA, has a critical role in X chromosome inactivation. Targeted inactivation of Xist in female hematopoietic cells leads to an aggressive MDS/MPN phenotype caused by coordinated derepression of several X-chromosomal genes.[45] This model thus represents the prototype of how alteration of a single epigenetic regulator can suffice to cause a myeloid malignancy, through coordinated deregulation of several key target genes. Other lnRNAs with potential relevance to MDS include the HOTAIR family that directly regulates *HOX* gene expression through recruitment of PRC2.[46] Finally, other classes of non-coding RNAs may in the future be involved in MDS. For instance, antisense RNAs are naturally expressed at many loci, including TSG, and can initiate epigenetic silencing.[47] This process could contribute to the aberrant methylation of p15/INK4B in MDS. Interestingly, expression of antisense RNAs at TSG loci have been reported to be regulated by the CTCF/cohesin complex.[48]

Splicing

Recurrent, mutually exclusive mutations affecting genes encoding components of the spliceosome (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2* and so on) have been reported in myeloid malignancies and appear more specific of MDS than the aforementioned mutation classes.[49] Whether splicing variants are maintained during cell division, and thus represent *bona fide* 'epigenetic' information, remains unknown. Splicing is essentially coupled to transcription, and there is increasing evidence that the splicing machinery and epigenetic regulators closely interact to regulate gene expression, DNA repair and alternative splicing.[50] The chromatin state can regulate splicing in two ways. First, the rate of RNA Pol II elongation, which is dependent on the degree of chromatin compaction, influences alternative splicing patterns: gene body methylation stimulates transcription elongation splicing. Specific chromatin modifications can also directly interact with the

spliceosome machinery. Conversely, aberrant splicing induced by spliceosome gene mutations could also contribute to epigenetic deregulation: for instance, some isoforms of DNMT3B lead to increased variability of methylation profiles.[51]

WHAT IS THE ORIGIN OF EPIGENETIC ABERRATIONS IN MDS?

A simplistic view would assume that epigenetic profiles of MDS result from the combination of gene mutations in epigenetic regulators or in oncogenes that indirectly control epigenetic regulators. For instance, $JAK2^{V617F}$, present in ~5% of MDS, directly downregulates the arginine methyltransferase PRMT5, an epigenetic writer that contributes to the erythroid phenotype of $JAK2^{V617F}$ mutations.[52] Evidence supporting such a 'deterministic' model come from AML[10] and are supported by the direct link between *ASXL1* mutations and deregulation of Hox genes, in particular upregulation of leukemogenic *HOXA* genes, such as *HOXA9*.[26] Conversely, no recurrent methylation or gene expression profile has been associated to *DNMT3A* mutations.[53] The key target genes of TET2 in adult hematopoiesis and leukemogenesis remain elusive.[14,27–31] Candidates most relevant to myeloid oncogenesis include the retinoic acid receptor pathway[30] and the *HOXA* cluster: contrary to *ASXL1* mutations, *TET2* knockdown has been associated with downregulation of proximal *HOXA* genes (*HOXA3*, *HOXA4* and *HOXA5*).[31]

Studies comparing epigenomes and transcriptomes have yielded inconclusive results so far, possibly because of methodological issues or of their dependence on cellular context. These mutations may also induce subtle changes in several unconnected target genes and be thus uncaptured by traditional analyses focusing on the strongest deregulations or 'pathway' analysis. They could also lead to transient effects, compensated later during leukemogenesis. Finally, these mutations may behave as 'epigenetic mutators', enhancing the intraclonal epigenetic variability of cancer cells.[54]

Another model, which is not exclusive from the previous 'deterministic' one, has been proposed to account for the aberrant epigenomes of MDS and other cancers. This 'stochastic' model postulates that epigenetic changes are randomly acquired and then selected for in a Darwinian fashion, leading to phenotypic diversification of otherwise genetically identical populations. Such an 'epigenetic instability' has been noted in hematopoietic or cancer cells *in vitro*. The intrinsic 'infidelity' of epigenetic information makes this hypothesis plausible: the error rate in maintenance of DNA methylation is orders of magnitude higher than DNA replication fidelity.[54] Compared with genetic mutations, such an epigenetic flexibility could provide enhanced adaptability, a key feature for cancer cells. Murine and human HSC accumulate epigenetic aberrations with aging, especially in polycomb-repressed genes, leading to deregulated gene expression of key pathways, such as stress response and loss of self-renewal potential.[55–57] This observation raises the intriguing possibility that mutations affecting the epigenetic status of polycomb-repressed genes (TET2, ASXL1, EZH2 and so on) may confer a competitive advantage to HSC by limiting this age-related methylation-mediated loss of fitness. This scenario, dubbed 'adaptive oncogenesis', would explain the high prevalence of mutations in those genes in older patients but not in children. Similar findings have been made with respect to p15 methylation.[58]

Not only can aberrant methylation spread in the MDS clone through Darwinian selection but also positive feedback loops elicited by epigenetic alterations can contribute to their fixation. For instance, innate immune signaling triggers the nuclear factor-κB-dependent expression of JMJD3, a histone demethylase that removes repressive H3K27me3 marks, and JMJD3 in turn upregulates the expression of several genes involved in innate immune signaling.[4,59] The mild chronic inflammatory state of aging could also trigger this mechanism.[60]

The functional result of aberrant promoter methylation can vary. Biallelic promoter methylation results in complete silencing of TSG, such as p15/INK4B. Methylation can also be heterozygous, and result in haplo-insufficiency, which can be sufficient for tightly regulated TSG to be oncogenic.[19] Hemizygous methylation can also complement an interstitial deletion occurring on the other allele, resulting in complete loss of function according to the classic 'two-hit' model. This has been proposed for *FZD9*, located in 7q, and *CTNNA1*, located in 5q, both genes encoding proteins of the Wnt signaling pathway.[3,61]

Finally, another model implicating DNA methylation as an oncogenic trigger has recently emerged. This model involves gene imprinting, one of the best studied systems of physiological DNA methylation. Imprinting corresponds to the programmed methylation of the maternal allele of a gene, resulting in monoallelic expression. It has recently been shown that del 20q, one of the most frequent interstitial deletions in MDS and MPN, preferentially targets the paternal chromosome, resulting in complete loss of function of two imprinted TSG, *L3MBTL1* and *SGK2*. These two genes are themselves epigenetic regulators whose coordinated repression is required for del 20q oncogenesis.[62] Further work is required to study the role of imprinting in myeloid leukemogenesis: for instance, the H19-IGF2 locus, one the best studied imprinted locus has recently been involved in HSC self-renewal,[63] and CTCF, the cohesin-complex partner, has been shown to regulate imprinting at this locus.[37]

CAN ABERRANT EPIGENETIC PROFILES CONTRIBUTE TO THE INCREASED SELF-RENEWAL OF MDS-INITIATING CELLS?

As most mutations in epigenetic regulators are common to all myeloid malignancies, it can be postulated that they mainly contribute to the common feature of those malignancies, namely enhanced self-renewal of leukemia-initiating cells, which in MDS have been shown to be HSC.[7] This assumption is confirmed by murine models. Knockdown of *Dnmt3a* leads to a massive expansion of HSCs with only subtle differentiation defects and no overt leukemia.[64] Abrogation of *Tet2* leads to enhanced HSC self-renewal and triggers an age-dependent chronic myelomonocytic leukemia phenotype (reviewed in Solary *et al.*[9]). Inactivation of the PRC1 component *Bmi1* results in HSC exhaustion through p16-mediated senescence, whereas the role of PRC2 components, especially *Ezh2*, in adult HSC self-renewal is unclear, perhaps because of functional redundancy. *Ezh2* functionality has even been shown to be required for maximal leukemogenicity in some models.[65] Initial reports suggested that HSC from *Asxl1* knockout mice had normal self-renewal, perhaps because of compensation early in life.[66] Preliminary results from a conditional inactivation model suggested that abrogation of *Asxl1* was deleterious for HSC self-renewal.[67] These findings may seem in contrast with the report that downregulation of *Asxl1* by an shRNA cooperates with oncogenic *Nras* mutations in myeloid leukemogenesis.[26] These seemingly contradictory findings may reflect the fact that *ASXL1* mutations are only oncogenic in cooperation with other lesions.[68]

Several models have been proposed to account for epigenetic deregulation of HSC self-renewal. First, epigenetic modifications may directly regulate genes of the cell cycle and survival machinery. For instance, Bmi1 directly downregulates a p16/INK4A-mediated HSC senescence program. Abrogation of p15 is also sufficient to trigger MDS/MPN in mice.[6] Second, epigenetic modifications may deregulate master regulators of the HSC self-renewal program, such as the *HOX* gene cluster,[15] as suggested for ASXL1 and TET2.[26,31] Evidence supporting an oncogenic role for *HOX* genes overexpression in MDS mainly comes from the NUP98-HOXD13 mouse model, which faithfully recapitulates many traits of MDS pathogenesis.

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Epigenetic deregulation can also target signaling pathways involved in self-renewal, such as the Wnt pathway. Aberrant methylation of Wnt pathway genes has been repeatedly reported in MDS[2,3] and is corroborated by deregulation of gene expression, especially during progression of MDS to AML.[69] Methylation of both activators and repressors of the Wnt pathway have been reported, but recent studies have reconciled these conflicting data by showing the key role of fine tuning of Wnt signaling in HSC self-renewal and myeloid differentiation.[70] Indeed, mice harboring a hypomorphic *Apc* allele, a key negative regulator of the canonical Wnt–β-catenin pathway, have a MDS/MPN phenotype.[71] Also of interest is the finding that IDAX, the TET2 DNA-binding partner, is a known regulator of Wnt signaling.[72]

CAN ABERRANT EPIGENETIC PROFILES CONTRIBUTE TO THE DIFFERENTIATION DEFECT OF MDS?

Both DNA methylation and histone modifications are dynamically regulated during hematopoietic differentiation.[56] In particular, polycomb complexes are at the crossroads of self-renewal and differentiation of HSC. Key developmental genes are characterized by bivalent chromatin domains, enriched in both active H3K4me3 and repressive H3K27me3 marks. These genes are primed for prompt expression upon induction of differentiation. Repressive marks prevent HSC exhaustion by inhibiting expression of these 'poised' differentiation genes. TET activity also seems preponderant at these promoters.[73] It is therefore likely that impairment of polycomb modifications or of hydroxymethylation will result in concomitant deregulation of HSC self-renewal and differentiation, therefore recapitulating the key features of MDS pathogenesis. For instance, mice expressing a hypomorphic *Dntm1* allele have impaired self-renewal and skewed myeloid differentiation,[74] and loss of Ezh2 function impairs lymphoid commitment.[22] The epigenetic status of one of these 'poised genes', the *PU.1* transcription factor that regulates myeloid commitment, has been studied in detail in MDS. Aberrant H3K27me3 marks have been shown to prevent its expression in RCMD, impairing myeloid differentiation.[75] Silencing of *ASXL1* has also been reported to impair myeloid differentiation *in vitro*.[76] Methylation of GATA1, the master regulator of erythropoiesis has also been proposed to contribute to MDS dyserythropoiesis.[77] Ribosomal stress triggered by RPS14 haploinsufficiency has recently been involved in the dyserythropoiesis of 5q- syndromes. A similar mechanism, this time resulting from aberrant methylation of ribosomal RNAs, could also be at work in non-del 5q MDS.[78] Finally, a newly discovered mode of epigenetic regulation may contribute to abnormal differentiation in MDS: nonsense-mediated decay is a pathway involved in elimination of mis-spliced transcripts. This pathway has now been shown to regulate gene expression in terminal granulocytic differentiation,[79] raising the intriguing possibility that MDS-specific spliceosome mutations may impair myeloid differentiation by perturbing physiological mis-splicing.

CAN EPIGENETIC MODIFICATIONS CONTRIBUTE TO THE PROGRESSION OF MDS TO AML?

Accumulation of abnormal methylation is a dynamic process in MDS, with increasing levels of methylation associated with progression to RAEB and AML.[3] This observation could be the mere result of accumulation of HSC cell cycles. Alternatively, it is likely that aberrant methylation actively contributes to MDS progression, notably by enhancing the stemness of leukemia-initiating cells as previously discussed. Accumulation of epigenetic aberrations also influences genetic stability: methylation has a key role for heterochromatin stability by repressing translocations induced by transcription of endogenous transposons and avoiding guanine quadruplexes-induced DNA breaks. Gene-body methylation may also be mutagenic through deamination of methylcytosines. Epigenetic instability, a newly recognized hallmark of cancer cells,[54] may also contribute to the oligoclonality of MDS, a key feature of progression.[80] Recent progress in single-cell methylation profiling may help uncover its role in MDS.[81] Finally, treatment with HMAs delays AML transformation in MDS patients, definitively establishing a link between aberrant methylation and MDS progression.[82]

WHAT IS THE MOLECULAR AND CELLULAR BASIS OF 'HMAS' ACTIVITY IN MDS?

Paralleling these important breakthroughs in the molecular biology of MDS, clinical research has highlighted the role of HMA in the treatment of MDS.[82] Azacitidine and decitabine are both cytosine analogs with unmodified sugar moiety, thereby lacking the replication fork-stalling effect of cytarabine or gemcitabine upon incorporation into DNA. DNA incorporation is cell cycle dependent, and prolonged exposure to even very low doses of HMA trigger a transient hypomethylation by trapping and depleting the DNMT1 pool. The canonical view of HMA's mode of action is that DNA hypomethylation allows re-expression of TSGs triggering the death or differentiation of MDS cells. However, definitive evidence supporting this model, especially *in vivo*, is still lacking. A number of alternative mechanisms have instead been suggested, but most studies exploring HMA pharmacodynamics have been carried out at high doses and/or with limited drug exposure on AML cell lines rather than MDS primary cells and must therefore be interpreted with caution. High doses of HMA are cytotoxic, possibly because stalled DNMT1s generate bulky adducts and trigger the DNA damage response pathway.[83]

HMA induce genome-wide demethylation, without region specificity, preferentially affecting the most heavily methylated genes.[84] There is limited correlation between demethylation and re-expression patterns, because additional repressive modifications prevent chromatin reopening.[85] Comparison of clinical trials,[82] as well as gene expression studies,[86,87] have stressed the possible differences between azacitidine and decitabine. Decitabine is completely incorporated into DNA, whereas only 20% of azacitidine is incorporated into DNA, after conversion to 5-aza-2′-deoxycytidine-5′-triphosphate by ribonucleotide reductase.[87] The remaining 80% is incorporated in RNA. The consequences of the latter are only beginning to be understood: it leads to alterations in protein synthesis, possibly through t-RNA depletion. One notable target of this effect are the ribonucleotide reductase enzymes themselves, thus not only generating further cellular stress through depletion of nucleotide pools, but also modulating azacitidine DNA incorporation.[88] Recently available technologies will allow the precise mapping of HMA incorporation into RNA.[89]

The cellular consequences of HMA hypomethyation on the MDS clone also remain elusive. Clinical evidence supporting significant clonal suppression by HMA is scarce. First, the CR rate is limited to 15–20%, and CR achievement has been repeatedly shown not to be required for prolonged survival with HMA.[90] Cytogenetic responses, observed in one-third of the patients, also do not correlate with prolonged survival.[91] In addition, the majority of patients have normal karyotype and are thus not informative for cytogenetic response, but several studies have shown that HMA fail to eradicate immunophenotypically defined leukemia-initiating cells.[7,92,93] Conversely, elegant *in vitro* and *in vivo* studies on AML cell lines have shown that prolonged treatment with HMA at non-cytotoxic, 'hypomethylating' doses inhibits the self-renewal of leukemic cells,[94] in keeping with the clinical observation that HMA delay progression to AML. Interestingly, the H19-Igf2 imprinted locus has recently been shown to be strongly demethylated by decitabine in AML cells,[84] and this locus seems to contribute to the hematopoietic self-renewal program.[63]

Conversely, HMA have been shown to increase the self-renewal of normal HSC.[95] Further work on primary cells is required to confirm these key observations and elucidate the molecular mechanisms of such a differential activity. A working hypothesis is that AML leukemia-initiating cells arise from differentiated progenitors[96] and are thus susceptible to induction of differentiation by HMA.[97,98] Whether this hypothesis holds true in MDS, where leukemia-initiating cells are presumably *bona fide* HSC[7] is currently unknown. *In vitro* experiments suggested that HMA may allow differentiation of the MDS clone through derepression of PU.1.[99] Treatment of MDS with HMA allows improvement of cytopenias, and this contributes to their survival benefit.[82] Though it is likely that in many cases such improvement is non-clonal,[100] elucidating the clonal origin of such 'hematological improvements' is a necessary first step before detailed understanding of the epigenetics at work in this restored or enhanced hematopoietic differentiation. Among the non-clonal effects of HMA, several reports have stressed their immunomodulatory properties in MDS. HMA can induce the re-expression of cancer antigens, inducing cytotoxic T-cell responses.[101] FoxP3, the master regulator of Tregs, is silenced by methylation in normal CD4+ cells, and treatment with HMA leads to phenotypic Treg expansion,[102–104] though the functional properties of these HMA-induced Tregs remains unclear.[105] HMA may also modulate other immune subsets.[106,107] These properties may be of particular relevance in the post-transplant setting, by modulating graft-versus-host and graft-versus-leukemia effects.

Mirroring the limited understanding of HMA mode of action, biomarkers of HMA activity are currently lacking. Preliminary works suggested that mutations in epigenetic regulators such as *TET2*[108–110] or *DNTM3A*[110,111] were associated with improved response rates to HMA, though not conferring a significant survival benefit. These observations warrant validation on larger, homogeneously and prospectively treated cohorts and looking at gene combinations. A number of studies have proposed candidate gene promoter baseline methylation,[109,112–115] miRNA[116] expression or surface protein expression[117] as biomarkers of HMA activity. Those studies relied on bisulfate pyrosequencing or methylation-specific PCR, and none has received independent validation.[118] Biomarkers of HMA metabolism have also been proposed.[119] Whether whole-genome expression and/or methylation profiling can define a signature predicting response to HMA has yet to be investigated, especially with next-generation sequencing technology. These studies should be performed on prospective cohorts uniformly treated, as different HMAs and different regimens may affect epigenetic profiles.

Rational strategies have been proposed to optimize the clinical efficacy of HMA. The bioavailability of oral azacitidine shows significant inter-individual variability, but this convenient route could allow prolonged HMA exposure. Current 5–7 day per month subcutaneous schedules of AZA or DAC in fact lead to potentially deleterious remethylation at the end of each cycle.[82] Combination therapy with synergistic action on DNMT depletion has been proposed, based on the inhibiting potential of mir-29b on DNMT expression.[116] Bortezomib[120] and HDAC inhibitors (HDACi)[121] have been shown to upregulate mir-29b expression. New HMAs are also in early development. The finding that promoter demethylation is not always sufficient to reinduce gene expression[85] provided the rationale for epigenetic therapeutic combinations, especially with HDACi.[122] Detailed *in vitro* studies investigating the mechanisms of a potential HMA/HDACi synergism in myeloid neoplasms are lacking. In particular, the sequence and timing of each drug's administration may be critical in achieving synergy.[122] Another limitation of HDACi is their lack of specificity for chromatin-bound deacetylases, in part because the latter are often embedded in multiprotein complexes limiting drug availability. This results in significant hematological and non-hematological toxicity. As a result, clinical evidence of the superiority of combination therapies over HMA alone is so far lacking.[82] In fact, the most promising approaches today, such as azacitidine–lenalidomide combinations, remain empirically based.[123] The 'druggable' potential of several histone 'writers' (for example, histone methyltransferases) and 'readers' (bromodomain-containing proteins)[124] will also offer future therapeutic potential in MDS, as exemplified by recent advances in MLL-rearranged AML.[125]

CONCLUSION

Expanding from the long-established notion of aberrant gene promoter methylation, new studies have revealed the extent of aberrant DNA methylation and histone modifications in MDS. Further work is required to integrate these epigenomic maps with transcription profiles, in order to identify the key pathways that are recurrently deregulated in MDS. These could include the Wnt pathway and the HOX gene cluster, both of which are involved in the self-renewal of normal and leukemic HSCs. Detailed functional studies will be required to validate their implication in MDS pathogenesis. Mutations in genes encoding epigenetic regulators involved in DNA methylation and hydroxymethylation, or in covalent histone modifications, also have a key role in MDS. Though data on the molecular function of these proteins is rapidly emerging, the functional consequences of their inactivation in MDS cells remain elusive. Future work will also probably highlight the role of aging in the emergence and selection of random epigenetic variations in HSC and pre-leukemic clones. HMAs are the mainstay of therapy for higher-risk MDS. Nonetheless, their precise mode of action remains unclear. Biomarkers of their clinical activity have also failed to emerge: recent data suggest the importance of intraclonal epigenetic heterogeneity in cancer. This notion may render vain the study of epigenetic profiles as prognostic tools or biomarkers. Mirroring the recent interest in the clonal genetic heterogeneity of MDS, single-cell studies will likely be required to unravel the epigenetic instability of MDS. For instance, it could be that variability of methylation at a given locus, not mean methylation value, is the best predictor of outcome.[126] Current prospects to improve the clinical results obtained with HMAs are mostly based on randomized comparisons adding empirically chosen compounds. Given the high complexity and dynamics of epigenetic remodeling, the rational design of epigenetic therapeutic combinations is likely to remain a distant goal.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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