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Transcriptional targeting by microRNA-Polycomb complexes

A novel route in cell fate determination

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Advances in the understanding of the epigenetic events underlying the regulation of developmental genes expression and cell lineage commitment are revealing novel regulatory networks. These also involve distinct components of the epigenetic pathways, including chromatin histone modification, DNA methylation, repression by polycomb complexes and microRNAs. Changes in chromatin structure, DNA methylation status and microRNA expression levels represent flexible, reversible and heritable mechanisms for the maintenance of stem cell states and cell fate decisions. We recently provided novel evidence showing that microRNAs, besides determining the post-transcriptional gene silencing of their targets, also bind to evolutionarily conserved complementary genomic seed-matches present on target gene promoters. At these sites, microRNAs can function as a critical interface between chromatin remodeling complexes and the genome for transcriptional gene silencing. Here, we discuss our novel findings supporting a role of the transcriptional chromatin targeting by polycomb-microRNA complexes in lineage fate determination of human hematopoietic cells.

Keywords: microRNA, chromatin remodeling, Polycomb complexes, microRNA-DNA interaction, transcriptional gene silencing

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Epigenetic Mechanisms of Cell Fate Determination

The maintenance of stem cell identity, lineage commitment and cell differentiation is assured by specific gene expression patterns temporally and spatially organized by regulatory processes. These processes involve transcription factors that affect the lineage-specific gene expression programs and epigenetic mechanisms which contribute to their stabilization. The importance of the establishment of specific gene expression patterns during development and adult life is well acknowledged. However, the mechanisms and pathways underlying cell fate choices are still incompletely defined.

Polycomb (PcGs) proteins are transcriptional repressors, which have recently received much attention as modulators of stem cell differentiation in mammals.[1-3] PcGs control transcriptional programs that preserve the epigenetic memory and identity of each cell type throughout the lifetime of organisms.[4,5] PcGs proteins are evolutionarily conserved and exist in at least two separate protein complexes; the PcG repressive complex 1 (PRC1) and the PcG repressive complex 2–4 (PRC2/3/4). PRC1 is a large complex including several proteins such as YY1, BMI1, HPH1–3, HPC proteins and RING1A-1B.[6,7] The core components of PRC2/3/4 are EZH2, various isoforms of EED, SUZ12 and the histone-binding proteins RbAp48/46.[8] Although the composition of PcG complexes can vary among different cell types and organisms,[9,10] they all modify chromatin structure by covalent modification of histone proteins.[3,11,12] PRC2 catalyzes trimethylation of lysine 27 on histone H3 (H3K27me2/3), while PRC1 mono-ubiquitinates histone H2A on lysine 119 (H2AK119Ub1).[13,14]

Genome-wide mapping of PcGs and chromatin marks revealed the coexistence of repressive H3K27me3 and activating

H3 lysine 4 tri-methylation (H3K4me3) marks at a number of genomic loci. These loci, termed "bivalent domains," were found in mouse and human ES cells as well as in a broad range of adult stem cells, including hematopoietic stem cells (HSCs).[9,15-17] Bivalent domains keep developmentally regulated and tissue-specific genes transcriptionally poised.[16-22] The co-localization of these marks appears well-centered around the genes' transcriptional start site; the H3K4me3 modification localizes ± 1 Kb around the transcriptional start site, whereas H3K27me3 affects a larger area of ± 2.5 Kb.[19,21,23] Bivalent domains can bind to both the PRC1 and PRC2 complexes at the same time or exclusively to the PRC2 complex.[24] Indeed, bivalent domains are divided into two classes depending on the PcG complex that binds them. The "PRC1-positive" bivalent domains are evolutionarily conserved, more likely retain H3K27me3 upon differentiation and account for developmental regulator genes. "PRC1-negative" bivalent domains, exclusively bound by PRC2, are less conserved, retain H3K27me3 poorly and correspond to membrane protein genes or genes with unknown functions.[24] Genes presenting bivalent domains show low or intermediate expression levels in ES cells and HSCs. When ES cells or HSCs are induced to differentiate, bivalent domains are generally resolved into either repressive H3K27me3 or activating H3K4me3 marks.[18,19] However, which molecular mechanisms drive the resolution of the bivalent domains and their inheritance through cell divisions is still an open question. The recent discovery of histone demethylases adds new complexity. Among the Jumonji C domain-containing proteins, Jarid1 de-methylates H3K4me2/3, whereas JMJD3 and UTX de-methylate H3K27me2/3.[25-32] These interactions may integrate the activity of PcG and Trithorax complexes. They modulate the inhibition or activation of genes required for lineage-specific determination.[31,33,34]

On the other hand, CDK1 and CDK2 phosphorylate EZH2 at threonine 350 during the S phase of cell cycle.[35,36] At target loci in cells, this modification activates EZH2 binding to PRC2 recruiters (such as non-coding RNAs).[37] This novel regulatory mechanism may be relevant for the maintenance of H3K27me3 marks through cell division.[35,36]

Overexpression of EZH2 and its aberrant recruitment on target gene sites have been implicated in cancer initiation and progression. In prostate cancer, overexpression of EZH2 can be driven by the upregulation of MYC protein via transcriptional (direct targeting of EZH2 promoter) and post-transcriptional (downregulation miR-26a and -26b) mechanisms.[38] In acute myeloid leukemia cells, Evi1 physically interacts with EZH2 and recruits EZH2 and other PcG proteins to the PTEN promoter region.[39,40] This induces histone modifications (H3K27me3), which represses PTEN transcription and activates the PI3KAKT/mTOR signaling pathway. This mechanism also contributes to leukemogenesis.[39,40] Interestingly, Evi1 is expressed in 10% of cases of acute myeloid leukemias associated with extremely poor prognosis.[41]

Resolution of Nuclear Factor I-A Chromatin Signature by Polycomb and RNAi Machinery Components Directs Human Granulopoiesis

We initially identified the Nuclear factor I-A (NFI-A) as a post-transcriptional miR-223 target, which was functionally involved in a regulatory circuitry that directed human granulopoiesis.[42]

NFI-A belongs to the Nuclear Factor I (NFI) family of transcriptional factors. This family is composed of four independent genes (NFI-A, -B, -C and X). Its members are involved in the cell growth, replication of adenoviral DNA, oncogenic processes and disease state.[43] NFI-A binds as a dimer or heterodimer, along with other members of the NF-I family or other transcriptional factors, to the dyad symmetric consensus sequence TTGGC(N5)GCCAA on DNA.[43] Binding sites for NFIs have been identified in genes expressed in virtually every tissue of vertebrates.

In human primary hematopoietic stem/progenitor cells (HSC/HPCs), upregulation of NFI-A levels induces differentiation along the erythroid lineage, while its downregulation shifts HSC/HPC fate toward the granulocytic lineage. Therefore, NFI-A acts as a regulator of human hematopoietic stem cell/progenitor lineage choice.[44-46]

Dissecting the epigenetic mechanisms that regulate NFI-A gene expression in HSC/HPCs, we observed, that in human ES cells, the NFI-A promoter region is marked by an H3K27me3/H3K4me3 bivalent domain.[20] We showed that, in hematopoietic cells, the resolution of the NFI-A bivalent domain is required to direct granulopoiesis or erythropoiesis.[46] During granulocytic differentiation of primary HSC/HPCs and myeloid precursor cell lines, NFI-A gene silencing is caused by the recruitment of PcG proteins YY1 and Suz12. These trigger the resolution of the bivalent domain (H3K27me3 increased, whereas H3K4me3 decreased) and induce nearby heterochromatin formation. A stable Suz12-knockdown in myeloid progenitor HL60 cells impeded the bivalent domain resolution, increased the expression levels of NFI-A and impaired retinoic acid (RA)-induced granulocytic differentiation.[46]

YY1 is the only PcG protein possessing a sequence-specific element (CCATnTT) for DNA binding, where it can recruit other PRCs.[47] A bio-informatic analysis of NFI-A promoter region showed that it is rich in consensus YY1 binding sites and GAGAG recurrent motifs, recently confirmed as Polycomb Responsive Elements (PREs).[7,48]

PcGs also own RNA binding properties and ncRNAs may be required for PcG recruitment to DNA, promoting chromatin remodeling and transcriptional gene silencing (TGS).[49,50]

We then hypothesized that a leading role in PRC promoter targeting is played by non-coding RNA (ncRNA) or by RNAi machinery components. We found that, in HL60 cells undergoing RA-induced granulocytic differentiation, YY1 associates with Dicer1.[46] These two proteins share a localization at the nuclear periphery, a site of preferential heterochromatin localization.[51] Moreover, YY1, Suz12, Dicer1 and transiently Argonaute (Ago) 1 accumulate on NFI-A promoter to form a repressive complex.[46] Accordingly, the silencing of both Dicer1 and Ago1 in HL60 cells significantly impaired the RA-induced bivalent domain resolution on NFI-A promoter. This affected

NFI-A-expression and cell differentiation response, thus supporting a direct role of these RNAi components in the transcriptional regulation of this gene.

These findings are also in agreement with emerging data attributing to component of RNAi machinery, such as Dicer and Ago proteins, a direct role in determining chromatin modifications. Dicer2 and Ago2 were found associated to chromatin and contribute to transcriptional regulation in Drosophila.[52] In *Schizosaccharomyces pombe*, Dicer1 physically associates with chromatin, providing direct evidence for RNAi-mediated heterochromatin formation in cis at centromeric repeats.[53] Moreover, Giles et al.[54] have shown that RNAi components Dicer1 and Ago2 are recruited at the 16 Kb chicken β-globin locus. The chromatin landscape of this locus is altered in siDicer1- and siAgo2–6C2 cells (lowered H3K9me2 levels, increased H4acetylation and H3K9 acetylation levels). Ahlenstiel et al.[55] also investigated the subcellular co-localization of Ago proteins with promoter-targeted siRNAs during TGS of SIV and HIV-1 infection. Their study revealed that Ago1 co-localizes with siRNA in the nucleus at the sites of TGS, while Ago2 co-localized with siRNA in the inner nuclear envelope. Overall, these and our results directly implicate Dicer1 and Ago proteins in epigenetic marking, heterochromatin formation and transcriptional activity of target genes, although some discrepancy regarding the specific role of Ago1 and Ago2 proteins still exists. How Polycomb and RNAi components can be guided to the controlled genomic loci is still an open question. We hypothesized that a newly discovered nuclear roles of small or long non-coding RNA also involve their direct binding of target genes genomic loci.

Gene Transcriptional Control by MicroRNAs

Emerging evidence suggest that noncoding RNA (ncRNAs), have important regulatory functions in the control of transcriptional programs. Besides determining the post-transcriptional gene silencing of their targets, ncRNAs may act as sensors and integrators of a wide variety of transcriptional responses and, probably, epigenetic events too.[56]

MicroRNA (miRNAs) genes transcribe small ncRNAs ~22 nucleotides long, which repress the expression of proteins involved in development, differentiation, proliferation and apoptosis. They mainly act through limited base-pairing to complementary mRNA sequences. Proper miRNAs' functioning requires their assembly into an RNA-induced silencing complex (RISC), which includes Dicer, members of the Argonaute protein family, the RNA binding protein TRBP and other proteins. In a RISC, miRNAs serve as the specificity guide for target mRNA recognition.[57,58] MiRNA expression is tissue-specific and highly regulated according to the cell's developmental lineage and stage.[57,58]

MiRNAs are also present in integrated transcriptional regulatory circuitries. They can change the expression levels of lineage-specific transcription factors and/or members of the epigenetic machinery and are, in turn, regulated by these factor activities.[59,60]

Recently it has been shown that miRNAs expression and activity can be also modulated by the cell type- and allele-specific expression of snpRNAs (single nucleotide polymorphism RNAs), which represent allele-specific "decoy" targets of miRNAs. MiRNA may act as intrinsic regulatory components of snpRNA networks that contribute to maintenance of the epigenetic state in cells.[61]

MiRNAs are therefore ideal intermediate in transcriptional regulation, as base pairing with target sequences can account for specificity and ensure the robustness of cell fate decision.

Growing evidence indicates that miRNAs also have nuclear functions. The canonical miRNA biogenesis implies Exportin 5-mediated transfer of the pre-miRNA from nucleus to cytoplasm.[57,58,62] However, a number of studies indicate that miRNAs may shuttle between cytoplasm and nucleus. This was first revealed in HeLa cells for miR-29b, which displays a mostly nuclear localization, determined by a 3' terminal hexanucleotide.[63] Further studies have shown that both CRM1 and Importin 8 contribute to intracellular miRNA transport.[64,65] These shuttling proteins can conduct nuclear importation for both miRNA and Ago proteins. Accordingly, a number of mature miRNAs were identified by microarray and RT-qPCR analysis in purified nuclei of colon cancer cells and in the nucleolus of rat myoblasts.[66,67] A clear demonstration of the nuclear distribution of miRNAs came from a deep-sequencing analysis of small RNAs in nuclear and cytoplasmic fractions derived from a human nasopharyngeal carcinoma cell line.[68] Three hundred miRNAs were identified both in the nucleus and in the cytoplasm, whereas 39 miRNAs displayed a preferential nuclear localization.[68] Overall, the nuclear distribution of miRNAs seems to be solid evidence. Indeed, besides their cytoplasmic activity, miRNAs may associate with RISC components in the nucleus to regulate transcription, contributing to both transcription activation and repression of genes. However, the presence of miRNAs in a transcription regulating molecular complex on the promoter of target genes and the biological relevance of miRNA-mediate transcriptional regulation was not analyzed in depth.

MiRNA and RNAi Components are Present on Promoters in Protein Complexes with PcGs to Regulate Target Gene Transcription

A role for miR-223 in driving TGS by PcGs-RNAi complexes was suggested by the presence on the NFI-A promoter region of two DNA sequences 5'-AAACTG-3' complementary to the miR-223 seed sequence (3'-UUUGACUGU-5'). These sequences are located at a distance of 1–2 nucleosomes from the YY1-Dicer1-Ago1 binding site, where the epigenetic modifications controlling NFI-A expression occurred. Indeed, we found that during human granulopoiesis, miR-223 translocates to the nucleus and localizes at its complementary sequences on NFI-A promoter.[46] We identified nuclear localization and NFI-A promoter targeting of miR-223 by multiple approaches, including confocal microscopy of transfected fluorescent miR-mimics and in situ hybridization to the endogenous miRNA with fluorescent oligonucleotides complementary to the sequence of miR-223. We could also detect

miR-223-specific signals on chromosome spreads from cells undergoing myeloid differentiation, but not undifferentiated cells. The signals were often detectable on symmetrical regions of sister chromatids, thus suggesting a gene-specific localization. Since, during metaphase mRNA synthesis is blocked, this finding may support a view whereby miR-223 directly binds to DNA on specific sequences. In agreement, by combining transfection of a Cy5-labeled miR-223 with anti-Cy5 chromatin immunoprecipitation we were able to demonstrate the presence of miR-223 on the NFI-A promoter DNA. Here, miR-223 is part of a ribonucleoprotein repressive complex involving YY1, Dicer-1 and transiently Ago1. The localization of this complex on the NFIA promoter is RNA-dependent, since it is prevented by RNase treatment and is enhanced by miR223 overexpression.[46]

A similar miRNA-dependent complex on DNA has been recently reported on the promoter of CDC2 and CDCA8, two E2F/Rb1 target genes, which contain a miRNA-complementary sequence in the antisense and sense strand, respectively.[69] In this case the miRNA involved is let-7f and the RISC component is Ago2. This complex involves the Rb1 and E2F protein and its aggregation is dependent on both Ago2 and let-7f.[69] Functionally, both miR-223 and let-7f containing nuclear complexes repress gene transcription. We found that miR-223 contributes, together with the Polycomb/RNAi complex, to the resolution of H3K27me3/H3K4me3 chromatin bivalent domains on the NFI-A promoter, which induces heterochromatin formation and transcriptional gene silencing.[46] Nuclear shifting of miR-223 and miR-223 overexpression increase-histone repressive marks and induce granulocytic differentiation, whereas blocking miR-223 activity using a miR-ZIP vector has the opposite effect (increases the activation histone mark H3K4me3 and blocks granulopoiesis).[46] In a similar way, the transcriptional complex assembled by let-7f, Ago2, and pRb1 on E2F target genes induces heterochromatin histone marks, with increased H3K27me3, H3K9me3 and decreased H3K4me3 and H3K9ac.[69] The activity of let-7f seems to contribute to the process of cellular senescence. Thus, by cooperating with Rb1 in the transcriptional silencing of E2F target genes let-7f participates in a fundamental biological response. Actually, antagomiRs against let-7f restore proliferation in senescent fibroblasts and extend their lifespan.[69]

MiRNAs Can Both Repress and Activate Gene Transcription

Several other reports have investigated transcriptional repression by miRNAs. MiR-320 transfection in fibroblasts induces TGS of the POLR3D gene, a subunit of RNA polymerase III, whose promoter contains a conserved miR-320 complementary sequence.[70] Concurrently Ago1, PcG component EZH2, and H3K27me3 associate on the gene promoter. These data and our findings suggest that miRNAs may repress transcription by driving a repressor protein complex to the promoter of target genes. Transcriptional repression has been also observed on the Progesterone Receptor gene upon transfection of double strand miR-mimic sequences selected for their partial complementarities to promoter-generated antisense small ncRNA.[71] TGS was accompanied by DNA methylation and H3K9me2 enrichment at the gene promoter and association of Ago2 with PR promoter generated ncRNA, suggesting that miR-423–5p may operate by complementarity to ncRNA. A robust TGS was also obtained on the HOXD4 gene by transfection of miR-10b in breast cancer cell lines.[72] This effect was inhibited by Ago1- and Ago3-siRNA and was possibly mediated by miR-10b interaction with ncRNA transcribed at the promoter. Anti-miR-10b transfection caused a strong increase in HOXD4 expression indicating that TGS by the endogenous miR-10b may be operating stably in vivo.[72] miR17–5p and miR-20 also induced heterochromatin features in promoters (TBCEL, RASA2, RHPN2 and WHSC1), which possess sequence complementarity to these miRNAs.[73] Overall, these observations and our data compose a picture whereby RISC components, PcG proteins and miRNAs associate on DNA to induce TGS. On the other hand, miRNA may be able to activate transcription. The genes encoding E-cadherin and cold-shock domain-containing protein C2 (CSDC2) have miR-373 complementary sequences in their promoter. Transfection of miR-373 into prostate and colorectal cancer cell lines induced their expression.[74] MiR-744 induced Cyclin B1 expression and enrichment of Ago1, RNA PolII and H3K4me3 at the gene promoter, where miR-744 complementary sequences were found.[75]

Sequence-specific Evolutionary Conservation of MiRNA Targeting Activity

In our study, the integrity of two miR-223 complementary seed-matches sequences on NFI-A promoter was required for TGS of NFI-A promoter reporter constructs during granulopoiesis.[46] Both the NFI-A promoter binding sites for miR-223 sequence are evolutionarily conserved. Specifically, the conservation for both these sites is higher in mammalians presenting adult hematopoiesis centered in the bone marrow, whereas it is lost in marsupials, birds and fish, all of whose hematopoiesis differ significantly. The pre-miRNA sequence outside the mature miR-223 has a low homology with the NFI-A promoter sequence, thus, theoretically, preventing its hybridization to nascent RNA.[46] The highest conservation between the two putative miR-223 binding sites on NFI-A promoter involves nucleotides of the 5' miR-223 "seed" region, which is the most important determinant for mRNA target recognition and base pairing.[76] Interestingly, miRNA target sites on mRNA are highly conserved among species. Non-conserved seed targets, however, are also regulated by miRNAs.[76,77] mRNAs with conserved miRNA target sites tend to be expressed in the same tissues as the related miRNA, whereas non-conserved miRNA target sites are often expressed in tissues in which the cognate miRNA is not expressed. Usually, changes in mRNA levels closely reflect the impact of miRNAs on gene expression. As a result, conserved targets are expressed at much lower levels in tissues that express the miRNA.[78] The opposite relationship between the expression patterns of miRNAs and their conserved mRNA targets underlies the role

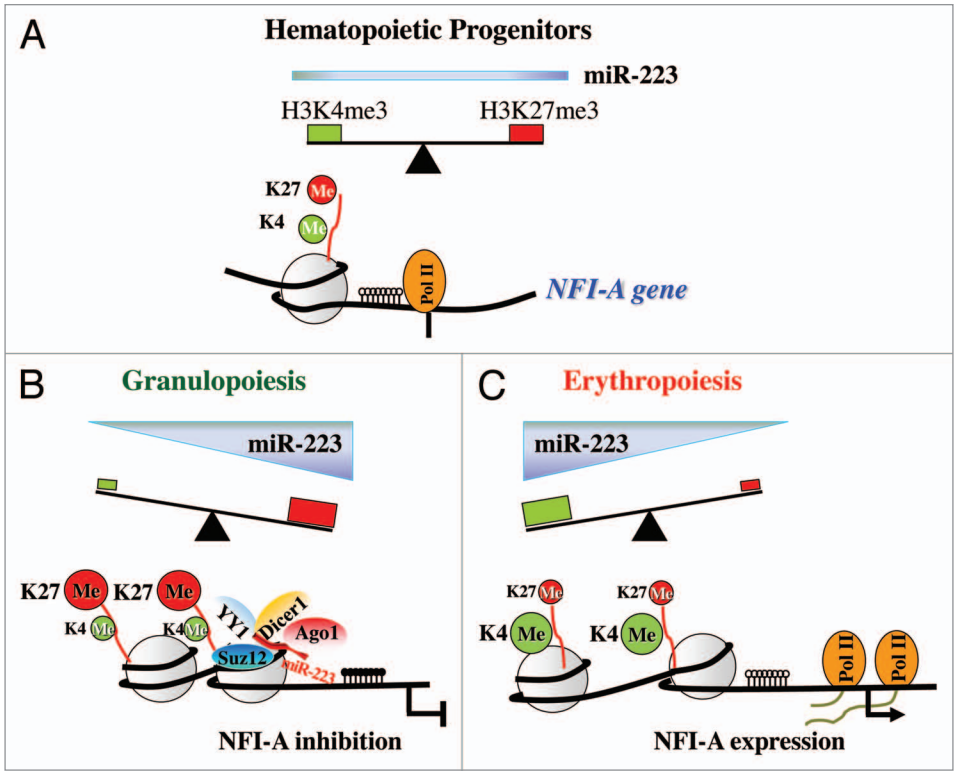

Figure 1. Transcriptional regulation of the NFI-A gene by a miR-223/Polycomb/RISC complex governs hematopoietic progenitors cell fate. (A) The undifferentiated hematopoietic progenitors display bivalent nucleosomal histone modifications on the NFI-A gene promoter, where H3K4me3 activating mark (green) and H3K27me3 repressive mark (red) are balanced. CpG islands are not methylated (white small circles). The expression levels of both miR-223 and NFI-A mRNA are low. (B) Upon granulocytic differentiation the expression of miR-223 markedly increases and bivalent domains are resolved: the repressive H3K27me3 mark accumulates whereas the activating H3K4me3 decreases and methylated CpGs increase (black small circles). This is due to the aggregation on the promoter of the NFI-A gene of a ribonuclear complex involving miR-223, the RISC components Ago1 and Dicer1 and the PcG proteins YY1 and Suz12. The complex is driven by miR-223 to miR-complementary sequences on the NFI-A promoter. The result is transcriptional repression of NFI-A gene. (C) During erythropoiesis the expression of miR-223 decreases and bivalent domains are resolved in the opposite direction. The NFI-A gene promoter acquires a "permissive chromatin status," with high levels of the activating histone marks H3K4me3, unmethylated CpGs and low levels of the repressive histone mark H3K27me3. NFI-A expression is high. Gray spheres indicate the octamer of nucleosomal histones with the double strand DNA wrapped around them.

of miRNAs in development and maintenance of tissue identity. Interestingly, miR-223 is almost exclusively expressed in bone marrow[79] and an inverse correlation between the expression patterns of miR-223 and NFI-A mRNA was detectable in normal and leukemic HSC/HPCs.[46] Thus, a cooperation between intimately connected post-transcriptional and transcriptional pathways may regulate the expression levels of developmental genes, such as miR-223 and NFI-A, which act as life-long key determinants of HSC/HPCs lineage restriction.

Concluding Remarks

Epigenetic marks are inheritable through cell divisions, however in response to differentiation-inducing cues these marks can be easily modified allowing developmental genes to be in a "poised state." Once a cell is committed to accept a certain fate, permanent repression or activation of developmental genes must take place to allow definitive cell fate decisions. We have now obtained evidence for a novel cooperative pathway of site-specific gene expression regulation. This pathway is triggered by the promoter recognition and transcriptional targeting activity of miR-223 and PcGs (YY1 and Suz12). Biologically, this mechanism is relevant for HSC/HPCs granulocytic lineage determination (**Fig. 1**). Here, we discussed data that support a key contribution of DNA targeting by miRNAs in the generation of an epigenetic "memory" of transcriptional programs. MiRNA base pairing with DNA sequences may provide robustness to regulatory epigenetic networks allowing definitive lineage fate decision.

Although a general picture of miRNAs-mediated nuclear pathways is emerging, many questions remain. Further studies need to be performed to elucidate whether, besides post-transcriptional gene silencing, endogenous miRNAs induce TGS of their targets via direct binding to DNA. Otherwise, the association of RISC components with members of the epigenetic machinery may alters DNA and chromatin structure.

Although the complementarity requirements for miRNA interaction with promoter sequences (either nascent RNA or DNA) are presently unknown, the three dimensional conformation of the DNA molecule may well determine these requirements, which may be different from

those defined over the years for mRNA in the cytoplasm. The next challenge will be to identify other miRNAs that may be active in cell nuclei as transcriptional regulators of genes requiring permanent transcriptional inhibition to allow definitive cell fate decisions.

New insight into the functioning of genetic and epigenetic regulatory associations is essential for the development of novel strategies to maintain stem cells, reprogram differentiated cells or direct cell differentiation. In addition, aberrant interactions between these factors may promote cell transformation and contribute to malignancy. Thus, a new vision of miRNA's range of activities may provide new clues into cancer pathogenesis, generating novel approaches to cancer treatment.

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