

Transcriptional fine-tuning of microRNA-223 levels directs lineage choice of human hematopoietic progenitors

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MicroRNAs (miRNAs) regulate cell proliferation, differentiation and death during development and postnatal life. The expression level of mature miRNAs results from complex molecular mechanisms, including the transcriptional regulation of their genes. MiR-223 is a hematopoietic-specific miRNA participating in regulatory signaling networks involving lineage-specific transcription factors (TFs). However, the transcriptional mechanisms governing its expression levels and its functional role in lineage fate decision of human hematopoietic progenitors (HPCs) have not yet been clarified. We found that in CD34⁺ HPCs undergoing unilineage differentiation/maturation, *miR-223* is upregulated more than 10-fold during granulopoiesis, 3-fold during monocytopoiesis and maintained at low levels during erythropoiesis. Chromatin immunoprecipitation and promoter luciferase assays showed that the lineage-specific expression level of mature miR-223 is controlled by the coordinated binding of TFs to their DNA-responsive elements located in 'distal' and 'proximal' regulatory regions of the *miR-223* gene, differentially regulating the transcription of two primary transcripts (pri-miRs). All this drives myeloid progenitor maturation into specific lineages. Accordingly, modulation of miR-223 activity in CD34⁺ HPCs and myeloid cell lines significantly affects their differentiation/maturation into erythroid, granulocytic and monocytic/macrophagic lineages. MiR-223 overexpression increases granulopoiesis and impairs erythroid and monocytic/macrophagic differentiation. Its knockdown, meanwhile, impairs granulopoiesis and facilitates erythropoiesis and monocytic/macrophagic differentiation. Overall, our data reveal that transcriptional pathways acting on the differential regulation of two pri-miR transcripts results in the fine-tuning of a single mature miRNA expression level, which dictates the lineage fate decision of hematopoietic myeloid progenitors.

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Hematopoiesis depends on changes in gene expression mediated by transcriptional regulators.^{1–3} By binding *cis*-acting elements within their target gene promoters, lineage-specific transcription factors (TFs) affect self-renewal and differentiation/maturation of hematopoietic stem/progenitor cells (HPCs).^{1,2,4} MicroRNAs (miRNAs) affect key developmental hematopoietic programs. Their aberrant expression or function has been associated with hematopoietic diseases.^{5–7}

MiRNAs exhibit stage- and tissue-specific expression patterns, finely regulated during development and adult life.^{8–12} MiRNAs primarily silence gene expression at the post-transcriptional level.^{10,13} Individual miRNA affect

cell-type-specific protein profiles by targeting hundreds of mRNAs^{14–17} or by directing transcriptional gene silencing.^{18,19}

MiRNAs are mainly transcribed by RNA polymerase II (Pol-II) into primary transcripts (pri-miRNAs).²⁰ MiRNAs are integrated in regulatory circuitries, which can change the expression levels of lineage-specific TFs and are in turn regulated by TF activities.^{21–25} However, the structural features of *miRNA* promoters and factors responsible for transcriptional regulation of *miRNA* genes remain poorly understood. Similarly unexplored is the role of multiple pri-miR transcripts in generating the final levels of mature miRNA.

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Abbreviations: AML, acute myeloid leukemia; Ara-C, 1- β -D-arabinofuranosyl cytosine; BFU-E, burst-forming unit-erythroid; C/EBP α , CCAAT/enhancer-binding protein α ; C/EBP β , CCAAT/enhancer-binding protein β ; CFU-GEMM, granulocyte, erythroid, macrophage, megakaryocyte colony-forming unit; CFU-GM, granulocyte, macrophage colony-forming unit; Epo, erythropoietin; Flt3 ligand, fms-related tyrosine kinase 3 ligand; GAPDH, glyceraldehyde phosphate dehydrogenase; GATA-1, GATA-binding protein 1; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HPC, human hematopoietic progenitor; HSC, hematopoietic stem cell; IL-3, interleukin-3; IL-6, interleukin-6; LMO2, LIM domain only 2; M-CSF, macrophage colony-stimulating factor; miRNA, microRNA; NFI-A, nuclear factor IA; PU.1, spleen focus-forming virus proviral integration 1; RA, retinoic acid; SCF, stem cell factor; TAL1, T-cell acute lymphocytic leukemia 1; TF, transcription factor; TPO, thrombopoietin; TSS, transcription start sites; VitD3, vitamin D₃

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The information for transcription and processing of miRNAs is present in the upstream regions of their genes, which contain more regulatory motifs than protein-coding gene promoters.²⁶ Binding sites for TFs are located upstream of the precursor miRNA hairpin (pre-miRNA) gene sequences and/or nearby *miRNA* transcription start sites (TSSs).²⁷ Moreover, human intergenic *miRNAs* often possess two predicted TSS located within ~2 and ~10 kb upstream of pre-miRNA sequences.^{27,28}

MiR-223 is an intergenic *miRNA* expressed at high levels in human peripheral blood (PB) granulocytes and bone marrow (BM) committed/mature myeloid precursors.^{18,25} Epigenetic regulation of *miR-223* correlates with myeloid differentiation and acute myeloid leukemia (AML) leukemogenesis.^{18,25,29,30}

Two regulatory regions may direct *miR-223* transcription during myelopoiesis: one involving *cis*-regulatory elements for myeloid TFs PU.1 (spleen focus-forming virus proviral integration 1) and CCAAT/enhancer-binding protein β (C/EBP β) at about 3400 bp relative to the 5' end of the pre-miR-223,³¹ and the other involving the competitive binding of CCAAT-box-binding TFs, the nuclear factor IA (NFI-A) and CCAAT/enhancer-binding protein α (C/EBP α), for the same CAAT element located at about 1000 bp upstream of the pre-miR-223.²⁵ The significance and the transcriptional regulation of these regulatory regions with regard to hematopoietic lineage specificity are still open questions. Moreover, the physiological roles of the changes in mature miR-223 levels generated by these transcriptional mechanisms in hematopoietic cells are unknown.

Here, we show that the coordinated recruitment and function of different lineage-specific TFs on two regulatory regions of the *miR-223* finely modulate the transcription of two pri-miR-223. Our results indicate the differential transcriptional regulation of pri-miR transcripts as an additional mechanism directing hematopoietic specificity. Moreover, we show that variations in the expression levels of miR-223 can direct HPC differentiation fate.

Results

Expression pattern of mature miR-223 during erythromyelopoiesis. Figure 1a shows the expression of mature miR-223 in PB nucleated cells (NCs), CD34⁺HPCs from normal donors and blasts from the PB and/or BM of 40 newly diagnosed non-acute promyelocytic leukemia (AML) patients. We excluded t(8;21) leukemias, in which miR-223 expression is epigenetically downregulated by AML1/ETO.³⁰ In comparison to mature granulocytes, the expression level of miR-223 is silenced in CD34⁺HPCs. In AML samples, miR-223 is expressed at low levels in undifferentiated AML subtypes (M0/M1) and erythroid leukemias (FAB-M6). In myeloblastic leukemia (FAB-M2), miR-223 is higher than in the AML subtypes along the myelomonocytic lineage (FAB-M4) and, particularly, monocytic lineage (FAB-M5). Overall, miR-223 appears mostly suppressed in AML samples with respect to PB NCs and its level correlated with the stage of myeloid differentiation.

We then analyzed mature miR-223 expression levels in RNA samples collected at sequential stages of unilineage differentiation/maturation of CD34⁺HPCs toward the

erythroid (E), granulocytic (G) or monocytic/macrophagic (Mo) lineages (Figure 1b). Cell growth, morphology, decrease in CD34 expression and increase in lineage-specific surface antigens are shown in Supplementary Figures S1A–C.

Mature miR-223 is maintained at low levels in CD34⁺HPCs undergoing erythroid differentiation, whereas a strong upregulation (10- to 15-fold compared with CD34⁺HPCs) is observed in cells induced to granulopoiesis (Figure 1b). During monocytic/macrophagic differentiation, a moderate and transient upregulation of *miR-223* is measurable at 7 days of cultures (reaching approximately threefold accumulation compared with CD34⁺HPCs) (Figure 1b).

We used HL60 cells as a differentiation model of bipotential myeloid precursors. Retinoic acid (RA)-induced granulocytopenesis and vitamin D₃ (VitD3)-induced monocytic differentiation³² were revealed by morphological evaluation (not shown) and immunophenotypical analysis of the myelomonocytic markers CD11b and CD14 (Figure 1c and Supplementary Figure S1D).

In RA-treated HL60 cells, mature miR-223 reached a five- to sixfold accumulation at 72–96 h. In VitD3-treated cells, miR-223 only reached about two- to threefold accumulation compared with untreated cells (Figure 1c).

Changes in miR-223 levels were not appreciably detectable in K562 cells induced by 0.5 μ M 1- β -D-arabinofuranosyl cytosine (Ara-C) treatment³³ into an erythroid pathway, as measured by the levels of the erythroid surface antigen CD235a and the percentage of benzidine-stained cells (Figure 1c and Supplementary Figure S1E).

Pri-miR-223 transcriptional variants are differentially regulated during hematopoiesis.

MiR-223 is composed of two intron and three exon sequences. The pre-miR-223 and mature miR-223 sequences are located in the third exon (Figure 1d). We measured, by qRT-PCR, the expression levels of the longest pri-miR-223 transcript (V1)³¹ in CD34⁺HPCs undergoing unilineage granulocytic, monocytic and erythroid differentiation, using primers located on exon 1 (oligo #1; Figure 1d). In CD34⁺HPCs, V1 is more efficiently upregulated during granulocytopenesis than monocytopenesis and a decline occurred during erythroid differentiation (Figure 1e). By amplifying the exon 3 and 26 nucleotides (nts) of the upstream intron 2 (oligo #2), we identified another pri-miR-223 transcript variant (V2), strongly upregulated upon granulocytic, but not monocytic or erythroid differentiation of CD34⁺HPCs (Figures 1d and f).

In HL60 cells, the V1 transcript is again more efficiently upregulated by RA-induced granulocytic than VitD3-induced monocytic differentiation (Figure 1g). The V2 transcript is upregulated exclusively by RA-induced granulopoiesis (Figure 1h). In K562 cells, the basal levels of V1 and V2 are lower than in HL60 cells. No substantial differences in these variant levels are measurable in cells primed to erythroid differentiation by Ara-C treatment with respect to control cells (Figures 1g and h).

Thus, the two pri-miR-223s are differentially modulated upon lineage differentiation of CD34⁺HPCs. As both these transcripts possess the stem-loop secondary structure, their expression determine the final levels of mature miR-223 (Supplementary Figure S2).

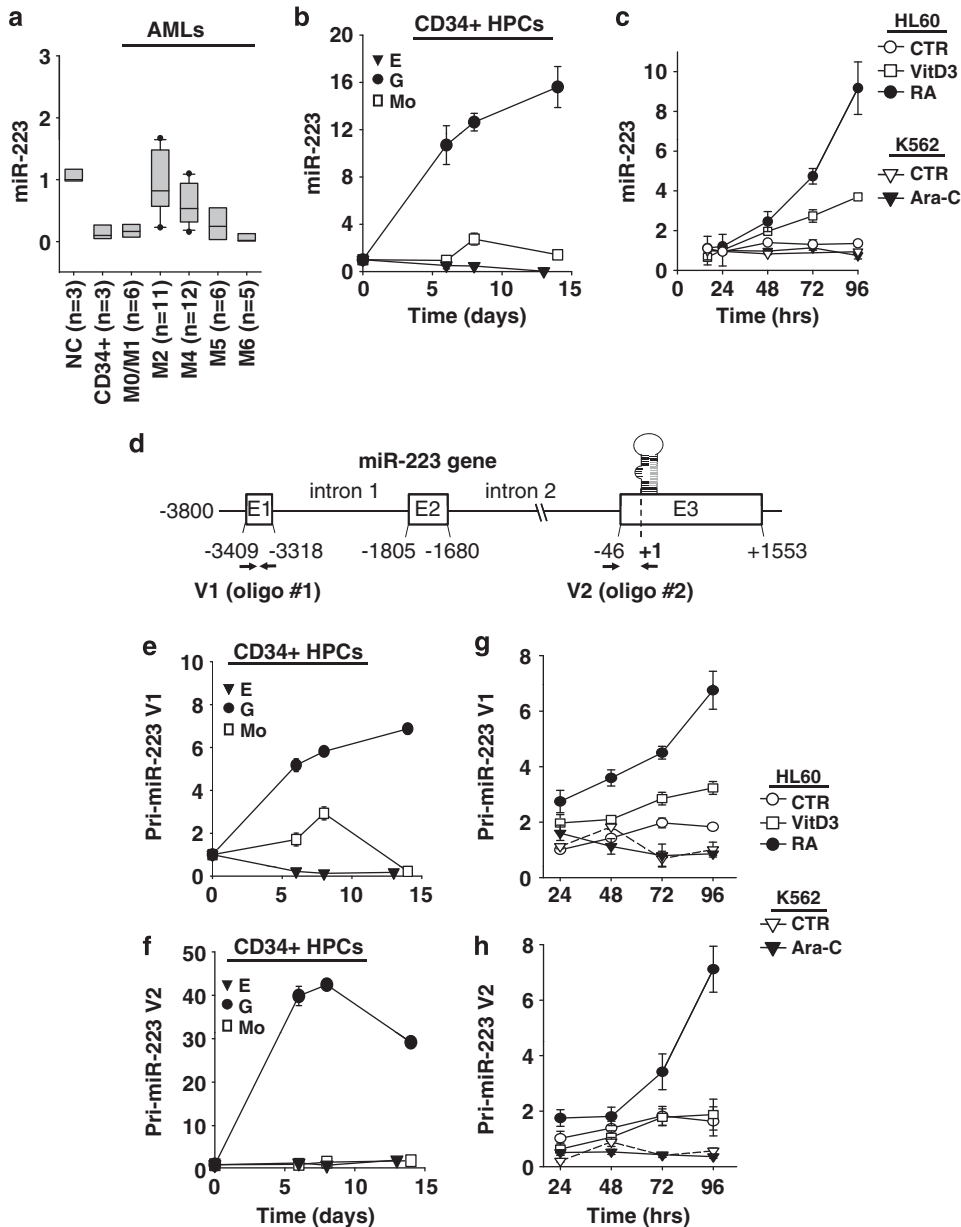


Figure 1 Expression levels of mature miR-223 and pri-miR-223 precursor variants in AML blasts in CD34⁺ HPCs cultures and in myeloid cell lines undergoing erythroid, granulocytic and monocytic differentiation/maturation. (a) Box-plot diagram showing the distribution of mature miR-223 expression in NCs, CD34⁺ HPCs from PB or BM of healthy donors and 40 AML patient blasts classified according with the FAB subtypes. Relative qRT-PCR quantification of miR-223 expression levels. The median (—) and outlier values (●) are indicated. qRT-PCRs measuring the expression levels of mature miR-223 (b), pri-miR-223 transcript variant V1 (e) and pri-miR-223 transcript variant V2 (f) in CD34⁺ HPCs undergoing erythroid (▼), granulocytic (●) and monocytic (□) unilineage differentiation/maturation; qRT-PCRs measuring the expression levels of mature miR-223 (c) pri-miR-223 transcript variants V1 (g) and pri-miR-223 transcript variant V2 (h) in HL60 untreated (○), treated with 250 ng/ml VitD3 (□) or 1 μ M RA (●) and K562 untreated (▽) or treated with 0.5 μ M Ara-C (▼) at the indicated time points. Data are normalized on RNU24 or GAPDH expression levels and represent the mean \pm S.D. of three independent evaluations. (d) Schematic representation of the *miR-223* (not in scale). The location of the exon (E) and intron sequences along the gene is indicated. Numbers are the nucleotides relative to the 5' end of the pre-miR223 sequence (nt + 1). Arrows indicate the position of the primers used to amplify pri-miR223 transcript variants V1 (oligo #1 nt - 3408/ - 3323) and V2 (oligo #2 nt - 70/ + 91)

The pri-miR-223 variants originate from a major transcriptional start site (TSS1) located at exon 1 and a second start site in intron 2 (TSS2)³¹ (Figure 2a). We investigated the differential regulation of these TSS by RNAse protection assay (RPA) on RNA extracted from HL60 cells undergoing RA-induced granulocytic and VitD3-induced monocytic differentiation.

A riboprobe complementary to the first exon (probe #1) produced a 91-nt protected fragment, whose expression reached a maximum induction of about 5- and 2.5-fold upon 96 h of RA and VitD3 treatment, respectively, as normalized with a β -tubulin riboprobe (V1; Figures 2b and d). Probe #2, comprising the end of the second intron and part of the third exon, measured in the same samples both the transcripts

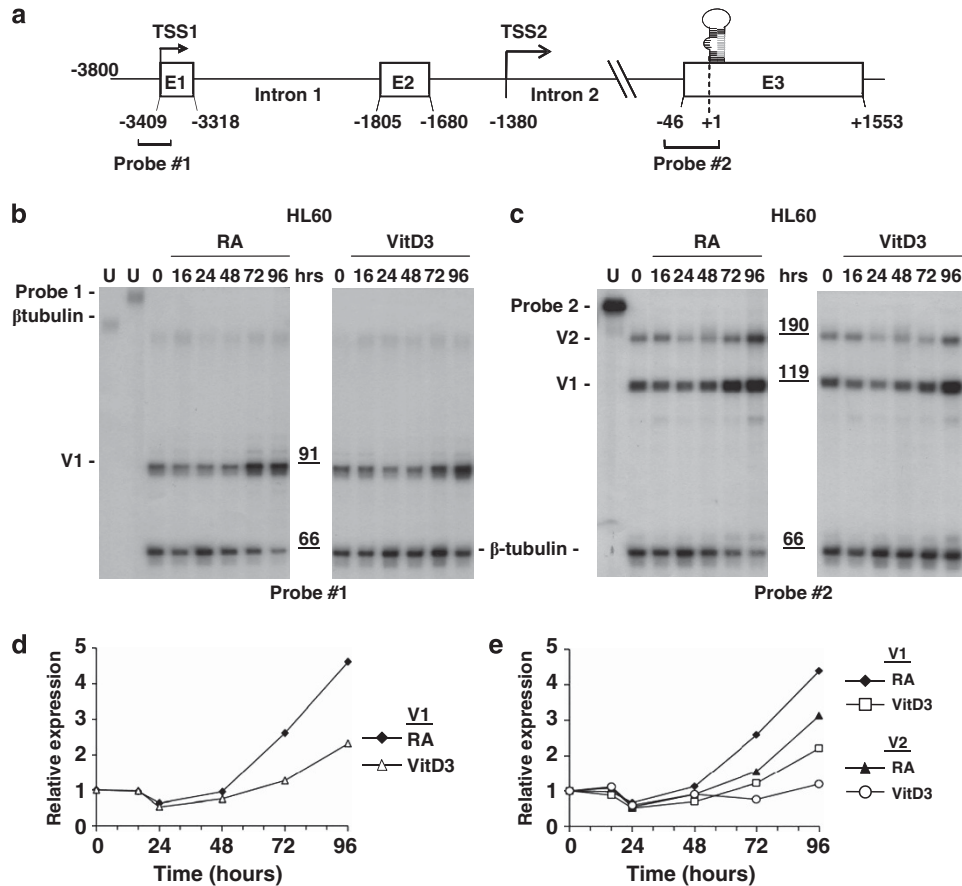


Figure 2 Characterization of human pri-miR-223 precursors expression by RPA assay. (a) Schematic representation of the *miR-223* (not in scale). The location of the two transcriptional start sites^{25,31} (TSS1 and TSS2) is indicated. Numbers are the nucleotides relative to the 5' end of the pre-miR-223 sequence (nt + 1). Probe #1 and 2 indicate the position of the riboprobes used for the RPA assay. (b and c) RPA assay performed with the indicated riboprobes, on RNA extracted from HL60 cells, untreated or treated with 1 μ M RA or 250 ng/ml VitD3 for the indicated time. U: control undigested probe #1, probe #2 and β -tubulin riboprobe. V1 and V2: protected fragments generated by the two transcripts; β -tubulin: protected fragment generated by the β -tubulin riboprobe used as quantitative control. (d and e) Densitometric scanning of the signals shown in (b and c), respectively; the values are relative to the β -tubulin control

derived from TSS1 (V1) and TSS2 (V2), respectively, as 119 and 190 nts protected fragments (Figure 2c). The V1 fragment was induced about fivefold, similarly to the transcript identified by probe #1. The V2 transcript was induced about 3.5-fold upon RA treatment, but only slightly modified by VitD3 (Figures 2c and e). The maximum amount of the V2 transcript was about 30% of the transcript originating from exon 1, thus significantly contributing to total pri-miR-223 levels. Overall, both pri-miR-223 variants contribute to increase in miR-223 levels during granulopoiesis.

TF occupancy of *miR-223* regulatory regions. We analyzed the role of TFs in the regulation of the *miR-223* transcription. This gene has two regulatory regions located at about 3.5 kb (distal) and 1 kb (proximal) relative to the pre-miR-223 sequence (Figure 3a and Supplementary Figure S3A).^{25,31} TFs regulating myeloid lineage commitment GATA-binding protein 1 (GATA-1), C/EBP β and PU.1 locate on the distal regulatory region, whereas C/EBP α and NFI-A are present on *miR-223* proximal regulatory region.^{25,31} A bioinformatic search revealed previously unreported DNA binding sites for TFs GATA-1, LMO2/TAL1 (LIM domain only

2/T-cell acute lymphocytic leukemia 1), PU.1 and C/EBP β in the proximal *miR-223* regulatory region (Supplementary Figure S3A).

We measured the expression levels of these TFs by immunoblotting (Supplementary Figures S3B and C). In HL60 cells, both RA and VitD3 treatments decreased the expression of NFI-A. In contrast, the p42 kDa active isoform of C/EBP α , but not truncated C/EBP α -p30,^{34,35} was upregulated at 16–48 h by both RA and VitD3 (Supplementary Figure S3B). The C/EBP β -long 30 kDa activator (LAP) and short 20 kDa inhibitor (LIP) isoforms³⁶ appear induced after 48 h VitD3 treatment, whereas LAP increased modestly in RA-treated HL60 cells. PU.1 expression is not significantly affected by RA or VitD3 treatment (Supplementary Figure S3B).

During Ara-C-induced erythroid differentiation of K562 cells, no significant changes in NFI-A, LMO2, PU.1 and C/EBP β isoform levels were measurable, whereas the erythroid TFs GATA-1 and TAL1 were upmodulated. C/EBP α was undetectable (Supplementary Figure S3C).

We next addressed the presence of these TFs at their putative binding sites on *miR-223* regulatory regions

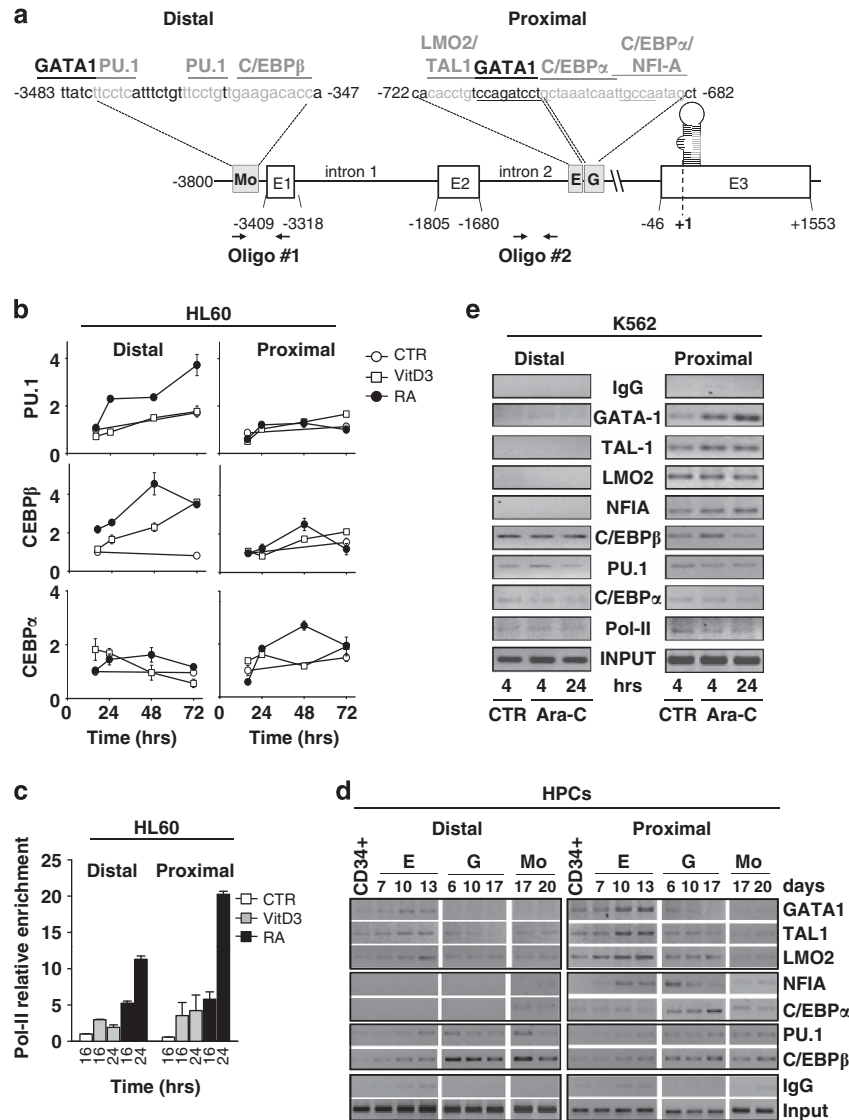


Figure 3 Recruitment of lineage-specific TFs on the *miR-223* regulatory regions. (a) Schematic representation of lineage-specific TF binding sites on *miR223* distal and proximal regulatory regions (not in scale). Numbers are the nucleotides relative to the 5' end of the pre-*miR223* sequence (nt + 1). Arrows indicate the position of the primers used in ChIP assays (oligo #1 nt - 3510/ - 3324 and oligo #2 nt - 1006/ - 808). ChIP assays were performed on chromatin immunoprecipitated using the indicated antibodies and semiquantitative or qRT-PCR amplification in: (b and c) HL60 cell lines at the indicated times and treatments; (d) primary CD34⁺ HPCs undergoing erythroid (E), granulocytic (G) and monocytic (Mo) unilineage cell differentiation; (e) K562 cell lines at the indicated times and treatments. Chromatins were immunoprecipitated using antibodies recognizing the indicated TFs. Control amplifications were carried out on input chromatin before immunoprecipitation (Input) and on mock-immunoprecipitated chromatin (IgG). Data represent the mean \pm S.D. of three independent evaluations

(Figure 3a) in HL60, K562 and in CD34⁺ HPCs undergoing differentiation/maturation. Chromatin immunoprecipitation (ChIP) analysis showed an increased binding of PU.1 and C/EBP β , and to a lesser extent of C/EBP α , on distal *miR-223* region during RA-induced granulocytic differentiation of HL60 cells (Figure 3b). CEBP β was also enriched in the distal regulatory region after 48 h of VitD3 treatment. However, no specific changes of PU.1 and C/EBP β occupancy occurred at the proximal region of *miR-223* in HL60 cells treated with RA or VitD3. In contrast, C/EBP α occupancy on proximal regulatory region of *miR-223* was markedly increased by RA treatment but not by VitD3 (Figure 3b). At both the distal and proximal *miR-223* regulatory regions, RA treatment increased the occupancy

of RNA Pol-II (Figure 3c), indicating the functional relevance of these results.

To prove the physiological role of the identified TFs, we studied their binding to *miR-223* in CD34⁺ HPCs induced to granulocytopoiesis. C/EBP α replaces NFI-A on the proximal regulatory region, as previously reported in myeloid cell lines,²⁵ but is undetectable on distal promoter (Figure 3d). This is probably due to the different sensitivity of the methodologies used (qRT-PCR and semiquantitative PCR). During granulocytic and monocytic differentiation, PU.1 and C/EBP β appear recruited mainly on the distal *miR-223* regulatory region, but released at late stages of monocytic differentiation, explaining the reported decreased expression of *miR-223* in macrophages.^{37,38}

During erythroid differentiation of CD34⁺HPC, the TFs GATA-1, TAL-1, LMO2 and NFI-A progressively increased the occupancy of their sites on the proximal miR-223 regulatory region (Figure 3d).

These data were validated in K562 cells, where Ara-C-induced erythroid differentiation increased the occupancy of GATA-1 and TAL1 sites on the *miR-223* proximal regulatory region. PU.1, C/EBP β factors and Pol-II enriched both distal and proximal regulatory regions of miR-223. C/EBP α was undetectable (Figure 3e).

Altogether, these findings (i) supported the coordinated presence of myeloid and/or erythroid regulatory factors on *miR-223* regulatory regions; (ii) point at c/EBPs and PU.1 TFs as positive regulators and (iii) GATA/TAL1/LMO2 as negative regulators of *miR-223* transcription during myelopoiesis and erythropoiesis, respectively.

Different transcriptional regulation of distal and proximal *miR-223* regulatory regions. To validate functionally our findings, we set up luciferase-based transcriptional assays in HL60 cells stably carrying a luciferase reporter gene driven by either the proximal or the distal *miR-223* regulatory regions (Figure 4a). In these cells, we measured the luciferase activity upon RA-induced granulocytic or VitD3-induced monocytic differentiation. RA treatment caused a marked dose- and time-dependent increase in the activity of both the proximal and distal regulatory regions (Figure 4b, left and right panel, respectively). However, the effect of VitD3 treatment was less pronounced, and mostly affected the activity of the distal regulatory region. These findings match the expression data reported above and indicate the diverse regulation of the two regions in different myeloid lineages.

To address the role of TFs, we transiently transfected in U937 and K562 cells luciferase reporter constructs driven by mutants of the two regulatory regions (Figures 4c and d). The cells were treated with RA or VitD3 or Ara-C for 48 h before measuring the luciferase activity on cell lysates. The results indicate that mutation of the C/EBP β (M1, M3) and the PU.1 (M2, M3) binding sites markedly impairs RA- or VitD3-induced activity of the distal regulatory region (Figure 4c, left panel). RA- or VitD3-mediated activation of the proximal regulatory region was blocked by deleting the binding sites for C/EBP β s (Δ 2), but not those for erythroid factors GATA-1 and TAL1/LMO2 (Δ 1) (Figure 4c, right panel). These data functionally prove the relevance of TFs binding to different *miR-223* regulatory regions during granulocytic and monocytic differentiation.

In K562 cells, the distal regulatory region is modestly affected by Ara-C-induced erythroid differentiation only when the C/EBP β (M1, M3) and the PU.1 binding sites (M2, M3) are mutated (Figure 4d, left panel). In contrast, the activity of the proximal region significantly decreased upon Ara-C treatment. The deletion of the LMO2/TAL1 and GATA-1 binding sites (Δ 1) counteracted this effect (Figure 4d, right panel), suggesting that *miR-223* repression may be mediated by a GATA-1/LMO2/TAL1-containing transcriptional complex.

To confirm this view, we co-transfected in 293T cells the reporter constructs containing the proximal or the distal regulatory regions and expression vectors for the TFs

GATA-1, LMO2, TAL1 and E2A, which form a complex regulating erythropoiesis.³⁹ The coexpression of these TFs had relatively little effect on the distal region activity, whereas the expression of at least three erythroid factors significantly repressed the activity of the proximal regulatory region, reaching a maximum repression when the whole complex of the four TF was transfected (Figure 4e).

Thus, erythroid TFs silence the contribution of the proximal regulatory region to the overall pri-miR-223 transcription, maintaining stable low levels of mature miR-223 in the erythroid lineage.

Modulation of miR-223 activity in myeloid progenitors affects their differentiation status and response to differentiation agents. We hypothesize that miR-223 activity must be finely regulated during lineage fate decision of human hematopoietic stem cell (HSC)/HPCs. Therefore, we transfected synthetic oligonucleotides to mimic miR-223 activity (mimic-223) or control oligonucleotides (mimic-CTR) in CD34⁺HPCs grown in multilineage erythroid, granulocytic and monocytic/macrophagic liquid suspension cultures. After 14 days of culture, the morphological analysis of cell differentiation showed that, with respect to control cells, the transfection of two doses of mimic-223 reduced the percentage of erythroid and monocytic/macrophagic cells, whereas it increased the percentage of granulocytic cells. Synthetic oligonucleotides inhibiting miR-223 activity (inhibitor-223) gave opposite results (Figures 5a and b).

Clonogenic studies on transfected CD34⁺HPCs plated in methylcellulose confirmed these observations. Indeed, overexpression of miR-223 significantly induced the granulocyte, macrophage colony-forming unit (CFU-GM) and decreased erythroid burst-forming unit-erythroid (BFU-E) colonies, whereas inhibition of miR-223 activity increased BFU-E and decreased CFU-GM colonies. The percentage of multipotent granulocyte, erythroid, macrophage, megakaryocyte colony-forming unit (CFU-GEMM) remained unchanged (Figure 5c).

We next used miR-Zip lentiviral technique to inhibit endogenous miR-223 activity (miR-Zip-223) in myeloid cell lines. An miR-scrambled vector was used as a control (miR-Zip-Scr). In HL60 wild-type and miR-Zip-Scr cells, NFI-A protein, a validated miR-223 target,²⁵ is downmodulated by RA and, to a lesser extent, by VitD3 after 72 h treatment (Figure 6a). This is due to the different levels of endogenous miR-223 induction (Figure 1c). However, NFI-A suppression is not seen in miR-Zip-223 cells indicating an effective inhibition of miR-223 activity (Figure 6a).

MiR-223 knockdown in HL60 cells reduced, by about twofold, the differentiation efficacy of both RA or VitD3 treatments. This was revealed by the lower proportion of cells displaying the granulocytic/monocytic surface marker CD11b in RA-treated miR-Zip-223 cells and both the CD11b and CD14 markers in VitD3-treated miR-Zip-223 cells, when compared with miR-Zip-Scr cells (Figure 6b). Remarkably, RA-treated miR-Zip-223 HL60 cells acquired monocytoid instead of granulocytic morphological features (Figure 6c). Monocytic differentiation was confirmed by a sharp increase in the expression of CD14 in RA-treated miR-Zip-223 HL60 cells, but not in miR-Zip-Scr cells (Figure 6b, right panel). Furthermore, in RA-treated miR-Zip-223 HL60 cells, the

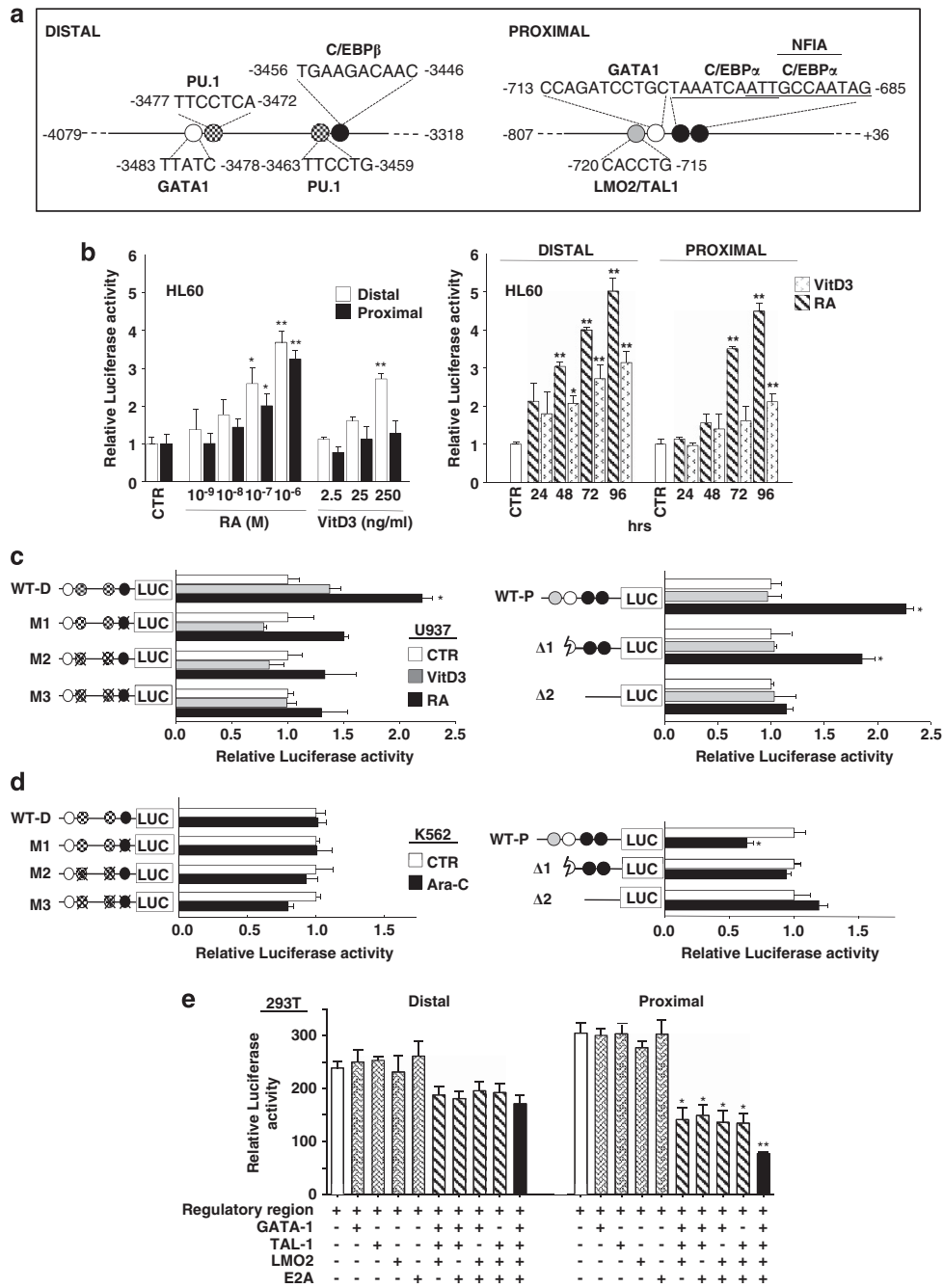


Figure 4 Evaluation of the activity of the *miR-223* regulatory regions by luciferase assays. (a) Schematic representation of the position and the sequence of relevant TFs binding sites on the distal and proximal *miR-223* regulatory regions. Numbers are the nucleotides relative to the 5' end of the pre-miR223 sequence (nt +1). (b) Luciferase activity measured on extracts of HL60 cells stably carrying the *miR-223* distal or the proximal regulatory region driving a luciferase reporter gene. Before lysis the cells were treated with RA or VitD3 for 72 h at the indicated concentrations (left panel) or 1 μ M RA or 250 ng/ml VitD3 for the indicated time (right panel). (c) Luciferase activity measured on extracts of U937 cells transiently transfected with the luciferase reporter constructs schematically depicted on the left side of the bars. The cells were treated 3 h after transfection with 1 μ M RA or 250 ng/ml VitD3 for 48 h, as indicated. Left panel: distal regulatory region and its mutants for the C/EBP β binding site (M1), PU.1 binding sites (M2) or all these binding sites (M3) are indicated. Right panel: proximal regulatory region and its mutants with deletion of the TAL1/LMO2/GATA-1 binding sites (Δ 1) or deletion of these sites and the C/EBP α and NFIA binding sites (Δ 2) are indicated. (d) Luciferase activity measured on extracts of K562 cells transiently transfected with luciferase reporter constructs as above. The cells were treated 3 h after transfection with 0.5 μ M Ara-C for 48 h, as indicated. Left panel: distal regulatory region and its mutants as in (c). Right panel: proximal regulatory region and its mutants as in (c). (e) Luciferase activity measured 48 h after transfection in lysates of 293T cells transiently cotransfected with luciferase reporters for the distal (left panel) or proximal (right panel) regulatory region and expression vectors for the indicated erythroid TFs. The bars represent the mean of two independent experiments performed in triplicate \pm S.D. Statistical significance was calculated between treated and untreated samples (panels b, c and d) or between mock-transfected and expression vector-transfected cells (panel e). * P < 0.05; ** P < 0.01

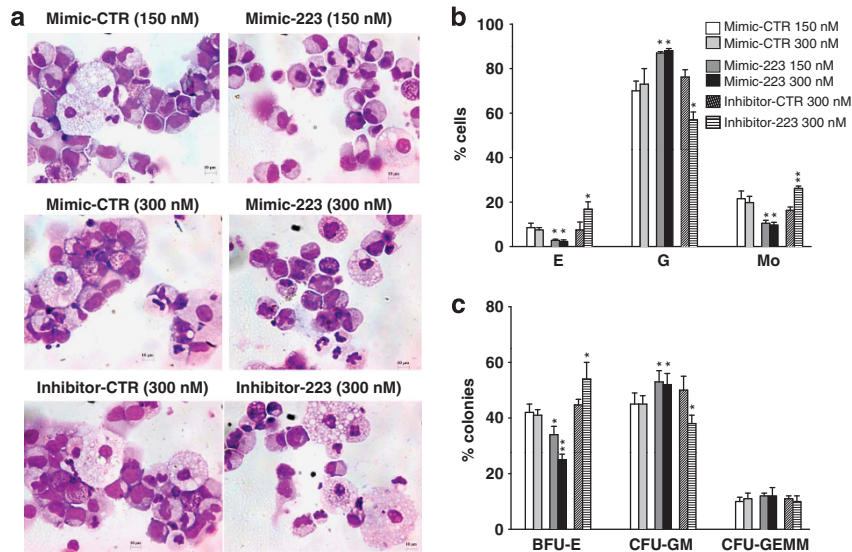


Figure 5 Modulation of miR-223 activity in CD34⁺ HSCs. CD34⁺ HSCs were transfected with the indicated concentrations of miR mimic negative control (mimic-CTR), miR-223 mimic (mimic-223), miRNA hairpin inhibitor negative control (Inhibitor-CTR) and miR-223 hairpin inhibitor (inhibitor-223). (a) Representative Wright–Giemsa staining of transfected cells undergoing multilineage cultures (day 14 of culture). Scale bar, 10 μ m. (b) Morphological evaluation of the percentage of erythroid (E), granulocytic (G) and monocytic/macrophagic (Mo) cells detectable after 14 days of multilineage liquid cultures. (c) Percentage of BFU-E, CFU-GM and CFU-GEMM growth in methylcellulose medium (14 days of culture). Error bars represent S.D. of three independent evaluations. * $P < 0.05$; ** $P < 0.001$

expression of the monocyte/macrophage serine esterase-1 (MSE) mRNA increased, when compared with miR-Zip-Scr cells (Figure 6d). Thus, the reduction of miR-223 function forces monocytic differentiation of myeloid precursors.

We then overexpressed the mature miR-223 or inhibited its activity by infecting K562 cells with lenti-223 and miR-Zip-223, respectively, or control vectors. Upon miR-Zip-223 infection, the expression of NFI-A was increased (Figure 6e). Several erythroid differentiation parameters, including ϵ -globin protein, percentage of benzidine histochemically stained cells and expression levels of glycophorin-A mRNA, were increased in K562 cells with inhibited miR-223 activity and further increased upon Ara-C-induced erythroid differentiation (Figures 6e and g, left panels and Supplementary Figure S4B). This indicates that a reduced miR-223 activity favors erythroid differentiation. Opposite results were obtained in K562 cells, where miR-223 was overexpressed (Supplementary Figure S4A). Indeed, ϵ -globin protein (Figure 6f), the percentage of benzidine-positive cells and glycophorin-A mRNA levels were slightly reduced in basal conditions and reached lower values upon Ara-C treatment with respect to control cells (Figure 6g, right panels and Supplementary Figure S4). Thus, erythroid differentiation is facilitated by low miR-223 levels, whereas it is inhibited by high miR-223 activity.

Discussion

Control of miRNAs' gene expression by transcriptional regulatory feed-back loops involving TFs is a common regulatory mechanism during development and cell fate determination.^{9,11,21,40} Several studies indicate that miR-223 has an important regulatory part in promoting myeloid cell differentiation and that its misexpression correlates with disease.^{18,25,30,31,41,42} Interestingly, a recent study performed

in 119 AML patients and in 121 healthy volunteers showed that miR-223 suppression in AML is not caused by DNA sequence alterations in the mature miR-223 or in the distal and proximal regulatory regions of *miR-223*, nor is it mediated by promoter hypermethylation.⁴³ These findings suggested that a dynamic relative regulation of pri-miR transcripts, determining mature miR-223 levels, could have a functional role during hematopoietic progenitors' differentiation.

MiR-223 affects the expression of a variety of known hematopoietic regulators, including LMO2, NFI-A, MEF2C (myocyte enhancer factor 2C) and E2F1 (adenovirus E2 promoter-binding factor 1). Most of these TFs are also present in transcriptional regulatory miR-223 circuitries necessary for the correct maturation of HSC/HPC.^{25,41–44} Therefore, in hematopoietic cells, miR-223 activity and TF networks appear extensively dynamic, thus calling for a tight regulation of *miR-223* levels during developmental transitions and lineage fate decisions.

Here, we provided evidence for a fine-tuning of mature miR-223 activity in 'lineage priming' of HPCs and myeloid precursors. Gain- and loss-of-function experiments performed in CD34⁺-HPCs and myeloid cell lines established that titrated variations in the activity of a single microRNA, miR-223, provide flexibility to cell fate commitment of myeloid progenitors into the erythroid, granulocytic or monocytic pathways of differentiation. Indeed, overexpression of miR-223 directs myeloid CFU-GM progenitors to granulopoiesis,^{25,31} whereas its silencing facilitates the erythroid and monocytic/macrophagic pathways. The negative regulation of *miR-223* transcription by LMO2, a validated miR-223 target⁴² identifies a novel transcriptional circuitry regulating erythroid development/maturation.

Findings reported in *miR-223* knockout mice imply that miR-223 negatively regulates HPC proliferation and granulocyte differentiation/activation.⁴¹ However, these mice were

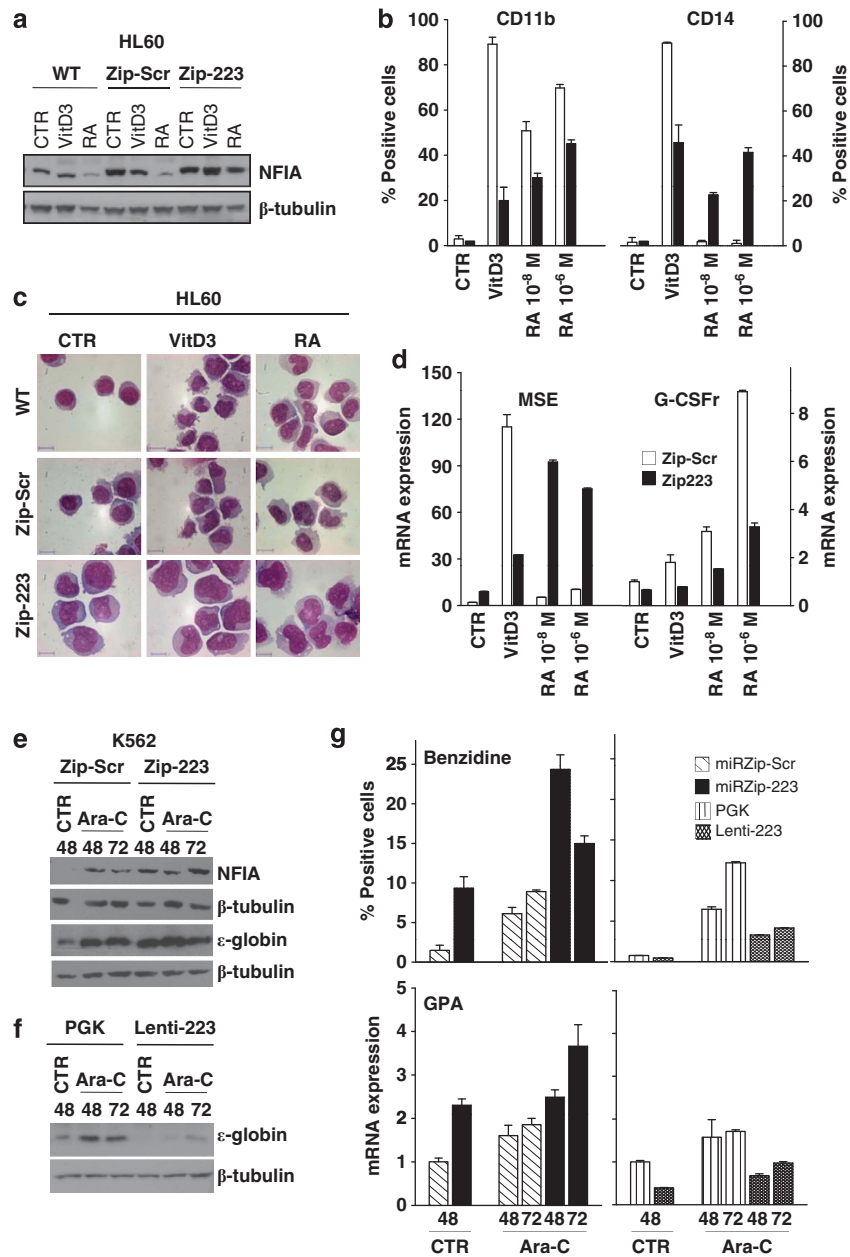


Figure 6 Knockdown of miR-223 in HL60 cells and miR-223 expression/silencing in K562 cells. HL60 cells were infected or not (WT) with the miR-Zip-223 anti-miR-223 microRNA (miR-Zip-223), or the scramble hairpin control (miR-Zip-Scr) constructs. (a) NFIA protein levels were measured by immunoblot analysis in cells treated or not (CTR) with 250 ng/ml VitD3 or 1 μ M RA for 72 h. β -Tubulin was used as a loading control. (b) Fluorescence-activated cell sorting (FACS) analysis of CD11b⁺ or CD14⁺ cells. (c) Changes in cell morphology as evaluated by light-field microscopy of Wright-Giemsa-stained cells (scale bar, 10 μ M). (d) Expression levels of genes related to granulocytic (G-CSFr) and monocytic (MSE) differentiation, measured by qRT-PCR analysis in cells treated with the indicated concentrations of VitD3 or RA for 72 h. The results represent the average of three independent evaluations \pm S.D. (e) NFIA and ϵ -globin protein levels measured by immunoblot analysis in K562 cells stably infected with empty lentiviral vector (miR-Zip-Scr) or lentiviral constructs knocking down miR-223 (miR-Zip-223) treated for the indicated times with 0.5 μ M Ara-C. β -Tubulin was used as a loading control. (f) Immunodetection of ϵ -globin in K562 cells ectopically expressing miR-223 (lenti-223) or empty vector (pgk) treated for the indicated times with 0.5 μ M Ara-C. β -Tubulin was used as a protein loading control. (g) K562 cells stably infected with empty lentiviral vectors (pgk or miR-Zip-Scr) or lentiviral constructs expressing miR-223 (lenti-223) or knocking down miR-223 (miR-Zip-223). Cells were treated for the indicated times with 0.5 μ M Ara-C and tested for the percentage of benzidine histochemically stained cells (upper panel), and expression levels of erythroid lineage-specific marker GPA measured by qRT-PCR analysis (lower panel). The results represent the average of three independent evaluations \pm S.D.

generated by *miR-223* locus deletion in embryonic stem cells. This gene defect might be circumvented to allow adult HSCs development. Inducible inactivation of *miR223* in BM progenitors would be required to fully elucidate the role of miR-223 in adult hematopoiesis. Nevertheless, this study

suggested the increased expression of miR-223 as a natural modulator of granulocytic differentiation, ultimately to prevent hyperinflammatory states. This supports the importance of the fine-tuning regulatory functions of miR-223 levels in hematopoiesis.

Our results show that in primary human hematopoietic cells and cell lines, the regulation of *miR-223* expression levels is sustained by the different usage of two regulatory regions present on its gene. This mechanism is widespread in eukaryotes for the regulation of developmental- and/or tissue-specific genes.⁴⁵ The high expression levels of mature miR-223 in granulocytic cells is determined by the increased levels of both the distal (V1) and proximal (V2) pri-miR-223 transcription variants. In contrast, only the pri-miR-223 V1 is induced during monocytic differentiation.

The detailed picture, obtained both in primary CD34⁺ HPCs and in classical cell line differentiation models, revealed a coordinated activity of TFs bound to the 'distal' and 'proximal' *miR-223* regulatory regions, resulting in a lineage-dependent regulated transcriptional activation of the *miR-223*. Interestingly, the upstream regions of miRNA genes often contain more regulatory motifs than protein-coding gene promoters.⁴⁶ Both the regulatory regions of the *miR-223* possess multisite binding targets for various hematopoietic lineage-specific TFs. The recruitment of C/EBP β and PU.1 myeloid TFs on the distal regulatory region appears to drive its expression levels during monocytic differentiation, whereas the recruitment of C/EBP α to the proximal *miR-223* regulatory region relates to strong induction of mature miR-223 measurable during granulocytogenesis. On the contrary, the binding of TAL-1-LMO2 and GATA-1 at their sites on proximal regulatory *miR-223* region represses miR-223 expression during erythroid differentiation.

The expression of miRNA genes may require a combination of tissue-specific TFs and distinct regulatory motifs. One single regulatory region may not be sufficient to accommodate all the required information. Multiple regulatory regions on miRNA genes may expand the potential of transcriptional circuitries initiated by lineage-specific TFs, thus adding an additional means for the correct temporal and spatial control of the expression patterns of miRNAs during lineage specification. Transcriptional circuits can generate threshold levels of mature miR-223 that are instrumental in determining cell fate decisions among three alternative types of myeloid progenitors (erythroid, granulocytic and monocytic).

In conclusion, our data indicate the usage of different miRNA gene regulatory regions as an additional way to create cellular diversity. Such regulation has an important role in the cell context-specific functions of miRNAs and supports fine-tuning roles for mature miRNAs levels in establishing hematopoietic lineage fate determination. Deregulation of this novel regulatory pathway may be relevant for the pathogenesis of hematopoietic disease.

Materials and Methods

Reagents. Reagents included all-*trans*-RA, Ara-C, VitD3 (Sigma-Aldrich, St. Louis, MO, USA), human cytokines (PeproTech Inc., Rocky Hill, NJ, USA) and human erythropoietin (Epo) (Amgen, Thousand Oaks, CA, USA).

AML samples, CD34⁺ human HPCs' purification culture and unilineage differentiation. BM and/or PB were obtained from 40 informed, newly diagnosed AML patients. Cases were classified according to the French-American-British (FAB) classification and showed an initial percentage of circulating blasts $\geq 80\%$. CD34⁺ cells' collection, isolation, unilineage cultures and morphological analysis were performed as described^{18,25,30,45-49} according to institutional guidelines. For erythroid unilineage culture, the medium was

supplemented with: human Epo (3 U/ml), interleukin-3 (IL-3; 0.01 U/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.001 ng/ml). For granulocytic unilineage culture, the medium was supplemented with IL-3 (1 U/ml), GM-CSF (0.1 ng/ml) and granulocyte colony-stimulating factor G-CSF (500 U/ml). For monocytic unilineage, the culture medium was supplemented with IL-6 (1 ng/ml), and fms-related tyrosine kinase 3 ligand (Flt3 ligand) (100 ng/ml) combined with plateau levels of macrophage colony-stimulating factor (M-CSF) (50 ng/ml). For multilineage cultures, CD34⁺-HPCs were grown overnight in serum-free media supplemented with stem cell factor (SCF) 50 ng/ml, Flt3 ligand 50 ng/ml, IL-3 20 ng/ml and thrombopoietin (TPO) 20 ng/ml and then transduced with miRIDIAN miR-223 Mimic, miR-223 hairpin inhibitor or negative controls (hairpin inhibitor and miRIDIAN microRNA Mimic controls) (Thermo Fisher Scientific, Dharmacon Waltham, MA, USA). An FITC-conjugated double-strand RNA was co-transfected (in a 10:1 ratio), following TransIT-TKO transfection reagent instructions. Twenty-four hours after transfection, FITC-positive cells were sorted by a FACSAria instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and cultured in a multilineage serum-free medium containing: de-lipidated BSA (10 mg/ml), saturated human transferrin (Tf; 700 μ g/ml), human LDL (40 μ g/ml), Flt3 ligand 100 ng/ml, kit ligand 100 ng/ml, IL-3 10 ng/ml, GM-CSF 10 ng/ml, TPO 100 ng/ml, Epo 3 U/ml, G-CSF 500 U/ml, M-CSF 250 U/ml, IL-6 10 ng/ml and basic fibroblast growth factor 10 ng/ml.

Clonogenic assay. Twenty-four hours after transfection, FITC-positive cells (3×10^5) were suspended in 1 ml methylcellulose medium supplemented with SCF, GM-CSF, IL-3 and EPO⁴⁸ and plated in triplicate culture dishes. After 14 days incubation, each plate was scored for BFU-E, CFU-GM and CFU-GEMM growth.

Cell lines culture and differentiation. HL60, U937 and K562 cell lines were maintained in RPMI 1640 GlutaMAX medium supplemented with 50 IU/ml of penicillin, 50 μ g/ml streptomycin and 10% FCS (Invitrogen, Carlsbad, CA, USA). The 293T cells were maintained in D-MEM with the same supplementation. Differentiation of HL60 and K562 cells was obtained by adding to the medium the dose of RA or VitD3 or Ara-C indicated in the text.

Immunoblot analysis. Proteins were fractionated by electrophoresis, electroblotted to nitrocellulose membrane (Protran, Whatman S&S, Maidstone, UK) and probed with the antibodies reported in Supplementary Table S1 and with an anti- β -tubulin antibody to ensure the equal loading of the samples. Immunoreactivity was measured using the ECL method (Amersham Biosciences, Fairfield, CT, USA).

Immunophenotypic analysis. Immunophenotypic assays were performed as described^{18,30,47} (phycoerythrin (PE)-conjugated anti-human CD34, CD11b, CD235a and allophycocyanin-conjugated anti-human CD14 antibodies were purchased from BD Biosciences (San Jose, CA, USA). A minimum of 50 000 events were recorded for each sample by a FACScan flow cytometer using CellFit software (Becton Dickinson).

Benzidine staining and morphological evaluation. Benzidine staining was performed as described.^{18,30,48} The percentage of benzidine-positive cells (blue) was determined by light microscopic examination of at least 500 cells per sample. Morphology was evaluated in conventional light-field microscopy of Wright-Giemsa-stained cytopins.

RNA extraction and RT-PCR analysis. Total RNA was extracted using the TRIzol (Invitrogen) according to the manufacturer's instructions. Mature miR-223 levels were measured using the TaqMan MicroRNA-specific assay (Applied Biosystem, Carlsbad, CA, USA) or by northern blot analysis as described.^{18,25} Glyceraldehyde phosphate dehydrogenase (GAPDH) and granulocyte colony-stimulating factor receptor mRNAs were determined by qRT-PCR using TaqMan oligonucleotides (Assay on Demand; Applied Biosystem). Pri-miR-223 precursors, glycophorin-A, monocyte/macrophage serine esterase-1 and GAPDH mRNAs were measured by qRT-PCR using SYBR green dye detection method. Primer sequences are listed in Supplementary Table S2. All reactions were performed in triplicate and analyzed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystem). The relative quantity of miRNAs and mRNAs was determined by the comparative 2 ^{$\Delta\Delta$ CT} method using RNU24 or GAPDH mRNA levels, respectively.

RNAse protection assay. RPA was performed on total RNA according to the standard protocol using the Riboprobe *in vitro* Transcription Systems

(Promega, Madison, WI, USA). RNA (15 μ g) was isolated from each sample and hybridized with 3×10^5 c.p.m. of labeled probes listed in Supplementary Table S2 at 65 °C for 12–16 h (hybridization buffer: formamide 80%, PIPES 40 mM, NaCl 400 mM, EDTA 1 mM). Probes for RPA were generated by PCR amplified from human genomic DNA using the primers listed in Supplementary Table S2. Probe #1 is a 100 bp fragment (–3413 to –3313 nt with respect to pre-miR-223 sequence); probe #2 is a 190 bp fragment (–117 to +73 nt); a fragment of 66 bp of the housekeeping gene β -tubulin was used for normalization. All the PCR fragments were cloned in pCR 2.1-TOPO vector (Invitrogen) under the control of T7 promoter, and linearized by digestion with *Bam*HI enzyme for riboprobe production. The samples were treated with RNase A/T1 for 30 min at 30 °C followed by proteinase K digestion for 15 min at 37 °C. The RNase-protected fragments were analyzed on a 8% denaturing urea–polyacrylamide gel followed by autoradiography. The intensity of the signals was quantitated using the ImageQuant system (GE Healthcare, Piscataway, NJ, USA) and normalized.

Chromatin immunoprecipitation. ChIP assays were performed as described.^{18,25,30} TF binding sites within the distal and proximal regulatory regions of *miR-223* were predicted by the MatInspector Professional (<http://www.genomatix.de/>) software packages and Alibaba 2.149⁵⁰ using the TRANSFAC database of TFs (<http://www.gene-regulation.com/pub/programs.html>). Genomic regions on *miR-223* were amplified using primers designed by the Primer express software (Applied Biosystem). Quantification of co-immunoprecipitated fragments was performed in triplicate samples using the SYBR green dye detection method with oligonucleotides listed in Supplementary Table S2. $\Delta\Delta C_t$ values of each immunoprecipitated sample were normalized with those obtained from the amplification of their respective input and by subtracting the values obtained in the corresponding samples with normal rabbit serum as described.^{25,30}

Lentiviral vectors construction. The lentivirus that express or knock down miR-223 (lenti-223 and miR-Zip-223, respectively) have been described.^{18,25} The miR-Zip-223 vector generates from its hairpin the following single-stranded RNA sequence, 5'-UGGGGUUUUGACAAACUGACA-3', which is perfectly complementary to miR-223. For transcriptional assays, a 761 bp 'distal' region and a 843 bp 'proximal' region were PCR amplified from human genomic DNA using the primers described in Supplementary Table S2. Deletion mutants of the proximal region nt –493 ($\Delta 1$) and nt –708 ($\Delta 2$) to nt +36 relative to the 5' end of the pre-miR-223 were produced by PCR amplification of human genomic DNA, using primers listed in Supplementary Table S2. C/EBP β (*atgaca*) (M1) or PU.1 (*ttctca*) (M2) or C/EBP β + PU.1 (M3) mutants of the distal regulatory region were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA), according to the manufacturer's instructions, using the primers described in Supplementary Table S2. All the fragments obtained were digested with *Kpn*I–*Xho*I and cloned into pGL4.10 (*luc2*) vector (Promega) and used for transient transfection and luciferase assays. Coding cDNA fragments corresponding to GATA-1 and TAL-1 erythroid TF genes were inserted in the PINCO retroviral expression vector.⁵¹ Coding cDNA fragments corresponding to LMO2 and E2A (TCF3) erythroid TFs³⁹ were cloned in pCDNA3 plasmid (Invitrogen).

Luciferase assays. Human embryonic kidney 293T cells (3×10^5) were transiently co-transfected with 0.5 μ g of response element ('distal' or 'proximal' regulatory region inserted in the pGL2 reporter vector, upstream of the luciferase open reading frame), 0.5 μ g of GATA-1 and TAL-1 constructs, 0.25 μ g of LMO2 and E2A expression vectors.³⁹ A co-transfected pCMV- β -Gal vector (Promega) (0.5 μ g) was used as an internal control for normalization of luciferase activity in each sample. U937 cells and K562 cells were transfected by electroporation as reported using Gene-pulser (Bio-Rad, Segrate, Italy).^{18,30,48} Cells were harvested 48 h post-transfection and assayed with Luciferase assay (Promega) and β -Gal assay (Promega) according to the manufacturer's instructions.

To generate stable HL60 cells carrying a luciferase gene containing *miR-223* regulatory regions, the cells were transfected with 20 μ g of each reporter construct and puromycin-selected (0.5 μ g/ml). After selection, HL60 cells were treated with RA and VitD3, harvested and assayed with Luciferase assay as described.^{18,30,48}

Statistical analysis. Data represented as mean \pm S.D. were derived from at least three independent experiments. Statistical significance between means was assessed by Student's *t*-test where $P < 0.05$ was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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