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Epigenetic role of miRNAs in normal and leukemic hematopoiesis

Hematopoiesis is a regulated multistep process, whereby transcriptional and epigenetic events contribute to progenitor fate determination. miRNAs have emerged as key players in hematopoietic cell development, differentiation and malignant transformation. From embryonic development through to adult life, miRNAs cooperate with, or are regulated, by epigenetic factors. Moreover, recent findings suggest that they contribute to chromatin structural modification, and the functional relevance of this 'epigenetic–miRNA axis' will be discussed in this article. Finally, emerging evidence has highlighted that miRNAs have functional control in human hematopoietic cells, involving targeted recruitment of epigenetic complexes to evolutionarily conserved complementary genomic loci. We propose the existence of epigenetic–miRNA loops that are able to organize the whole gene expression profile in hematopoietic cells.

KEYWORDS: differentiation ▪ epigenetics ▪ hematopoiesis ▪ leukemia ▪ miRNA

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Epigenetic mechanisms provide a plastic system for regulating gene expression, and are extensively implicated in hematopoiesis and leukemia development and progression [1]. In this framework, a new class of regulators, the miRNAs, have recently emerged as players in the extensive changes responsible for hematopoietic cell development, differentiation and malignant transformation. This class of noncoding RNA, classically linked to transcription factor networks [2,3], is now being integrated into epigenetics [4–6]. miRNAs control gene expression, cooperate with or are regulated by epigenetic factors, including DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and polycomb group (PcG) family proteins [4,5,7,8]. Therefore, miRNAs take part in chromatin structural modifications in a direct or indirect way. miRNAs can affect the expression of epigenetic factors in many systems, providing evidence for the role of these molecules as indirect players in the epigenetic dynamism of the cells.

We will describe the complicated networking of miRNAs and epigenetic pathways, proposing the existence of epigenetic–miRNA loops that are able to organize the whole gene expression profile in every cell. We will then highlight disruptions in this regulatory loop, and how physiological functions are likely to be interfered with, possibly contributing to the establishment of disease [6]. Finally, we will discuss emerging evidence suggesting that in human hematopoietic cells miRNAs functionally control gene transcription by the targeted recruitment of epigenetic complexes to evolutionarily-conserved complementary loci in the genome.

The aim of this review is to encompass the most recent findings underlying the functional relevance of the epigenetic–miRNA axis in normal and malignant hematopoiesis, highlighting the synergistic action of 'classical' epigenetic machineries and miRNAs.

Hematopoiesis

Hematopoiesis is a dynamic and highly complex developmental process that gives rise to a multitude of cell types circulating in the blood of multicellular organisms. Blood cells provide tissues with oxygen and guard against infection. They also prevent bleeding by clotting and mediate inflammatory reactions. As the hematopoietic system plays a central role in human physiology and diseases, such as anemia, infection, bleeding disorders, cancer and autoimmunity, it has been intensely studied for more than a century.

The wide variety of differentiated elements present in animal blood is generated from one unique hematopoietic stem cell (HSC). The hematopoietic system is organized in a hierarchical manner and includes clear-cut nodal progenitor cells that have intermediate levels of lineage potency. The design of this network apparently serves to provide stable substations, perpetually programmed to supply new downstream lineage cells whenever needed. In this scenario, the orchestration of epigenetic events tightly regulates the system [9] and, at the same time, drives the transcriptional activity and expression of genes underlying lineage commitment, whose deregulation can result in cancer onset and progression [10].

The differentiation/maturation of HSCs leads to sequential developmentally restricted states. Initially, they give rise to multipotent progenitors (MPPs), which have lost the ability to self-renew, but still maintain a full-lineage differentiation potential. MPPs can differentiate into lymphoid-primed MPPs, common lymphoid progenitors and common myeloid progenitors. Common lymphoid progenitors give rise to lymphocytes and natural killer cells, whereas common myeloid progenitors differentiate into granulocyte–monocyte and megakaryocyte–erythrocyte progenitors [10,11]. From embryonic development through to adult life, hematopoiesis is tightly regulated by transcriptional and epigenetic events, which prime and commit HSCs and progenitors to their definitive destiny [9,10].

Epigenetics

In the hematopoietic context, epigenetic regulation is required not only for development, but also for tissue homeostasis via the self-renewal and differentiation of somatic stem cells. Accumulating evidence suggests that epigenetic regulators play critical roles in HSCs multipotency and self-renewal ability. Therefore, these properties must be precisely balanced to preserve the multipotent cell pool throughout life [12]. Using microarray technology, the genome-wide DNA methylation pattern has been studied throughout the different stages of hematopoietic differentiation. Promoters of different genes, initially methylated in HSCs or MPPs, are found to be progressively demethylated in a tissue- or lineage-specific way, reflecting a progressive activation of lineage-specific genes. Among these genes, we can recognize those usually considered to be 'signatures' of each hematopoietic cell type, such as myeloperoxidase in granulocyte–monocyte progenitors, globin genes in erythroid precursors, FoxP3 transcription factor in T-regulatory cells differentiation, G-CSF receptor in granulocyte precursors and GP6 during megakaryocyte differentiation [13]. More recently, a potential role for promoter DNA demethylation was investigated by combining genome-wide promoter methylation with gene expression approaches, comparing a variety of somatic tissues. Interestingly, this study revealed that promoter demethylation-associated gene regulation is more frequent in the hematopoietic system than in nonhematopoietic tissues (463 demethylated genes in blood vs an average of 241 demethylated genes in nonhematopoietic samples), supporting the overall strong influence of epigenetic signals in driving the tightly time- and step-controlled cell fate determination during hematopoiesis [13]. The demethylation process is quite complex and involves proteins, such as the TET family of proteins, which are frequently mutated in neoplastic hematopoietic diseases [14].

In parallel, during late commitment of HSCs, other subsets of genes are affected by *de novo* methylation, allowing a definitive switch to a unique developmental/differentiation program. Similarly, the global methylation status of HSCs undergoes large variations during aging, reflecting the reduced self-renewal and differentiation capability of stem cells [15]. Moreover, *de novo* methylation has been observed for a subset of genes associated with PcG complexes during aging and tumorigenesis. These events are usually associated with gene silencing and could thus contribute to the reduced phenotypic plasticity and self-renewal of aged stem cells [16]. Besides DNA methylation status, histone tail modifications play a fundamental role in the regulation of chromatin assembly.

Although acetylation of histone 3 (H3) lysine 9 and 14 always correlates with accessible euchromatin and therefore promotes gene transcription, histone methylation correlates with both permissive and nonpermissive chromatin states, and consequently with either transcriptional activation or repression. Indeed, coexistence of H3K27me3 and activating H3K4me3 marks the same DNA region defining the so-called 'bivalent domains', which are usually located at regulatory regions of gene promoters. In the hematopoietic context, these chromatin modifications reflect parallel changes in gene expression and transcriptional competence of developmentally regulated genes [10,17,18]. This bivalency allows cell commitment to be postponed and, contemporarily, progenitor cells to be 'poised' within alternative lineage fates; chromatin resolution into either the active or repressed state represents a definitive cell fate decision [17,18]. Evolutionarily conserved PcGs and trithorax protein groups are responsible for the trimethylation of H3K27 and H3K4, respectively and preserve the epigenetic memory of each cell type. This ensures the correct execution of key developmental programs, including self-renewal and commitment of hematopoietic cells [10,19,20].

A thorough coverage of the abovementioned epigenetic changes can be found in outstanding papers recently reviewed by Dawson and Kouzarides [21].

miRNA biogenesis & function

In 1993, small regulatory RNAs were recognized in *Caenorhabditis elegans* [22], and later were discovered in plants and mammals [23–25], these RNAs were designated 'miRNA' [26]. The miRNA world has rapidly expanded and now counts 2042 mature human entries registered in the miRBase database [27]. miRNAs are short molecules, 19–25 nucleotides in length, and belong to a class of conserved noncoding transcripts that finely regulate gene expression by an RNA-mediated gene-silencing mechanism, involving translational repression and mRNA degradation [28]. In animals, target recognition occurs mainly through incomplete base-pairing of the miRNA and the target gene 3′ untranslated region. While complementarity at the 5′ end of the miRNA, designated as the 'seed' region [29], appears to be essential for target recognition, a certain flexibility is permitted at the 3′ end for successful binding to the target mRNA [29,30]. These small regulatory RNAs function as part of the effector RNA-induced silencing complex, together with members of the Argonaute (Ago) family of proteins [31].

miRNA genes are found in intergenic regions as well as in introns of protein-coding genes. When located within introns, in some cases, their expression is coregulated with their host genes [32]. The majority of mammalian miRNA genes are transcribed by RNA polymerase II as long 5′-capped and 3′-polyadenilated precursors. Primary miRNA transcripts can be hundreds of bases to several kilobases in length and might contain one or several miRNAs [33]. The biogenesis of miRNAs involves two processing steps whereby the long primary miRNA is first cotranscriptionally cleaved by the nuclear 'microprocessor' complex [34,35], containing Drosha, an RNase III-like enzyme, and its cofactor DGCR8 [36,37]. Then a short stem-looped precursor, the pre-miRNA, is released and exported to the cytoplasm where it is further processed by a second complex containing the RNase III-like enzyme, Dicer. This mechanism liberates the mature miRNA duplexes for loading into the RNA-induced silencing complex [38]. The relatively simple mechanism for target recognition, together with the small portion of the miRNA sequence required for efficient binding to the cognate target mRNA, provides a combinatorial system for gene expression regulation [3].

miRNA-mediated regulation has indeed been described as a central hub in cellular and differentiation pathways and it is extensively involved in hematopoiesis and hematological malignancies [39,40]. The homozygous Dicer deletion in mice is incompatible with a functional HSC state and leads to a marked defect in hematopoietic progenitor competitive repopulation assays [41]. Moreover, haploinsufficiency of Dicer in B cells failed to promote B-cell malignancy or accelerate Myc-induced B-cell lymphomagenesis in mice, thus suggesting a role for Dicer in B-cell lymphoma development and survival [42]. The deletion of Ago2 also leads to severe defects in erythroid and B-cell development [43].

The role of miRNAs in hematopoiesis was largely determined from profiling studies that revealed how several miRNAs are expressed at various stages of hematopoietic development and are specific for each lineage [44]. A recent report encompasses the expression of known hematopoietic miRNAs throughout the mouse hematopoietic system, providing a comprehensive atlas of miRNA expression, from early stem cells through the differentiation stages to mature blood elements [45].

Many miRNAs are also involved in malignant transformation, and sometimes prove to be good diagnostic markers for the different leukemia subtypes. Since comprehensive reviews listing miRNAs involved in myeloid leukemias and myelodysplastic syndromes (MDS) are now available [46,47], we focused on miRNAs connecting the epigenetic myelodysplastic machinery to these diseases. Indeed, miRNAs affect the expression of epigenetic factors in many systems, including hematopoiesis, providing evidence for their role as indirect players in the epigenetic dynamism of the cell.

Epigenetic–miRNA loops in normal & malignant hematopoiesis

Epigenetic regulation of miRNA genes

Epigenetic regulation of miRNA gene transcription appears to be crucial as miRNAs are increasingly defined as critical molecules in normal and aberrant development, cell proliferation and commitment processes.

The primary regulatory mechanism for miRNA expression is probably their transcriptional control [6]. The initial evidence showing that miRNAs undergo epigenetic regulation was obtained in a variety of cancer models. In addition, either pharmacologic or genetic approaches unmasked hypermethylated miRNA genes [48,49]. Interestingly, an extensive analysis of genomic sequences of miRNAs have shown that they are frequently associated with

CpG islands [50]. The expression of miRNAs is affected by promoter hypermethylation or global hypomethylation [51].

Although the network complexity underlying aberrant miRNA genes epigenetic regulation in pathophysiological conditions is yet to be determined, single factors, such as cancer-specific fusion proteins or cytokines, have been implicated in driving epigenetic silencing of miRNA genes (Table 1) [52,53]. For instance, the AML1–ETO oncoprotein, resulting from the t(8;21) translocation of AML binds to the miR-223 gene promoter [52], where it recruits an epigenetic silencing complex consisting of HDACs, DNMTs, and methyl-CpG-binding proteins. Through CpG methylation, this complex inhibits pri-miR-223 expression and, in turn, sustains the block in myeloid differentiation underlying the pathogenesis of this acute myeloid leukemia (AML) subtype [52]. Similarly, AML1–ETO triggers the heterochromatic silencing of miR-193a by binding to AML1-binding sites and recruiting chromatin-remodeling enzymes (HDACs and DNMTs) on its gene promoter. Suppression of miR-193a expands the oncogenic activity of the fusion protein AML1–ETO [54].

In a recent report it was shown that in AMLs presenting the t(8;16) translocation, which fuses genes encoding two histone acetyltransferases, MYST3 (or MOZ) on chromosome 8 and CREBBP (or CBP) on chromosome 16, 90 miRNAs are downregulated and among those 29 present CpG islands in their promoter regions. Treatment of myeloid cell lines and primary patient cells with DNMT and HDAC inhibitors (5-Aza-deoxycytidine and Trichostatin A, respectively) rescued the expression of 27 out of 29 of these miRNAs suggesting that their suppression is indeed mediated by epigenetic silencing mechanisms [55]. In addition, an altered regulation of miR-203 was described in T cell lymphoma mouse model and human cells, where its promoter was found hypermethylated [56]. miR-203 also controls the activation

Table 1. Examples of epigenetic regulation of miRNAs.

Epigenetic factor/mechanism	miRNA	Target mRNA†	Cellular process/disease	Functional relevance	Ref.
Heterochromatic silencing by AML1–ETO/HDAC/DNMT complexes	miR-223	E2F1, LMO2, MEF2C, NFIA, IGF1-R	t(8;21)-AML	Contributes to myeloid differentiation block	[52]
	miR-193a	KIT, ETO, HDAC, DNMT3a, MDM2, CCDN1, MCL1	t(8;21)-AML	Contributes to leukemogenesis Hypermethylated in AMLs	[54,60]
Heterochromatic silencing by Sp1/NF-κB/HDAC complex	miR-29b	CDK6, DNMT3A, DNMT3B, TCL1A, TET1, Sp1	AML	Contributes to leukemogenesis	[59]
Aberrant promoter CpG methylation	miR-34b	CREB, E2F3, FOXP1, MAP2K1, ZAP70	AML	CREB overexpression and leukemic transformation from MDS	[61]
	miR-203	ABL1	T-cell lymphoma and CML	ABL1 activation and lymphomagenesis	[56,58]
			Ph-MPNs	No correlation with clinical demographic data or outcome	[57]
Aberrant CpG island methylation associated with EVI1	miR-124	CDK6, CEBPA	MDS	Contributes to disease establishment and progression	[62]
Chromatin silencing by Egr2/Jarid1b	miR-17–92	RUNX1, p21, BIM, CCL1, DNAJC27, FBXO31, GPR137B, NPAT, OBFC2A, YES1, ZNFX1	Hematopoiesis	Macrophage differentiation	[66]
PRDM5-dependent recruitment of HDACs and G9a HMTase	miR-21 miR-196b miR-135b	NFIB, REST HOX-B8 KLF4, MAFB	Hematopoiesis	Neutrophil differentiation	[67]
PcG-induced CpG island methylation and histone modification	miR-214 miR-200c	EZH2 BMI1	Hematopoiesis	Stem cell senescence	[71]

†*Experimentally validated target genes of hematopoietic or epigenetic regulators reported in the references or by miRTarBase database* [109]. *The studies addressing epigenetic regulators or mechanisms affecting miRNA expression are cited in the reference column.*
AML: Acute myeloid leukemia; CML: Chronic myelogenous leukemia; DNMT: DNA methyltransferase; HDAC: Histone deacetylase; MDS: Myelodysplastic syndrome; Ph-MPN: Philadelphia chromosome myeloproliferative neoplasm.

of *ABL1*, a classic oncogene that is extensively characterized in hematopoietic malignancies. miR-203 is epigenetically silenced in chronic myelogenous leukemias carrying the t(9;22) Philadelphia (Ph) translocation; this enhances the t(9;22)-fusion product *BCR–ABL1* activation, thus elevating the growth rate of chronic myeloid leukemia cells [56].

Interestingly, in hematopoietic malignancies, 12% of miRNAs are located in fragile genomic regions that only encompass 0.2% of the whole genome [4], and one of these regions hosts miR-203 [4]. miR-203 hypermethylation was also observed in cell lines and primary cells from Ph chromosome-negative myeloproliferative neoplasms (Ph^-MPNs), although there was no correlation between miRNA methylation and clinical demographic data or outcome [57]. Moreover, in a large screening (150 primary samples) for methylated miRNAs, miR-203 was found to be methylated in 5% of acute lymphoblastic leukemia (ALL), in 42% of chronic lymphocytic leukemia and 38% of non-Hodgkin lymphoma [58]. Thus, the oncogenic activity of this particular miRNA spreads across different leukemia subtypes (lymphoid and myeloid), but the mechanism leading to miR-203 promoter hypermethylation has not currently been determined.

miR-29b was reported to be a central hub in the gain-of-function mutations of KIT (product of oncogene *c-kit*) characterizing a specific subset of AMLs (i.e., core binding factor AMLs). In these AMLs, miR-29b-dependent KIT overexpression contributes to leukemia growth. miR-29b participates in a Sp1/NF-κB/HDAC/ regulatory network, which mediates *c-kit* overexpression and that can be successfully targeted by pharmacological disruption of the Sp1/NF-κB/ HDAC complex with HDAC inhibitors [59]. In addition, miR-193a was shown to be epigenetically silenced in primary AML blasts and AML-derived cell lines, by a promoter methylation at specific CpG islands. This contributes to *c-kit* overexpression, with *c-kit* being one of miR-193a validated targets [60].

One of the features of AML is also the overexpression of CREB factor, which is targeted by miR-34b [61]. This miRNA was shown to be repressed in primary AML samples by promoter hypermethylation, occurring at leukemia onset after a MDS [61]. These results demonstrate that epigenetic deregulation of miRNA genes is relevant in oncogene regulation and may represent a therapeutic target in specific subsets of human leukemias.

Specific miRNAs are downregulated in MDS, a group of hematopoietic malignancies characterized by ineffective hematopoiesis. Among these, miR-124 expression level is inversely correlated with the degree of its promoter methylation [46]. This observation was then supported by data from a mouse model where both miR-124 methylation and silencing trigger an MDS-like disease [62].

Downregulated miRNAs in MDS (e.g., miR-140, -378 and -632) are predominantly intragenic and show a similar expression pattern to their host genes, suggesting mechanisms of coregulation during myeloid maturation. Indeed, the increased methylation status of shared promoters induces the downregulation of several miRNAs and their host genes. Thus, epigenetic regulation in MDS involves both protein-coding genes and miRNAs, unifying the research fields of 'miRNAs in MDS' and 'epigenetic regulation in MDS' [63].

Notably, miR-124 expression was to be found downregulated in ALL patients' samples (59% of 353 analyzed samples), where its promoter was hypermethylated [64]. Moreover, functional studies showed upregulation of miR-124 after treatment of ALL cell lines with 5-aza-cytitdine, suggesting a role for CpG methylation in transcriptional silencing of this miRNA in ALL pathogenesis [65].

The epigenetic regulation of miRNAs was extensively investigated in aberrant hematopoiesis, which is a new putative target for therapeutic intervention. Moreover, some studies address its physiological importance in HSCs and differentiating precursors. It has been reported that, upon macrophage differentiation of myeloid progenitors, the transcription factor PU.1, which is essential for myeloid and lymphoid differentiation, induces the expression of Egr2 that, in turn, represses miR-17-92 polycistron. miR-17-92 is a cluster encoding six miRNAs, miR-17, -18a, -19a, -20a, -19b-1 and -92a; the repression of this miRNA cluster is mediated by the histone demethylase Jarid1b, leading to H3K4 demethylation within the CpG island at the miR-17-92 promoter. The reduced expression of the miR-17-92 cluster is a *conditio sine qua non* for macrophage differentiation [66].

Moreover, several miRNAs are epigenetically repressed by HDACs and G9 histone methyltransferase [67], with a mechanism mediated by zinc-finger repressor Gfi-1, a key intrinsic regulator of HSC self-renewal [68], and its interacting factor PRDM5, a zinc finger protein belonging to the PR (PRD1-BF1 and RIZ homology) domain-containing tumor suppressor protein family, acting in different pathways during hematopoiesis and leukemia [68,69].

miRNA-mediated regulation of epigenetic factors

The epigenetic signature of stem cells has been recently defined [70] and miRNA-mediated regulation has been included in this picture [71–73]. The epigenetic role of miRNAs was globally evaluated in two recent reports where the authors investigated the role for mammalian Dicer and Dicer-dependent small RNAs, particularly miRNAs, in mouse *Dicer1*-null embryonic stem cells (ESCs). Their findings showed that Dicer1 abrogation results in decreased expression of DNMTs [72,73]. Benetti and colleagues reported that Dicer ablation impairs telomere homeostasis [72]. Thus, miRNAs activity extends to chromosome structure and telomere length maintenance. They observed that in *Dicer1*-null ESC the decrease in Dnmt levels paralleled the significant increase of Rbl2, a known transcriptional inhibitor of Dnmt expression, and that the substantial downregulation of miR-290 cluster efficiently targets the Rbl2. The authors suggested a mechanism whereby the conserved mammalian miR-290 cluster regulates Rbl2 at the post-transcriptional level, leading to Dnmt3a and Dnmt3b transcriptional repression. This also induces DNA methylation defects within subtelomeric regions, where telomere recombination is increased and aberrant telomere elongation is detectable [72]. These results identify a novel mechanism by which miR-290 regulates Rbl2-dependent Dnmt expression, thus affecting telomere-length homeostasis. Sinkkonen and colleagues also demonstrated that defective DNA methylation can be rescued by ectopic expression of *de novo* Dnmts or by transfection of miRNAs belonging to the miR-290 cluster into *Dicer*$^{-/-}$ ESCs, further supporting the notion that miRNAs control *de novo* DNA methylation in ESCs [73].

The general miRNA-mediated mechanisms described for the control of DNMTs' function in pluripotent ESCs could possibly occur in other cellular contexts, such as in HSCs and progenitor cells, where the modulation of DNA methylation during lineage-specific differentiation plays a crucial role [74]. Indeed, So and colleagues reported that inhibition of DNMTs' activity or expression in human cord blood-derived multipotent stem cells by 5-azacytidine or RNAis, respectively, led to the induction of cellular senescence, cell cycle arrest and decreased multipotency, accompanied by a decrease in the expression of PcGs, such as EZH2 histone methyltransferase and PcG protein BMI1 [71]. The decrease of PcGs is dependent on miR-214 and -200c, which are known to target EZH2 in skeletal muscle and ESCs, and BMI1 in breast cancer stem cells, respectively [75,76]. Although the same analysis has not currently been performed in the HSC compartment, a central role for both EZH2 and BMI1 in these cells has been extensively described [77–79].

Throughout hematopoietic cell differentiation each transition requires the action of both epigenetic and transcription factors [80,81] as well as miRNAs [82,83]. Terminal erythroid cells differentiation involves a progressive heterochromatin formation and chromatin condensation, leading to enucleation in mammals [74]. Chromatin condensation preceding nucleus expulsion from orthochromatophilic erythroblasts is accompanied by nucleosomal histone modifications [84]. The requirement of HDACs activity in erythroblast chromatin condensation is suggested by the consequences of their inhibition. Treatment of mouse erythroblasts with HDAC inhibitor Trichostatin A, maintains histone acetylation and inhibits chromatin condensation and nuclear extrusion [85]. Recently, miRNAs have been thought to be indirectly involved in the late phases of erythroid differentiation. Downregulation of the developmentally regulated miR-191, identified by RNA deep sequencing performed in terminally differentiating CFU-E erythroid progenitors, is required for erythrocyte precursor chromatin condensation, global gene expression shutdown and enucleation processes [86]. In these events, the involvement of miR-191 is indirect; it targets the erythroid-enriched gene *Riok3*, which belongs to the RIO family of atypical protein kinases, and *Mxi1*, a well-known c-Myc antagonist. *Riok3* and *Mxi1* function in a complex mechanism eventually leading to the downregulation of the histone acetyltransferase Gcn5, which is necessary for proper erythrocyte maturation. Either knockdown of *Riok3* and *Mxi1*, or overexpression of miR-191, blocks Gcn5 downregulation and impairs enucleation [86]. Thus, in mammalian cells, miR-191 repression is essential for erythroid chromatin condensation and enucleation, by allowing upregulation of Riok3, Mxi1 and downregulation of Gcn5.

With regards to physiological differentiation patterns, accumulating data revealed how a combination of genetic and epigenetic abnormalities triggers cancer evolution. The most representative aberrations are CpG island hypermethylation at promoter genes and deacetylation or methylation of histone proteins. The action of miRNAs on the epigenetic network complicates this scenario, as many miRNAs have been found to be deregulated in leukemia [87]. In fact, miR-29b, involved in lung cancer epigenetic abnormalities [88], participates in the global hypomethylation observed in AML [7]. miR-29b hypomethylating role was tested during myeloid leukemogenesis; its expression promotes DNA hypomethylation through the direct targeting of DNMT-3A and -3B. miR-29b also decreased DNMT1 expression indirectly, via downregulation of Sp1, a known DNMT1 transactivating factor. This epigenetic activity of miR-29b was also confirmed in primary leukemic blasts, thus providing the basis for a future use of synthetic miR-29b in a miRNA-based intervention for AML therapy [7]. To date, miR-29b is the only leukemia-related miRNA linked to epigenetic changes by direct targeting of one component of the epigenetic machinery; however, it is likely that other miRNAs are involved in epigenetic factor regulation in solid tumors [89] and they may also have a role in leukemia (Table 2).

A new direct action of miRNA on chromatin?

Approximately half a century ago, Jacob and Monod proposed that base complementarity would allow specific RNA interaction with other nucleic acid sequences [90]. Besides forming RNA–RNA interactions, RNA can potentially bind to complementary DNA sequences, working as a 'guide' in the control and maintenance of chromatin status [91].

Although, the *in vivo* existence of RNA–DNA triplexes and their direct action on epigenetic remodeling have not yet been validated, some recent data provided evidence for a role for ncRNA in chromatin-based processes [8,91].

Interestingly, genome-wide analyses revealed an over-representation of putative triple-helix target at human gene promoters, highlighting the potential interaction of ncRNAs with the major groove of the DNA double helix to control gene expression [92,93].

Growing evidence now highlights that miRNAs localize to the nucleus. Despite the canonical miRNA biogenesis that implies Exportin-5-mediated transfer of pre-miRNA from nucleus to cytoplasm, a number of studies indicate that mature miRNAs can be shuttled from the cytoplasm back to the nucleus. This shuttling also involves Ago proteins nuclear import [94]. Moreover, deep-sequencing data revealed a complex subcellular distribution of miRNAs, with 300 of them being identified in both the nucleus and cytoplasm, and 39 having a preferential nuclear localization [95].

Initial evidence on the action of exogenous sequence-specific siRNAs in transcriptional gene silencing [96–100] triggered the idea of a role for endogenous small ncRNAs in evolutionarily conserved epigenetic gene silencing pathways [96,101]. In fact, the existence of endogenous miRNA-directed epigenetic processes in mammals has been reported [102–104].

miR-223 was described as a nucleation center for chromatin remodeling complex recruitment during myeloid lineage determination [8]. A role for miR-223 in driving transcriptional gene silencing via PcG–RNAi complexes was suggested by the presence of two DNA sequences complementary to the miR-223 seed on the promoter region of NFI-A, whose mRNA is also targeted by miR-223 [2]. Our group found that during human granulopoiesis, miR-223 translocates to the nucleus and localizes at its complementary sequences on the NFI-A promoter [8]. The nuclear localization of miR-223 was also observed during metaphase, when RNA synthesis is blocked,

Table 2. miRNA-mediated regulation of epigenetic factors.

miRNA	Targeted epigenetic factor	Related cellular process/disease	Functional relevance	Ref.
miR-290 cluster	Dnmt1, Dnmt3a, Dnmt3b	Mouse ESCs†	Telomere length homeostasis ESC proliferation, differentiation and cell cycle control	[72,73]
miR-214	EZH2	Skeletal muscle ESCs†	Stem cell senescence	[75]
miR-200c	BMI1	Breast cancer stem cells†	Stem cell senescence	[76]
miR-191	Gcn5 (via Riok3 targeting)	Hematopoiesis	Erythroid differentiation (enucleation)	[86]
miR-29b	DNMT3A, DNMT3B, DNMT1 (indirectly)	AML	Contributes to the DNA hypermethylation observed in AML	[7]

†Nonhematopoietic systems. However, these studies involve key genes known to have a function in hematopoiesis and/or hematological malignancies.
AML: Acute myeloid leukemia; ESC: Embryonic stem cell.

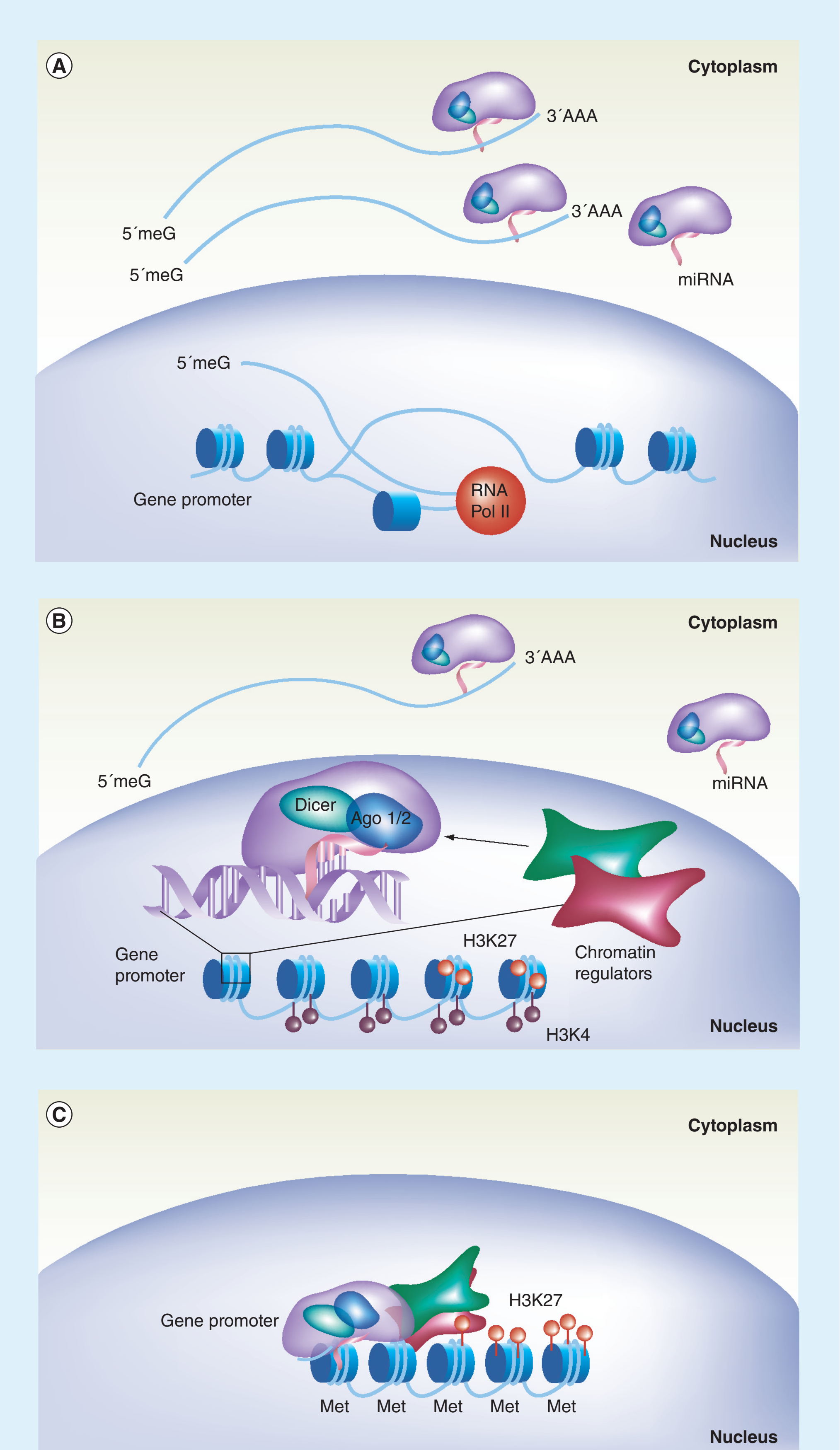
A
Cytoplasm
3´AAA
3´AAA
5´meG
5´meG
miRNA
5´meG
Gene promoter
RNA Pol II
Nucleus
B
Cytoplasm
3´AAA
5´meG
miRNA
Dicer
Ago 1/2
Gene promoter
H3K27
Chromatin regulators
H3K4
Nucleus
C
Cytoplasm
Gene promoter
H3K27
Met
Met
Met
Met
Met
Nucleus

Figure 1. Proposed double-step mechanism for miRNAs' direct action on chromatin (facing page). (A) Classical miRNA action on the regulation of gene expression. The miRNA (pink line) embedded into the RNA-induced silencing complex (RISC; purple). Mature miRNA guides the RISC complex to the 3´ untranslated region of a complementary capped (5'meG) and polyadenilated (3'AAA) mRNA (blue line), and is exported to the cytoplasm after its transcription into the nucleus by RNA Pol II (red sphere). **(B)** The action of miRNA on mRNA associates with a new mechanism whereby miRNA and RISC components Ago1/2 (blue) and Dicer (light green), once imported into the nucleus, mediate chromatin remodeling factor (red and dark green squares) recruitment on DNA. **(B)** A detailed magnified view of the proposed recruitment mechanism. Here a feedback loop is proposed where the same gene is regulated at both **(A & B)** post-transcriptional and **(B)** transcriptional levels. A bivalent domain is exemplified by the coexistence of both activating (H3K4me3; dark red circles) and inhibitory (H3K27me3; light red circles) histone marks. **(C)** This double-step regulation ensures long-term efficient silencing, by extensive chromatin remodeling with resolution of the bivalent domain into heterochromatin via extensive H3K27me3 and Met.
Met: DNA methylation; RNA Pol II: RNA polymerase II.

suggesting a direct miR-223 binding to DNA [8]. miR-223 is part of a ribonucleoprotein repressive complex formed by PcG proteins YY1 and Suz12, Dicer-1 and transiently Ago1, and the complex's localization on the NFI-A promoter is RNA dependent [8]. This complex is recruited on the NFI-A promoter region, marked by an H3K27me3/H3K4me3 bivalent domain, which is then resolved (H3K27me3 increase and H3K4me3 decrease); this induced nearby heterochromatin formation and NFI-A silencing.

An analogous miRNA-dependent complex assembly on DNA has been recently reported for the promoters of the retinoblastoma repressor E2F/Rb1 complex targets, CDC2 (also known as CDK1) and CDCA8. Similarly, a miRNA-complementary sequence was found for let-7f in both promoters, in the antisense and sense strand, respectively [102].

As miR-223 acts on NFI-A mRNA homeostasis and promoter chromatin accessibility, resulting in a more efficient and irreversible repression [2,8], we hypothesize a general double-step control mechanism that would initially ensure fine tuning and later, long-term efficient silencing of developmentally regulated genes during HSC differentiation along each hematopoietic lineage (Figure 1). Therefore, once progenitors are committed, one unique differentiation destiny is ensured, providing the organism with the proper blood elements according to developmental timing, blood cell replacement and environmental signals (e.g., inflammation, growth factors, hormones and wounds).

Conclusion: extending the concept of epigenetics to miRNA-mediated gene regulation

Epigenetic mechanisms play an important role in hematopoiesis since they allow time- and environment-dependent modulation of genes encoding designated key regulatory factors of differentiation, proliferation and function of different hematopoietic cell types [3].

The epigenetic modifications occurring on DNA or nucleosomal histones are not necessarily mutually exclusive processes. They occur by sequential recruitment of different enzymes and, represent dynamic processes affecting the transcriptional status of a specific gene [104].

Since hematopoietic cells can respond to a plethora of stimuli, the use of many different 'ways' to switch genes on and off, gradually or sharply, confers plasticity and a timely response to different signals. An altered balance of this intricate network can be one of the leading mechanisms for pathological conditions such as neoplastic transformation [105].

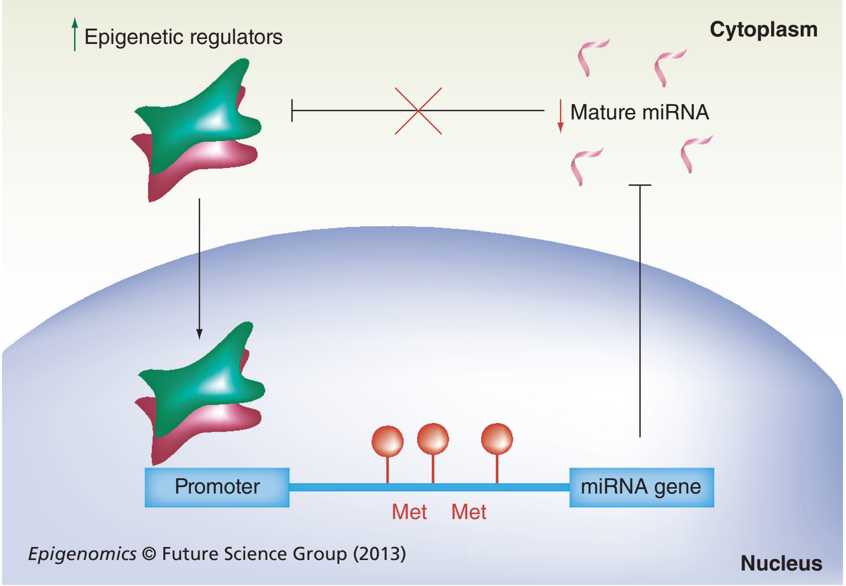

Figure 2. Epigenetic–miRNA loops. When the miRNA gene promoter is hypermethylated or modified in its chromatin structure (red circles) by the binding of epigenetic regulators (red and green squares), mature miRNA transcription is impaired (black line). Therefore, cytosolic miRNA levels are decreased (red arrow). Mature miRNA impairs the expression of epigenetic regulators (black line). However, decreased miRNA levels allow (red cross on black line) the increased expression of epigenetic regulators (green arrow) that bind to the miRNA gene promoter in the nucleus (black arrow).
Met: DNA methylation.

miRNAs can act as targets as well as effectors of this dynamic regulation [8,89,106], therefore, a monocausal relationship between miRNA action on gene expression and a unique specific function is unlikely [40].

As highlighted in this review, miRNAs can be regulated by epigenetic events such as DNA methylation or histone modifications (Table 1) [8,107] and they can silence members of the epigenetic machinery at post-transcriptional level (Table 2), therefore, creating 'epigenetic–miRNA loops', as exemplified in Figure 2. This points to a new level of complexity in chromatin remodeling and epigenetic regulation mediated by miRNAs, which complements the most studied regulatory circuits involving transcription factors in hematopoiesis [2,3,80]. These regulatory circuits are mostly repressing miRNA expression; however, the information regarding the epigenetic mechanisms involving miRNA is still limited.

Future perspective

Hematopoietic differentiation is often depicted as an intricate pattern, the 'hematopoietic tree', with its root in the HSC compartment and the branches in each committed progenitor and mature precursor cell populations. Each transition from one stage to the next during progenitor maturation is governed by the action of epigenetic and transcription factors [80,81] as well as miRNAs [82,83].

Chromatin remodeling factors and miRNAs functions often overlap and cross-regulate each other, making the epigenetic system even more dynamic and intricate.

Moreover, the connection between miRNA nuclear localization and function is remarkable and suggests new pathways for miRNA-mediated gene expression regulation, directly acting on chromatin structure.

Two tightly linked systems, chromatin remodeling factors and miRNA [83], exert a crucial role in HSC self-renewal, commitment and differentiation [108]; now these two main mechanisms of regulation of HSC fate may be brought together, as a direct action of miRNAs on chromatin has been discovered. Therefore, miRNAs can be considered to be part of a multilevel regulatory mechanism, that modulates gene expression from the level of the chromatin structure through to mRNA translation/stability.

The regulatory circuit involving epigenetic mechanisms and miRNAs in hematopoiesis and leukemia has been extensively described [108], but in the years to come, more focus should be put on the HSC compartment, where an interaction is likely, but has yet to be investigated in detail.

Filling in these gaps will help translational research to put new findings into clinical practice, given the high potential of miRNA in regenerative medicine [40]. The possible use of miRNAs as a new class of drug able to control the epigenetic machinery is fascinating [89]. As compared with the general action of chemical compounds, this would give researchers

Executive summary

Hematopoiesis

- Hematopoiesis is a complex and multistep process, whereby the wide variety of differentiated elements present in an animal's blood is generated from one unique stem cell. From embryonic development through to adult life, this process is tightly regulated by epigenetic events, which prime and commit progenitors to their definitive destiny.

Epigenetics & miRNA regulation crosstalk

- Epigenetic gene regulation has been extensively studied in hematopoiesis, in particular hematopoietic stem cells, and this recently extended to hematopoietic malignancies. In this framework, a new class of regulators, the miRNAs have recently emerged as a player for the extensive changes responsible for hematopoietic cells development, differentiation and malignant transformation.
- miRNAs are a species of short noncoding RNA that post-transcriptionally regulate gene expression. This class of noncoding RNA, classically linked to transcription factor networks, is now being integrated into epigenetics.
- Some miRNAs control gene expression, cooperate with or are regulated by important epigenetic factors, including DNA methyltransferases, histone deacetylases and polycomb group genes. Therefore, miRNAs take part in chromatin structural modification in a direct or indirect way.

miRNA–epigenetic factors' direct cooperation

- The complicated network linking miRNAs and epigenetic pathways suggests the existence of an epigenetic–miRNA loop, able to organize the whole gene expression profile in each and every cell.
- Disruption of this regulatory loop is likely to interfere with physiological functions, possibly contributing to the establishment of disease processes.
- A general double-step control mechanism could be envisioned ensuring fine tuning first and long-term efficient silencing later of key regulatory genes for differentiation along each hematopoietic lineage.

the chance to target specific effector enzymes, inhibiting their activities and affecting the expression of a wide range of epigenetically modulated genes.

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